Investigation of the immunological differences between granulocytes from healthy donors and breast cancer patients, with respect to the cancer testis antigen, *CTCFL*

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ABSTRACT

Granulocytes or Polymorphonuclear neutrophils (PMNs) are key players in the non-specific immune system against microbial infection as they express surface receptors for the recognition of general antigenic patterns found on pathogens. In addition PMN's also act as a mediator for the antigen-specific adaptive immunity and T-cells function. However, an anti-tumoral role of the PMN's was suggested over the recent years beside their well-studied innate function, opening the door for new studies in this area.

CTCFL *or* BORIS (Brother Of the Regulator of the Imprinting Site) is a paralogue protein to a ubiquitously expressed transcription factor, CTCF. The expression of CTCFL was detected exclusively in the spermatocytes. The role of BORIS was then supposed to be linked to the *de novo* DNA-methylation and re-establishment of methylation markers, however, a full understanding its function calls for further investigations. CTCFL was also recently established as a member of the cancer-testis (CT) gene family as its activation in somatic cells was found to be associated with the development of many types of cancer, including breast cancer. This presents it as an attractive biomarker for cancers diagnosis/prognosis and therapy target. Interestingly, CTCFL was also detected in PMNs obtained from breast cancer patients while being undetectable in the PMN's of healthy donors. Again this supports the prospect of using this protein as a biomarker in the early detection of cancer and treatment.

In this project we aimed to investigate the effect of CTCFL expression in PMNs of breast cancer patients on the physiological characteristic and immunological functions of these leukocytes. The first part of our experiments assessed phagocytic activity, oxidative burst function, survival and expression of immunity receptors in BORIS-positive PMN cell-line model. The second part investigated the origin of CTCFL activation by the incubation of peripheral human PMN's with the serum of breast cancer patients or the adenocarcinoma cell lines and observe the possible alteration in CTCFL levels and the corresponding levels of the immune system receptors. Finally, we attempted to manipulate the levels of CTCFL in PMN cell line model (knock down or over-expression) and the effect it may produce on the expression of immune system receptors.

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List of Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
APL	Acute Promeylocytic Leukaemia
C/EBP	CCAAT-Enhancer-Binding Protein
CAM	Cell Adhesion Molecule
CR	Complement Receptor
CTA	Cancer Testis Antigen
CTCF	CCTC-Binding Factor
CTCFL	CTCF-Like
DC	Dendritic Cell
DCIS	Ductal Carcinoma *in situ*
ECM	Extracellular Matrix
EGFP	Enhanced Green Fluorescent Protein
ER	Oestrogen Receptor
Fab	Antigen Binding Fragment
FACS	Fluorescent Activated Cell Sorting
FBS	Foetal Bovine Serum
Fc	Constant Fragment
FcR	Constant Fragment Receptor
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
ICAM	Intracellular Cell Adhesion Molecule
ICC	Immunocyctochemical
ICR	Imprinting Control Region
IDC	Invasive Ductal Carcinoma
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRS	Immunoreactive Score
Jak	Janus Kinase
kDa	Kilo Dalton
LB	Luria Betani
LHRH	Luteinising Hormone-Releasing Hormone
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
mL	Millilitre

mg	Milligrams
NET	Neutrophil Extracellular Trap
NO	Nitrous Oxide
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear Neutrophil
PR	Progesterone Receptor
PRR	Pattern Recognition Receptor
PS	Phosophatidyl Serine
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SERM	Selective Oestrogen Receptor Modulator
STAT	Signal Transducer And Activator Of Transcription
TEMED	Tetramethylethylenediamine
Th1	T Helper Lymphocytes
TLR	Toll-Like Receptor
TNBC	Triple-Negative Breast Cancer
TNF	Tumour Necrosis Factor
WB	Western Blot
ZF	Zinc Finger

Chapter 1 - **General Introduction**

1.1 Polymorphonuclear Neutrophils

1.1.1 Background

Polymorphonuclear neutrophils (PMNs) are a subset of granulocytes and one of the main cellular members of the innate (non-specific) immune system. The granulocytes subset is divided into three populations according to their cellular morphology and cytoplasmic staining characteristics; neutrophilic, eosinophilic, and basophilic granulocytes (Murphy et al., 2011). Both eosinophils and basophils usually have bi-lobed nuclei of different shapes and granulated cytoplasm, with basophils exhibiting more granulation than eosinophils, but eosinophils will stain red with the acidic dye eosin, while basophils will stain blue with the basic dye methylene blue. In contrast, neutrophils are large cells with an average diameter 12-15 μm, with a prominent, multi-lobed nucleus and a granulated cytoplasm which stains for both the acidic and basic dyes described above. PMNs are the most abundant leukocytes population and comprise 60% to 70% of the total circulating blood leukocytes, whilst eosinophils constitute only 1%–3% and basophils only 1% (Murphy et al., 2011).

Approximately 10^{11} neutrophils are normally produced in the bone marrow and released into the blood circulation daily and these numbers are sustained in the blood stream by homeostasis. Mature circulating neutrophils are short-lived in the blood stream when not activated lasting only 8-12 hours, before they undergo apoptosis (programmed cell death). In the presence of invading pathogen(s) and/or the release of specific cytokines, neutrophils are activated and their survival is extended to 2 -3 days (Brandt et al., 2000).

As members of the innate immune system, PMNs provide the first line of defence against infection and are central modulators for inflammation (Kumar and Sharma, 2010). Like most of the innate immune system members, PMNs are constantly present in the human body, where they inspect organs for possible pathogenic threat and 'non-self' antigens. Both cellular (e.g. macrophages) and molecular (e.g. complement) components of the innate immune system recognise common molecular patterns encountered frequently on pathogens. In contrast, the adaptive immune system (also known as the specific immune system) is activated after the innate immunity response, typically 5 to 6 days from the initial infection, and it provides a highly specific response against specific antigens that are uniquely expressed in the different pathogens.

1.1.2 Production of Polymorphonuclear Neutrophils

The process of granulocyte production is known as granulopoiesis. The daily production of PMNs is a continuous process and can reach up to $2x10^{11}$ cells. Like all blood cells of the adult human, granulocytes are generated in the bone marrow from a common haematopoietic stem cell (haemocytoblast) that is capable of self-renewal and has the potential to differentiate into myeloid or lymphoid linages (multi-potent or pluripotent cells). Granulopoiesis commences with common precursor cells that are 'committed' to the myeloid linage, known as common myeloid progenitors. These precursor cells differentiate in the bone marrow until they reach full maturation and are then released into the blood stream. While differentiation is a continuous process, it is useful to create reference points when studying the development of neutrophils within the bone marrow. Therefore, the maturation of neutrophils has been divided into six morphological stages: myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils, and finally, fully mature and functional segmented neutrophils, which are the version released into the blood stream (Glasser and Fiederlein, 1987; Murphy et al., 2011) (Figure 1.1).

During the development of PMNs they acquire intracellular vesicles, known as granules. These are classified into three main types based on the stage in which they are formed during the maturation process and also their protein content: primary granules, known as azurophils; secondary granules, known as specific; and tertiary granules. The formation of primary granules is first observed during the promyelocyte stage of differentiation, while the secondary and tertiary granules are formed at the meylocyte and metamyelocyte stages, respectively (Theilgaard-Mönch et al., 2005).

The granules in neutrophils contain many different proteins and these are utilised in the destruction of microbes through an oxygen-dependent mechanism known as oxidative burst (see Section 1.1.4.2 for further details). These include cytotoxic proteins, such as defensins, bactericidal/permeability increasing protein, and lactoferrin. They also acquire proteolytic enzymes, such as elastase, matrix metalloproteinases (MMPs), and lysozyme (see Table 1.1). During the metamyelocyte stage of PMN development, mitosis comes to a complete halt and cells are no longer able to divide and undergo terminal differentiation. The regulation of PMN production and the control of their differentiation is heavily controlled by chemokines, starting from commitment to the myeloid linage and through all the different stages of neutrophilic differentiation (Ono et al., 2003).

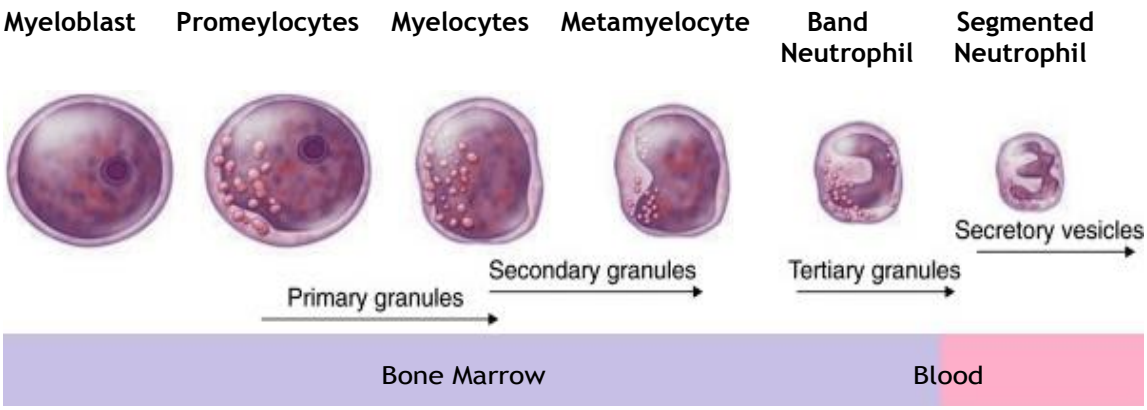

Figure 1.1: Neutrophilic granulocyte production in bone marrow (granulopoiesis)
The myeloblast is the first haematopoietic cell to 'commit' to the granulocytic-myeloid linage. The differentiation to the neutrophilic line or other lines of granulocytes (basophilic and eosinophilic) commences after the promyelocyte stage. These cells develop different types of granules as they progress into more differentiated stages, until eventually becoming fully mature PMNs containing primary, secondary, tertiary and secretory vesicles. In healthy individuals, all stages of differentiation occur in the bone marrow and only fully mature granulocytes are released into the blood stream (segmented neutrophils and rarely band neutrophils). Adapted from Goldman and Ausiello (2008).

Table 1.1: Proteins found in the granules of mature neutrophil
Source: (Kelley et al., 2012).

Component	Azurophil Granules (Primary) Peroxidase Positive	Specific Granules (Secondary) Peroxidase Negative	Gelatinase Granules (Tertiary) Peroxidase Negative	Secretory Vesicles
Relative Size	Largest	intermediate	intermediate	smallest
Antimicrobial proteins	Defensins Lysozyme Elastase BPI α_1-Antitrypsin Glucuronidase Myeloperoxidase Cathepsin G	Lysozyme Lactoferrin Gelatinase MMP-3 MMP-8 MMP-9 Lactoferrin β_2-Microglobulin	Lysozyme Gelatinase Acetyltransferase	
Membrane proteins and receptors	CD63 CD68 Alkaline phosphatase	CD11b/CD18 fMLP-R Cytochrome b558 CR3 CD66, CD67 Fibronectin receptor TNF receptor	CD11b/CD18 fMLP-R Cytochrome b558 CR3 Deacylating enzyme	CD11b/CD18 fMLP-R Cytochrome b558 CR1 CD10, CD14, CD15, CD45 Decay accelerating factor Alkaline phosphatase Uroplasminogen activator
Matrix proteins	β-Glucuronidase	Collagenase Gelatinase Laminin	Gelatinase	Albumin

BPI, bactericidal/permeability-increasing protein; FMLP, formyl-methionyl-leucyl-phenylalanine; MMP, matrix metalloproteinase; TNF, tumor necrosis factor.

The principal regulator of physiological granulopoiesis is granulocyte colony stimulating factor (G-CSF) (Furze and Rankin, 2008), while other chemokines include stem cell factor (SCF), interleukin-3 (IL-3), interleukin-6 (IL-6), and granulocyte macrophage colony stimulating factor (GM-CSF) amongst others (Kumar and Sharma, 2010; Theilgaard-Mönch et al., 2005). The process of granulopoiesis and differentiation is also regulated at the chromatin level (i.e. epigenetically). While much more remains to be elucidated, several studies have suggested that myeloid genes expressed during neutrophilic differentiation are controlled by several transcription factors in a timed and combinatorial manner, rather than by a single exclusive 'master' factor (Rosmarin et al., 2005). Transcription factor profiling in differentiating neutrophilic cell lines, as well as primary cells, has identified the following transcription factors as involved in granulopoiesis: the CCAAT-enhancer-binding protein (C/EBP) family (α, β, ε, ζ, δ, γ),

PU.1, Elf-1, CDP, AML-1, c-myb, GATA-1, c-fos and c-jun (Bjerregaard et al., 2003). In addition, other factors, such as growth factor independent-1 (Gfi-1) and NFκB have also been reported to be involved in the differentiation of neutrophils (Hock et al., 2004; Wang et al., 2009). It is worth noting that members of the C/EBP family are also found in other haematopoietic linages, the liver and in adipocytes (Rosmarin et al., 2005). PU.1 has been repeatedly proven to be necessary for commitment to the myeloid linage (Iwasaki and Akashi, 2007; Nerlov and Graf, 1998). The balance between C/EBPα and PU.1 determines commitment to monocytic or granulocytic differentiation (Laslo et al., 2006), as increased expression of PU.1 factors results in monocytic differentiation, while an elevation of C/EBPα directs the balance towards granulocytic differentiation (Radomska et al., 1998; Zhang et al., 1997).

1.1.3 Mature Polymorphonuclear Neutrophils

The mature PMN is terminally differentiated and the main morphological hallmarks of maturation are the segmentation of the nucleus, where the nuclear to cytoplasmic material ratio is decreased due to nuclear material condensation, and the predominant granulation of the cytoplasm. The surface of PMNs also undergoes several changes, such as the expression of new surface receptors, which enable PMNs to recognise inflammatory agonists. Several classes of receptors are expressed by PMNs, including G-coupled seven-transmembrane receptors, Fc receptors, adhesion molecules such as integrins and leukocytes selectins (L-selectins), cytokine receptors, and innate immunity receptors such as TLRs and C-type lectins. The binding of different agonists to these receptors regulates the many complex physiological and functional activities of PMNs, such as priming, activation, mobilisation, transmigration, and finally engulfment and destruction of foreign antigens (Abramson and Wheeler, 1993; Kelley et al., 2012). These receptors are either expressed constitutively on the surface of PMNs or are stored within the cytoplasmic

vesicles and are mobilised to the cell surface when PMNs are stimulated for an inflammatory response (Kindt et al., 2007). The mature activated PMN also becomes capable of *de novo* protein synthesis of receptors upon stimulation (Edwards, 2005). Among the receptors the mature PMN can selectively biosynthesise under certain stimulation are class II receptors of the major histocompatibility complex (MHC) and CD14 (Wagner et al., 2003). They are also able to produce a number of cytokines when stimulated, for example PMNs can produce TNFα and interleukin-12 (IL-12), and also chemokines such as IL-8 (Cassatella, 1999). Consequently, the population of mature PMNs is regarded as heterogenic in their acquired functional capabilities (Beyrau et al., 2012).

1.1.4 Activation of Polymorphonuclear Neutrophils

The activation of PMNs describes the collective processes which alters their morphology and physiology, leading to PMNs execution of their immunological function in pathogen destruction. These alterations include their adhesion and rolling, passing through the capillary endothelium (diapedesis), and migration to the source of inflammation stimuli and signals to eliminate pathogens (Kelley et al., 2012). The activation of PMNs is triggered and regulated by the binding of different activating stimuli, and will be discussed in Section 1.1.5. However, cytokines are major effectors in the initial activation of neutrophils. The signalling pathways of many cytokines, such as GM-SCF, interferons and several interleukins, are targeted in the therapy of many diseases (Girard, 2004). PMN activation is triggered via cytokines binding to specific receptors on the PMN surface. The ligation of the cytokine receptor induces gene expression and the production of inflammatory mediators by human neutrophils. The expression of targeted genes is preceded by the activation of transcription factor expression and the accumulation of corresponding mRNA transcripts of the targeted genes. It is noteworthy that cytokines

exhibit what is known as ‘cytokine pleiotropy’, the ability of a single cytokine to exert multiple immunological effects, and ‘cytokine redundancy, the ability for multiple cytokines to exert a similar reaction (Girard, 2004; Ozaki and Leonard, 2002).

1.1.4.1 Cytokine Receptors on PMNs

Neutrophils express several cytokine receptors, mainly from the interleukin-1(IL-1)/Toll-like receptor (TLR) family, tumour necrosis factor (TNF) receptor family, TGFβ, and the conventional cytokine receptor superfamily, which is subdivided into type I and II cytokine receptor families (Futosi et al., 2013). Most cytokine receptors expressed on PMNs belong to the conventional cytokine superfamily. Type I and II cytokine receptors are mostly dimeric molecules with tyrosine residues in their intracellular sequence. The most important cytokine receptors belonging to the type I receptors expressed on human neutrophils are interleukin-4 (IL-4), IL-6, IL-12, interleukin-15 (IL-15) receptors, as well as G-CSF and GM-CSF receptors. The main type II cytokine receptors found on neutrophils are receptors for the interferons, IFNα, IFNγ, and the inhibitory cytokine, interleukin-10 (IL-10). Both types of cytokine receptors lack tyrosine kinase catalytic activity, the addition of a phosphate group on the tyrosine residue within the receptor, thus they rely on Janus kinase (Jak) intracellular proteins to carry out the function of tyrosine phosphorylation (Futosi et al., 2013).

1.1.4.2 Jak/STAT signalling pathway in PMN

The Janus kinase /signal transducer and activator of transcription (JAK/STAT) pathway is a common signalling pathway used by many cytokines in the regulation of the immune system, including PMNs activation and function (Girard, 2004). The pathway involves two families of proteins in the signal transduction: the JAK protein family and the STAT protein family. JAKs are cytoplasmic tyrosine kinases that are constitutively associated with the proline-rich region in the intracellular domain of the cytokine receptor. The

STAT proteins are transcription factors found in the cytosol of many cells. The binding of the cytokine/ligand to its receptor leads to the cytokine receptor dimerization and bringing the two JAK protein closer to each other. This activates the associated JAK proteins, as the two JAKs phosphorylate each other (autophosphorylation). The JAK proteins also phosphorylate the tyrosine residue of the tyrosine residues of the cytokine receptor. The phosphorylated tyrosine residue serve as docking site for the SH2 domain on the STAT transcription factor (Girard, 2004; Ihle, 1996; Taniguchi, 1995). JAKs phosphorylate the docking STAT proteins, leading to their disassociation from the cytokine receptor then dimerization and translocation to the nucleus, where they regulates the transcription of targeted genes (Darnell, 1997; Levy and Darnell, 2002; Shuai and Liu, 2003). There are four members within the Jak family; Jak1, Jak2, Jak3, and tyrosine kinase 2 (Tyk2) (Schindler et al., 2007; Stark et al., 1998), and seven mammalian STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (Darnell, 1997). All Jak and STAT proteins have been shown to be expressed on human neutrophils (Al-Shami et al., 1998; Al-Shami and Naccache, 1999; Brizzi et al., 1996; Caldenhoven et al., 1999; Dogusan et al., 2001; Ryu et al., 2000). Many growth factors and cytokines activate Jak1, Jak2 and Tyk2 (Ghoreschi et al., 2009; O'Shea and Plenge, 2012), for example the cytokine GM-CSF, which influences granulocyte production and exerts several actions on human PMNs, has the ability to activate STAT proteins in neutrophils via Jak2, in particular STAT1 and 3, as well as STAT5B (Moraga et al., 2014). G-CSF, which is involved in granulocyte proliferation and maturation, similarly induces phosphorylation of STAT1 and STAT3 in human neutrophils. In contrast, Jak3 is only activated by cytokines when their receptor complex is comprised of the common γ chain (CD132), for example both IL-4 and IL-15 receptors share a common γ-chain and they use Jak1 and Jak3 to activate STAT5 or STAT6. Type II cytokine receptors for IFNs

and IL-10 primarily utilise Jak1, with a partial additional role for Jak2 and Tyk2, and they activate STAT1, STAT 2 or STAT 3 (Ghoreschi et al., 2009; O'Shea and Plenge, 2012). It is worth noting that the hormone prolactin has been found to induce STAT1 phosphorylation in neutrophils and in the same sense acts as a cytokine. The prolactin receptor also belongs the cytokine receptor type I family and is expressed on neutrophils (Dogusan et al., 2001).

1.1.5 Polymorphonuclear Neutrophils Adhesion, Transendothelial Migration and Extravasation

In order for a mature circulating PMN to reach an infected tissue they must first go through a multistep process whereby they migrate from the blood stream through the blood vessel endothelial wall and into the adjacent tissue (extravasation). PMNs are able to travel to the infected site by following a trail from a chemical attractant (or chemoattractants, e.g. microbial antigens) by a process known as chemotaxis (Kindt et al., 2007). The circulating PMNs do not bind to resting, non-inflamed vascular endothelial cells, and therefore do not exit the blood stream. However, when the vascular endothelium is activated (inflamed) by several cytokines and inflammatory mediators, PMNs can adhere to the endothelium then extravasate through the blood vessel and tissue neighbouring the inflammation site. Mediators of endothelial cell inflammation include bacterial lipopolysccharide (LPS), TNF-α, the complement fragment C5a, leukotriene B4, and histamine. These molecules induce vascular endothelial cells to release the cell adhesion molecules (CAMs) E- and P-selectins onto their surface within minutes following inflammation. Meanwhile, circulating neutrophils express their own set of CAMs, such as PSGL-1 (P-selectin glycoprotein ligand-1), which bind to the P- and E-selectins on the endothelium surface, and L-selectin, which bind to CD34, glycosylation dependent CAM (GlyCAM), and mucosal vascular addressin cell adhesion molecule

(MadCAM-1). This tethering motion slows the neutrophils, allowing chemokines expressed locally, either by the inflamed endothelium or later by other phagocytes (i.e. macrophages), to interact with their receptors present on the surface of rolling neutrophils. At this point, two main chemokines are involved in the activation of neutrophils, interleukin-8 (IL-8) and macrophage inflammatory protein-1β (MIP-1β). This interaction leads to a tighter adhesion between the two neutrophils and the endothelium, mediated via binding of intracellular cell adhesion molecules (ICAMs) on endothelial cells with β2 integrins (CD11a,b/CD18) on activated neutrophils. It is worth noting that ICAMs are also known as immunoglobulin (Ig) super adhesion molecules, as they contain a variable number of immunoglobulin-like domains within their structure and have been classified as members of the immunoglobulin family (Kindt et al., 2007). Following this tight adhesion, the neutrophils migrate to in-between two neighbouring endothelial cells without disrupting the integrity of the endothelial barrier, and then through the nearby tissue. Eventually, they follow a trace of an increasing chemoattractant (e.g. IL-8 released by local macrophages), which guides them to the site of infection (chemotaxis). Neutrophils then migrate into the tissue through the junctions between neighbouring endothelial cells (paracellular migration) using surface ligands, including ICAM-2, platelet endothelial-cell adhesion molecule-1 (PECAM-1) and proteins of the junctional adhesion molecule (JAM) (Woodfin et al., 2009). Transcellular transmigration may also occur under conditions of high ICAM-1 expression and density, with a small minority of neutrophils penetrating and passing through pores within the cytoplasm of endothelial cells (Woodfin et al., 2009; Yang et al., 2005).

1.1.5.1 Integrins

The integrins are a major family of adhesion receptors present on nucleated cells, including leukocytes. Members of this family are hetrodimeric proteins consisting of one

β subunit (CD18) which is non-covalently associated with an α subunit. Both subunits are type I transmembrane proteins and have a large extracellular domain and a short cytoplasmic domain (Springer and Wang, 2004). Integrin heterodimers are divided into different sub-families according to which α subunit and β subunit they possess (Humphries et al., 2006; Hynes, 2002); there are 18 α subunits and 8 β subunits which can be assembled into 24 different integrin heterodimers (Takada et al., 2007). Integrins are particularly important for mature PMNs and macrophages (i.e. phagocytes) as they transduce signals inside the cell which regulate most, if not all, of the functional characteristics of phagocytic cells. This includes the firm adhesion of PMNs onto the vascular endothelium, rearrangement of the actin cytoskeleton, cell spreading, transmigration to an infected site, the production of reactive oxygen intermediates (ROI), the release of antimicrobial granule proteins (degranulation), the production of various cytokines, cell proliferation, and survival/apoptosis (Berton and Lowell, 1999; Mayadas and Cullere, 2005). The expression of the β2 integrin sub-family is known to be restricted to leukocytes and they are largely expressed on neutrophils. Members of the β2 integrin sub-family known to be expressed on phagocytes include αL/β2 integrin (CD11a/CD18), αM/β2 integrin (CD11b/CD18), αx/β2 integrin (CD11c/CD18) and αD/β2 integrin (CD11d/CD18). Other members belonging to the β1 and β3 integrin sub-families are also expressed on phagocytic cells, such as α4/β1 integrin, α5/β1 integrin, α6/β1 integrin, and αv/β3 integrin (Berton and Lowell, 1999). Most of the β2 integrins are stored in vesicles within neutrophils and are fused to the plasma membrane when a mature neutrophil is activated by inflammatory agonists. The main ligands for leukocyte integrins are found either on vascular endothelial cells (see Section 1.1.3) or molecules of the extracellular matrix (EMC). For example, PMNs express complement receptor type 1 (CR1) (i.e. CD35) for the complement components C3b and C4b, and also complement receptor 3

(CR3) (CD11b/CD18) for iC3b. It is worth mentioning that CR3 belongs to the integrin family of receptors and it is also known as macrophage antigen-1 (Mac-1).

1.1.6 Function of Neutrophils in the Immune System

Innate immunity provides the first line of defence against infection, and most innate immune system members are constantly present in the human body where they inspect all the organs for possible pathogenic threat. Both cellular (e.g. macrophages) and molecular (e.g. complements) members of the innate immune system recognise common molecular patterns encountered frequently in pathogens. In contrast, the adaptive system provides a high degree of specific immunity, and unlike the innate immune system, develops a unique 'memory' of antibodies for each invading antigen, usually 5 to 6 days after an initial infection, delivering a stronger and often more effective response for killing the pathogen. The major agents of adaptive immunity are lymphocytes and their production of antibodies. Neutrophils or PMNs belong to the innate immune system. Following pathogen invasion or trauma, the bone marrow is induced to release a greater number of mature neutrophils into the blood circulation (leucocytosis) by several cytokines. These neutrophils are activated to migrate to the site of infection by following increasing concentrations of chemokines and inflammatory mediators. On reaching the inflamed tissue, the main objective of an activated neutrophil at this stage is the recognition, internalisation, digestion and destruction of invading pathogens. This is manifested in the immunological phenomenon known as phagocytosis, where professional phagocytes (i.e. monocytes/macrophages and neutrophils) accumulate at the site of an infection and start to rapidly uptake invading pathogens. Neutrophils are also equipped with oxygen-dependent and oxygen-independent mechanisms for killing bacteria. The neutrophil extracellular trap (NET) is another method used by neutrophils for eliminating pathogens (Kindt et al., 2007).

1.1.6.1 Phagocytosis

Phagocytosis is the engulfment and degradation of pathogens by cells known as phagocytes, which include macrophages, dendritic cells (DCs) and neutrophils. The process of phagocytosis is initiated by surface receptor on phagocytes, collectively named pattern recognition receptors (PRRs), which recognise and bind to an array of conserved molecules found on microbes and foreign elements, known as pathogen-associated molecular patterns (PAMPs). Two of the most common and well defined PAMPs on the surface of bacteria are LPS and peptidoglycans, whilst other PAMPs include viral, fungal and yeast molecular motifs. One of the main PRRs that recognises and binds to PAMPs is the TLR family, which is found mainly, but not exclusively, on phagocytes. So far, there are ten TLR member (designated from TLR 1 to TLR 10) are indentified in humans (Zeromski et al., 2008). Previous work has demonstrated that PMNs express mRNA for all TLRs except TLR-3 (Hayashi et al., 2003). Furthermore, agonists of all the expressed TLRs elicit an inflammatory reaction (Hayashi et al., 2003; Sabroe et al., 2003). The activation of TLRs through their binding to PAMPs mediates many defensive responses, including transcriptional activation, synthesis and secretion of cytokines to promote inflammation, and the attraction of other members of the innate immune system, such as macrophages and natural killer cells (Doan et al., 2012). Another PRR, and also a co-receptor for TLR-4, is CD14, which is the main receptor for bacterial LPS. CD14 is found on the membrane of PMNs and also in a soluble form within the blood stream, as it can be shed by membranes or released directly from vesicles into the cytoplasm. Despite the low expression of CD14 on the surface of resting PMNs, granulocytes comprising of intracellular pools of CD14 are translocated to the surface of PMN membranes upon binding to LPS (Rodeberg et al., 1997) or under stimulation with IFNγ or TNFα (Takaoka and Yanai, 2006; Takeshita et al., 1998). An indirect approach which amplifies

phagocytosis is opsonisation. In this process a microbe is coated with IgG antibody (isotype IgG1 or IgG3) or IgA, which facilitates its recognition by phagocytes (Kindt et al., 2007). PMNs express a surface receptor for the constant fragment (Fc) region of an IgG antibody while the antigen-binding fragment region (Fab) of the same antibody binds to the targeted antigen. An antibody receptor on a phagocyte is known as a Fc receptor (FcR). Several members of this family are expressed on PMNs, amongst them FcγRI (CD64), FcγRII and FcγRII (CD32), FcγRIII (CD16b) and FcαRI (CD89), the latter being a receptor for IgA. Binding of the antigen to these receptors will activate its internalisation and initiate degranulation or its fusion with lysosomes, and finally its annihilation. FcRs collaborate with complement receptors to bind simultaneously to the same antigen. While an FcR binds to an antibody tagging a targeted antigen, a complement receptor binds to the complement fragment attached to the same antigen, and therefore their receptors work synergistically to facilitate phagocytosis by PMNs.

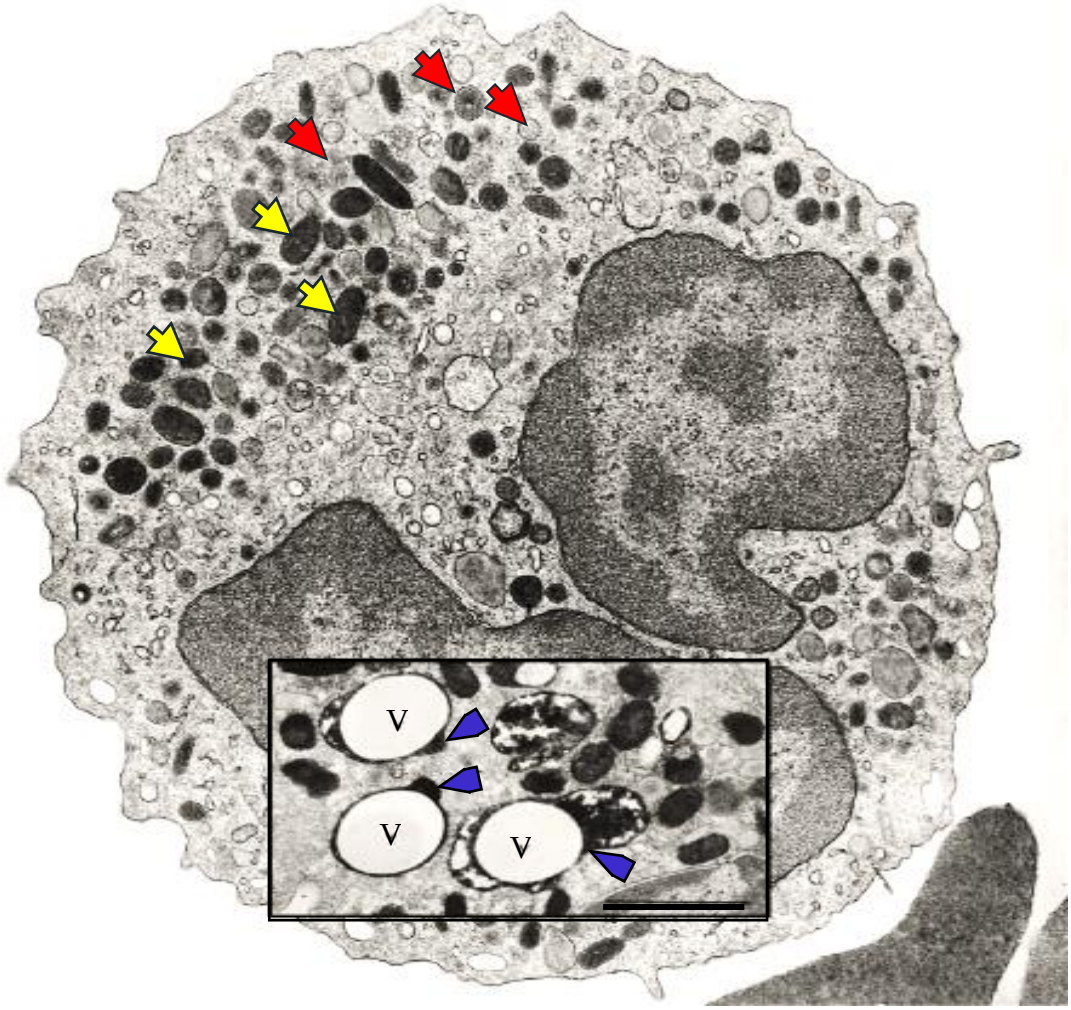

Figure 1.2: Transmission electron micrograph of a human neutrophil during phagocytosis
This image was taken 20 seconds after the neutrophils phagocytosed latex particles opsonised with immunoglobulin G (IgG). The section is stained for myeloperoxidase (MPO). The large dark clusters indicated with yellow arrow are the azurophils (primary granules), while light small clusters indicated with the red arrows are the secondary (specific) granules. The framed image represents degranulation of azurophils (dark clusters indicated with blue arrows) into phagosomes (white circles labelled with "V" for "vacuoles"). Bar = 1 μm (Segal et al., 1980).

The binding between PAMPs and PRRs activates the formation of plasma membrane protrusions (i.e. pseudopods), which is mediated by cytoskeletal proteins in cytoplasm. These pseudopods then surround the targeted microbe and enclose it inside the phagocyte. The vesicle enclosing the pathogen is called a phagosome, and this fuses with enzymes within intracellular vesicles (i.e. lysosomes) and proteolytic and cytotoxic proteins stored in primary and secondary granules and the forming phagolysosomes.

1.1.6.2 Antimicrobial and Cytotoxic Activity (Degranulation)

The destruction of the engulfed microbes or foreign elements by PMNs takes place within phagolysosomes, where multiple killing mechanisms are employed. Most of these mechanisms are oxygen-dependent, as they generate ROIs; superperoxide radicals ($O^{\cdot}{}_2^-$), hypochlorite anions ($HOCl^-$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and also reactive nitrogen intermediates, such as nitric oxide (NO), nitrogen dioxide (NO_2) and nitrous acid (HNO_2). These intermediates are highly lethal to the ingested microbes and even more damaging when utilised together. An example of this is the combination of the very potent antimicrobial agent NO with superperoxide anions yields peroxynitrite and a member of the reactive nitrogen species which have a much greater cytotoxic event against microorganisms. Most antimicrobial activity against bacteria, fungi and protozoa is due to nitric oxide and its derivatives (Kindt et al., 2007). The annihilation of microbes in this manner results in metabolically heightened levels of oxygen, thus the process is known as oxidative burst or respiratory burst. PMNs also have oxygen-independent killing mechanisms which are similarly highly cytotoxic to microorganisms. These include the degrading enzymes and proteins stored in the primary and secondary granules in the cytoplasm of PMNs.

1.1.6.3 PMN Modulation of an Inflammatory Response

When PMNs are activated at an infected site they release IL-8, which amongst its diverse effects as a chemokine, attracts other neutrophils and macrophages to the same site (Moraga et al., 2014). It also releases other chemokines, mainly MIP1$_\alpha$ and MIP1$_\beta$, which recruit T helper lymphocytes (Th1) to the inflammatory response (Scapini et al., 2000). In addition to MIP1$_\alpha$ and MIP1$_\beta$, degranulation of defensins attracts naïve T cells and immature DCs to the site of infection (Yang et al., 2000). The interaction of PMNs with DCs induces them to produce IL-12, which in turn induces Th1 polarisation (Bliss et al., 2000; Cassatella, 1995).

1.1.6.4 Apoptosis and Neutrophil Extracellular Traps

PMNs are part of a hard-and-fast response and therefore are programmed for self-death 24 -48 hours after entering an infected tissue. While inactivated circulating neutrophils are cleared via the spleen, activated neutrophils at the site of an infection undergo apoptosis after the phagocytosis of pathogens and degranulation. In addition, the release of ROIs also promotes the destruction of neutrophils and may also damage the surrounding tissue. Apoptotic neutrophils at the site of an infection are eventually engulfed by tissue macrophages; however, neutrophils have one last defence mechanism against pathogens before being cleared by macrophages. NETs are created once the self-destruct programme of a neutrophil has been activated. DNA, proteins and enzymes become fused together, and erupt out of an apoptotic neutrophil unleashing a web that traps and kills bacteria (Figure 1.3). This mechanism is effective against an array of different bacteria, from *Shigella*, which causes dysentery, to *Salmonella*, which is the infectious agent for typhoid fever (Brinkmann et al., 2004).

Figure 1.3: Scanning electron micrograph of a mouse lung infected with *Klebsiella pneumoniae*

The bacterium (pink) is snared in a neutrophil extracellular trap (green), a web of decondensed chromatin released by neutrophils to catch and kill pathogens. Papayannopoulos et al. (2010) revealed that neutrophil elastase degrades histones to promote chromatin decondensation and trap formation (Image by Volker Brinkman and Abdul Hakkim).

1.1.6.5 Regulation of the Role of Mature PMNs in Immunity

It is worth noting that under particular stimulation a mature PMN is capable of *de novo* protein synthesis. Neutrophils also synthesise and secrete small amounts of some cytokines, including IL-1, IL-6, IL-8, TNF-α, and GM-CSF. IL-1β is not stored within neutrophils but instead is rapidly synthesised *de novo* by processes requiring transcription and translation. An increase in IL-1 can be detected within an hour of human neutrophils being exposed to GM-CSF.

Many cytokines are generated during an inflammatory response, either by the infected tissue or other cells of the immune system. These cytokines have a significant role in directing the functional features of the immune system cells, as well as mature neutrophils and their different defence mechanisms. The pyrogenic cytokines, IL-1, TNF-α, and IL-6, all prime various pathways that contribute to the activation of NADPH oxidase. The pro-inflammatory cytokine IL-8 is an activating factor and a potent chemoattractant for mature neutrophils; IL-8 synergises with IFN-γ, TNF-α, GM-CSF, and G-CSF to amplify various neutrophil cytotoxic functions. Cytokines also increase the microbiostatic and killing capacities of neutrophils against bacteria, protozoa and fungi. IFN-γ and GM-CSF independently amplify neutrophil antibody-dependent cytotoxicity. The anti-inflammatory cytokines, IL-4 and IL-10, inhibit the production of IL-8 and the release of TNF-α and IL-1, which is reflected in the blockade of neutrophil activation. IL-1 is produced by stimulated monocytes and macrophages, blood-vessel endothelial cells, and muscle cells. The biological effect of IL-1 expression is the release of neutrophils from the bone marrow, increased thromboxane A2 release from neutrophils, and enhanced degranulation from neutrophils after stimulation with other agonists. IFN-γ induces the expression of FcγR1, which increases antibody dependent cytotoxicity, primes the ability to generate reactive oxidants, and selectively stimulates protein

biosynthesis; receptors for IFN-γ have not been characterised in detail. GM-CSF and its receptor prime the ability of mature neutrophils to generate oxidant secretions within cell suspensions when exposed to a second stimulus, and primed neutrophils generate greater levels of oxidants compared to non-primed neutrophils. However, adherent neutrophils (e.g. to a plastic surface) can be activated by GM-CSF alone. GM-CSF is initially a chemotactic for neutrophils, but after approximately 30 minutes of exposure chemotaxis is inhibited, probably in order to attract neutrophils to the site of GM-CSF production and then to immobilise them to the site of infection. The mechanism by which GM-CSF activates neutrophils has only partially been elucidated. GM-CSF receptors are coupled to G-proteins, and the activities of phospholipases A2 and D are necessary for the up-regulation of receptor expression and the priming of oxidase. G-CSF is a neutrophil-specific cytokine that enhances phagocytosis, primes the respiratory burst and membrane depolarisation upon subsequent stimulation, and also augments antibody-dependent cellular cytotoxicity of neutrophils towards tumour cells. By itself, it does not activate a respiratory burst in suspended neutrophils but instead primes oxidant production in response to stimulation by fMet-Leu-Phe. In contrast, G-CSF alone stimulates reactive oxidant production in adherent cells, and also increases the expression of CD11b (C3bi receptor) with a concomitant increase in the adhesion of neutrophils to surfaces. In addition, it can up-regulate the affinity of the neutrophil-endothelium 'homing' receptor leukocyte adhesion molecule-1 (LAM-1) without changing the expression of LAM-1 on the neutrophil surface. G-CSF induces the expression and secretion of alpha-interferon, which inhibits the formation of granulocyte colonies from progenitor cells as part of a feedback mechanism to regulate neutrophil formation at high levels of G-CSF.

1.1.7 Neutrophils and Cancer

Neutrophils are well-known 'fighters' against non-self, which in theory should include tumours harbouring altered genes, yet the anti-tumoural role of neutrophils remains controversial. While many factors have been argued by some to destroy cancer cells and can be used in anti-cancer therapy, others have advocated that many of the same factors are in fact promoting malignancy, angiogenesis (the development of new blood vessels from existing ones to feed a tumour site), and even metastasis, including many of the defence mechanisms which are used by neutrophils (Di Carlo et al., 2001). The concept of immunesurveillance claims that members of the immune system can detect tumour precursors and eliminate them before clinical symptoms become apparent. However, it is becoming increasingly likely now that in their diligent battle against the non-self, neutrophils may promote malignancy progression, albeit involuntarily in the case of tumourigenesis. The reaction of neutrophils towards tumours was found to be similar in many ways to the inflammatory response and wound healing process (Coussens and Werb, 2002). It has been argued by some (De Larco et al., 2004) that this similarity in behaviour could be due to the release of IL-8. IL-8 is one of the cytokines released at assault sites where it recruits neutrophils to the site for defence against microbes, and the release of IL-8 enhances the rate of tissue repair by supplying healing wounds with blood vessels. However, IL-8 has been found to be expressed by highly metastatic tumour cells, such as breast tumours (Green et al., 1997). In addition, IL-8 has even been suggested to be an angiogenic factor (Koch et al., 1992), as neutrophils respond to IL-8 by remodelling the extracellular matrix (ECM) through the release of proteases (i.e. matrix metalloproteinase, MMPs) and heparanase that hydrolyses ECM components, thereby creating channels to facilitate migration (De Larco et al., 2004) This remodelling of the ECM by neutrophils causes the release of growth factors, such as basic fibroblast growth

factor, which is a well-established potent angiogenic factor (Vlodavsky et al., 1991), and new blood vessels, which are generated through the pre-established channels by neutrophils. At the same time, remodelling of the ECM by neutrophils is also suggested to increase metastasis, as the released MMPs weaken cell-to-cell interactions and tumour cells become more inclined to leave the main tumour mass (Shamamian et al., 2001). However, the release of IL-8 cannot be the sole reason for tumour progression and metastasis, and it is more likely to be one of many phenotypic alterations acting in concert. One of the phenotypic features that implicate neutrophils in the progression of cancer is the release of reactive oxygen species (ROS) by neutrophils. The recognition of the association between chronic inflammation in different diseases and malignancy development is not new and has been documented since the 19th century (Balkwill and Mantovani, 2001; Kim and Karin, 2011). This relationship has been linked to the release of free radicals (i.e. ROS) as a by-product of the phagocytic activity of neutrophils alongside other phagocytes during inflammation. ROS are produced by NADPH oxidase and myeloperoxidase to give the final product hypochlorous acid (HOCl), a potent bactericidal agent (Rosen et al., 2002) and also a modifier of several ECM proteins (Vissers and Thomas, 1997). This modification decreases cell-matrix interactions, which may increase the tendency of tumour cells to stray away from the primary tumour (Di Carlo et al., 2001). It has also been demonstrated by many studies that the release of ROS has the potential to cause DNA damage and/or alteration (Haqqani et al., 2000; Sandhu et al., 2000), which means an increase in the mutational rate of cells undergoing malignancy (De Larco et al., 2004).

1.2 CTCF-Like Transcription Factor

1.2.1 Background

CTCF-like (CTCFL) is a transcription factor and a cancer testis antigen (CTA) which is a paralogue (homologue) of the 11-Zinc finger (ZF) transcription factor; CCTC-binding factor (CTCF) (Loukinov et al., 2002; Scanlan et al., 2004). It is also widely known as the Brother Of the Regulator of the Imprinted Site (BORIS), but throughout this thesis it will be referred to as CTCFL. Unlike CTCF which is expressed in all soma (Ohlsson, 2001), CTCFL expression has been found to be restricted to a subpopulation of spermatocytes within the testis (Lobanenkov et al., 1990; Loukinov et al., 2002). However, both CTCFL and CTCF have been found be conserved evolutionarily from Drosophila to humans (Klenova et al., 2002) While the mammalian CTCF is involved in several functions in soma, including transcription activation, repression and gene silencing (Bell et al., 1999), CTCFL is believed to be involved in epigenetic reprogramming of the male germ line (Loukinov et al., 2002). Thus, CTCFL has been suggested to be a pro-proliferative factor, while CTCF has been suggested to possess anti-proliferative activity.

1.2.2 The Gene Encoding CTCFL

The DNA sequence of the human *CTCFL* gene consists of 27,931 base pairs (bp) and 11 exons (Accession NC_000020), with 566 single nucleotide polymorphism (SNPs) reported by the National Centre for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/snp). The chromosomal locus for the *CTCFL* gene was discovered using fluorescent *in situ* hybridisation (FISH) at 20q13.2 (Loukinov et al., 2002). Comparing the *CTCFL* chromosomal locus with the human genome paralogy map (Popovici et al., 2001) has revealed it to be paralogous to chromosomal location 16q22.2, which is the *CTCF* gene. Furthermore, homology analysis of whole exon-intron structure

of *CTCFL* and *CTCF* has shown a remarkable similarity in the exons encoding the 11-ZFs in both genes. These findings suggested that both *CTCFL* and *CTCF* are descended from a common ancestral gene, and *CTCFL* has been generated by a gene duplication event (Klenova et al., 2002). The transcription of human *CTCFL* has been demonstrated to be regulated by three promoters, A, B and C. Promoter A is located at -2071 to 1276 bp, promoter B at -1106 to -996 bp, and promoter C at -821 to -622 bp upstream of the start site (Renaud et al., 2007). All three promoters have putative binding sites for SP1 and AP-2 transcription factors. In addition, promoter A was found to have putative binding sites for CREB, promoter B for NF-κB and N-myc, and promoter C for WT1 and EKLF (Renaud et al., 2007). Transcription of the *CTCFL* gene starts at 1447, 899 and 658 upstream of the first ATG sequence, and recently, 23 transcript variants of the *CTCFL* gene have been shown to encode for 17 different CTCFL protein isoforms (Pugacheva et al., 2010).

1.2.3 CTCFL Protein

The molecular weight of the CTCFL protein ranges between 75.5 kDa (Uniprot) and 83.3 kDa, and the protein sequence consists of 633 amino acids (aa). CTCFL belongs to the zinc-finger protein family, and has 11 zinc fingers (10 C_2H_2 zinc fingers and 1 C_2HC zinc finger) centred between the N-terminus and the C-terminus. Studying the protein sequence homology of CTCFL and CTCF demonstrated homology between the 11-zinc fingers between both proteins following optimal alignment of their aa sequences in humans, including all the major DNA base recognition residues at positions 1, 2, 3 and 6 within each finger (Loukinov et al., 2002). In contrast, the carboxy- and amino-termini which flank the 11-zinc finger region in CTCFL and CTCF are different and show no homology (Klenova et al., 2002). The similarity of zinc fingers in the CTCFL and CTCF proteins and the divergence in the flanking regions suggests that while both CTCFL and

CTCF recognise and bind to the same DNA-binding sites, they will result in different functional consequences (Klenova et al., 2002). Spatial configuration of the CTCFL protein is yet to be confirmed, although initial investigations using computer analysis predictions of the C-terminus reveals it to be disordered, i.e. lacking a 3D structure, and the same configuration has been deduced for the N-terminus (Campbell et al., 2010). The full length of the CTCFL protein, including the C- and the N-termini, has been shown to interact with PRMT-7 and Histone 1, 3 and 2A (Jelinic, 2006b). Furthermore, 16 different proteins have been found to date which exclusively interact with the N-terminus of CTCFL. These proteins are mainly classified as testis specific, helicase associated transcription factors, and chromatin associated proteins (Nguyen et al., 2008).

1.2.4 Role of CTCFL in Epigenetic Reprogramming

Epigenetic reprogramming occurs during the early stages of embryo development. Erasure of methylation marks takes place for all imprinted alleles, regardless of parental origin (Nakagawa et al., 2001). CTCFL expression has been found to be strictly testis-specific to the population of spermatocytes of humans and mice (Loukinov et al., 2002). Using immunostaining of mouse testes sections, CTCFL-positive spermatocytes that re-establish paternal DNA-methylation patterns and display genome-wide DNA-demethylation were found to be negative for CTCF (Loukinov et al., 2002). The first detectable expression of CTCFL is 14.5 days post coitum (dpc) in mitotically arrested gonocytes (Jelinic, 2006a). At later stages of spermatogenesis, CTCFL is silenced while CTCF is re-activated. CTCFL and CTCF expression patterns overlap very little and potentially not at all (Klenova et al., 2002; Loukinov et al., 2002). The up-regulation of CTCFL in CTCF-negative cells and of CTCF in CTCFL -negative cells occurs in association with erasure and re-establishment of methylation marks (Loukinov et al., 2002). A proposed mechanism for CTCFL re-establishment of male imprinting was

demonstrated in the case of the well-studied imprinted locus IGF/H19 (Jelinic, 2006b) and suggests that a regional marking (histone methylation) in the H19 Imprinting Control Region (ICR) of chromatin (histone H2A and H4) takes place by binding of CTCFL to the ICR and the recruitment of protein arginine methyltransferase (PRMT7), with the subsequent histone methylation responsible for *de novo* recruitment of DNA methyltransferases Dnmt3a and Dnmt3L (Jelinic, 2006a) (Figure 1.4).

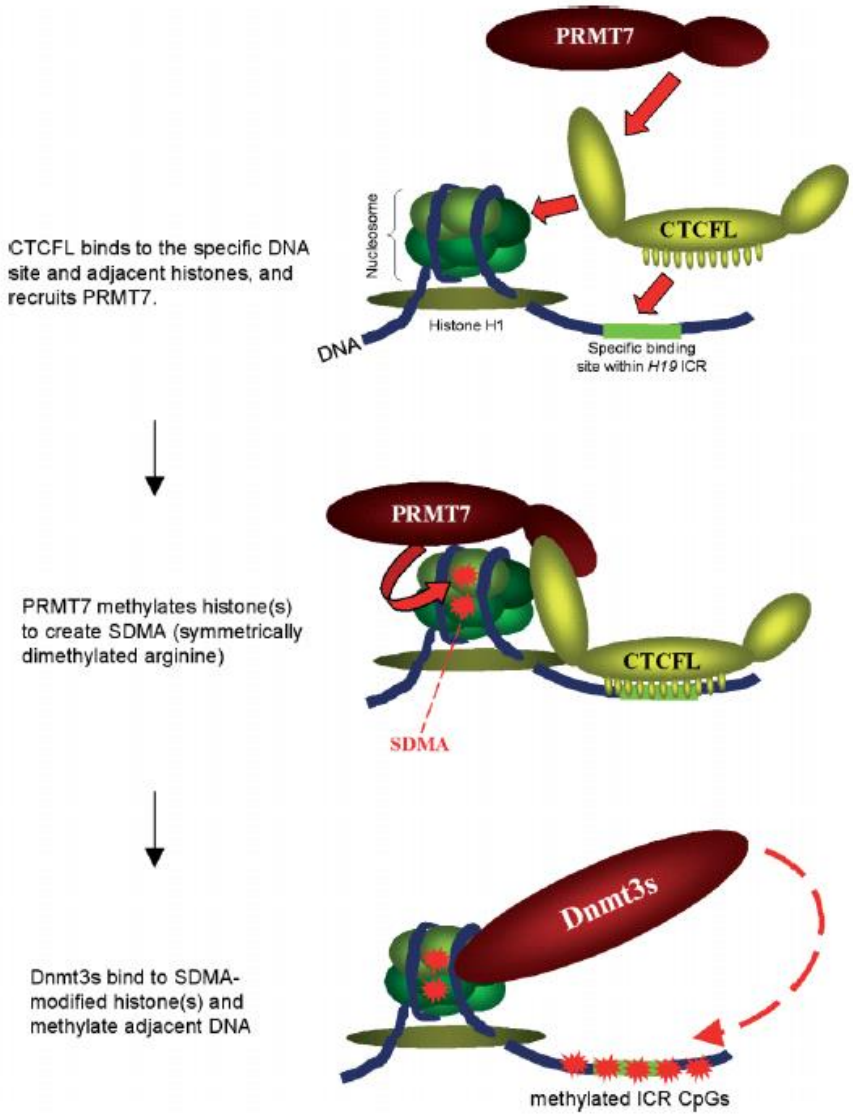

Figure 1.4: Proposed Model for the role of CTCFL in the establishment of male germ line imprinting marks

Histones are illustrated here as green spheres, while CTCFL zinc fingers are indicated as projections which recognise specific sequences within the *H19* ICR. Methylation of adjacent histones is indicated by red stars. The methylation of cytosine within the dinucleotide sequence CpG is indicated by red stars within the indicated ICR and the DNA strand is indicated in blue. SDMA = symmetrical dimethylated arginine; PRMT7 = protein arginine methyltransferase7; Dnmt3s = de novo methyltransferases 3a, b, and L (Jelinic, 2006b).

1.2.5 CTCFL and Breast Cancer

CTCFL is designated a member of the CTA family, and members normally demonstrate restricted expression within the testis although expression has also been shown to be activated in the soma of many types of cancer (Scanlan et al., 2004). While many of the CTA genes are located on the X-chromosome, some are not and these are known as non-X CTAs (Simpson et al., 2005). The functions of these CTAs are not fully understood although the expression of many of these genes has been found to coincide with meiosis (Simpson et al., 2005). CTCFL has been shown to be a downstream regulator for other CTA genes, as its expression leads to the demethylation and derepression of the CTAs MAGE-1 and NYESO-1 (Hong et al., 2005; Vatolin et al., 2005). Furthermore, the chromosomal localisation of CTCFL at 20q13.2 raised the suspicion of it being linked to cancer, as this chromosomal region is known for its amplification or gain of material in many human cancers and has always been suspected as hosting a transforming or immortalising gene or genes (Collins et al., 2001; Tanner et al., 1994). Indeed, CTCFL has been found to be activated abnormally in many types of cancer cell lines, as well as different types of tumours including female cancers (Hong et al., 2005; Loukinov et al., 2002; Vatolin et al., 2005). It has been reported that some types of cancer are manifested by a loss of imprinting (LOI), whereby a silent copy of a gene may be activated or vice versa due an epigenetic mis-regulation (Moulton et al., 1994; Randhawa et al., 1998). Given the proposed model of CTCFL action, this may raise the possibility that CTCFL, in conjunction with PRMT7, may be responsible for the methylation of the non-methylated maternal H19 ICR allele. This would result in biallelic ICR methylation and consequently LOI (Jelinic, 2006a). A recent investigation of CTCFL expression in 18 breast cancer cell lines, as well as primary breast tumours, has confirmed its aberrant expression in breast cancer (D'Arcy et al., 2008). Interestingly, CTCFL expression has

also been detected in PMNs of breast cancer patients (D'Arcy et al., 2006). CTCFL expression levels in PMNs were found to be elevated as the size of tumours increased in the studied breast cancer patients (D'Arcy et al., 2006). In addition, levels of CTCFL expression were found to positively correlate with levels of progesterone receptor (PR) and oestrogen receptor (ER); both are biomarkers used in therapy determination for breast cancer patients.

1.3 Overview of Breast Cancer

1.3.1 Background

Breast cancer is the most common cancer worldwide in women, and the second most common cancer overall, with more than 1,676,000 new cases diagnosed in 2012 (25% of all female cancer cases and 12% of the all cancer cases). Global statistics suggest that the incidence rate of breast cancer is highest in Western Europe and lowest in Middle Africa (Ferlay et al., 2014). In the UK, breast cancer has been the most common cancer in the UK since 1997, despite it occurring less frequently in men than women. Furthermore, breast cancer accounts for 30% of all new cases of cancer in females in the UK (Cancer Research UK, 2014). Breast cancer is recognised as a genetic disease which is initiated by the accumulation of genetic and epigenetic alterations. The existence of a familial history increases the risk by a factor of two or three. Aetiologically, there are several factors along with the genetic susceptibility of the individual which contribute to the initiation of breast cancer, including gender, age, exposure to pollutants and radiation, exposure to exogenous and endogenous hormones, lifestyle and dietary habits (Huang and Davidson, 2006). Despite many new advances in the treatment of breast cancer, the early detection of a tumour is still considered the cornerstone in the control of breast cancer and survival (Anderson et al., 2008).

1.3.2 Histopathology of Neoplastic Breast Tissue

Breast cancer tissue can be categorised according to its histological features and the extent of its spread into the breast tissue. *In situ* (non-invasive) breast cancer refers to a cancer where the cells have remained within their original area and have not spread to the breast tissue around a duct or lobule. The most common type of non-invasive breast cancer is ductal carcinoma *in situ* (DCIS), which is confined to the lining of the milk ducts, and the abnormal cells do not spread through the duct walls into the surrounding breast tissue (Charpentier and Aldaz, 2002; Huang and Davidson, 2006). In contrast, invasive (infiltrating) breast cancers spread outside the membrane that lines a duct or lobule, and invades the surrounding tissues. The cancer cells at this stage have a higher tendency for detachment and metastasis to other organs in the body (e.g. lymph nodes, lungs and bone marrow), travelling through the lymphatic system or bloodstream. An invasive carcinoma could commence invasion either from the milk ducts then break through the ductal walls and invade nearby breast tissue (invasive ductal carcinoma, IDC), or start from milk-producing lobules and then invade nearby breast tissue (invasive lobular carcinoma, ILC). More than 70% of all breast cancer cases are IDC. Less common types of cancer include forms which are neither lobular nor ductal and which originate from the fibrous supporting tissue, adjacent lymphatic nodes, or blood vessels. A cancer may also arise from another cancer metastasised to the breast tissue (Charpentier and Aldaz, 2002; Huang and Davidson, 2006).

1.3.3 Diagnosis and Prognosis of Breast Cancer

Today, the establishment of a diagnosis for breast cancer is acquired via the collaboration of multiple assessments, including a clinical examination (physical inspection), ultrasound and/or mammogram, and the histopathological assessment of a biopsy

obtained from a suspected tumour (Mincey and Perez, 2004). Clinical examination also takes into consideration the general health and menopausal status of a patient. Breast cancer cases are classified using the TNM staging system with respect to the size of the tumour (T), the status of cancer cells spread to lymph nodes (N), and the occurrence of metastasis to other organs (M). Cancers with similar stages usually have a similar prognosis and often have similar therapy plans. Breast cancer cases are grouped under the breast cancer stage grouping system based on information provided using the TNM system. The stage groups are expressed in Roman numerals from stage I (the least advanced stage) to stage IV (the most advanced stage), whilst non-invasive cancer is listed as stage 0 (National Cancer Institute, 2014). The histopathological examination also grades the extent or lack of tumour cells differentiation in comparison to normal breast tissue and the rate of growth, as this reflects the aggressiveness of a tumour and its tendency to expand and metastasise. A well-differentiated cell with close resemblance to normal breast tissue indicates a low grade tumour cell with a good prognosis for the patient, while a low level of differentiation indicates the opposite. Specific tumour markers can be used to profile an examined tumour biopsy and is a very important tool in the determination of a patient's prognosis and therapy plan. The main clinical markers currently used in breast cancer prognosis are the ER, PR and human epidermal growth factor receptor 2 (HER2/neu) (Duffy and Crown, 2008; Molina et al., 2005). HER2/neu positive breast tumours tend to grow faster and have a higher recurrence than HER2/neu negative tumours. A positive receptor assay suggests a better prognosis in terms of responding to treatments, as different and highly effective therapy plans directed against these receptors are available. In contrast, a triple-negative breast cancer (TNBC) is a breast tumour that tests negative for all three receptors. TNBC has a more difficult prognosis as it is insensitive to some of the most effective therapies available today, such

as hormone therapy, and requires a targeted therapy plan or a therapeutic trial (National Cancer Institute, 2014).

1.3.4 Treatment

Based on the stage of a tumour a therapy plan is recommended to a patient. Usually, therapy requires a combination of different treatment approaches, such as surgery, radiation therapy, chemotherapy, and hormone therapy or targeted therapy. Treatment of the disease aims to remove all tumour cells from a patient and avoid reoccurrence of the cancer or metastasis.

1.3.4.1 Surgery and Radiation

Most breast cancer cases have surgery employed at some point in order to remove a tumour from the breast. Breast cancer surgery either involves removal of a tumour and some of the surrounding normal tissue but not the breast itself (breast conserving surgery), or total removal of the breast with the tumour (total or simple mastectomy). Some lymph nodes may also be removed as a biopsy for histopathological assessment (lymph nodes dissection). Radiation administered to the breast is often given after breast-conserving surgery to help lower the chance that the cancer will return in the breast or the nearby lymph nodes. Radiation may also be recommended after a mastectomy in patients either with a cancer larger than 5 cm or when cancer is found in the lymph nodes. In addition, radiation is used to treat cancer that has spread to other areas, for example to the bones or brain (National Cancer Institute, 2014).

1.3.4.2 Chemotherapy

Chemotherapy employs cytotoxic drugs to kill cancerous cells in a systemic manner (affecting the entire body). Unfortunately, these drugs also affect non-cancerous cells and result in severe side effects. Consequently, chemotherapy needs to be carefully tailored to

the type of breast cancer and state of cancer cells in the lymph nodes in order to obtain a maximum recovery for a patient with fewer side effects. Chemotherapy is prescribed to advanced breast cancer cases and may also be prescribed to patients in the early stages of the disease. A number of chemotherapeutic agents are used in the treatment of breast cancer, including alkylating agents, antimetabolites, cytotoxic antibiotics, anti-microtubule agents and topoisomerase inhibitors. In many cases, chemotherapy drugs are more effective when given in combination, especially during the early stages. For example, anthracyclines, such as doxorubicin (antibiotics), and taxanes, such as docetaxel (an antimitotic drug), are given with certain other drugs, such as fluorouracil (5-FU) (antimetabolite) and cyclophosphamide (alkylating agent). Chemotherapy may be given to a patient prior to surgery (neoadjuvant therapy) in order to decrease the size of a tumour or restrict its growth to other areas of the breast thereby leading to less extensive surgery. However, chemotherapy may be prescribed to patients after total removal of a tumour by surgery (adjuvant therapy) to kill any remaining cancerous cells that went undetected and to ensure that there is no reoccurrence of the cancer. In this situation chemotherapy is administered to the patient in cycles, where a period of treatment is followed by a resting period in order for the body to recover from the medication side effects and before being repeated for a number of times.

1.3.4.3 Hormone Therapy

Hormonal therapy depends on a patients profile for ERs and PRs, as patients lacking hormone receptors (ER-negative and PR-negative) cannot benefit from this form of treatment. The statistics suggests that around 75% of breast cancer patients are oestrogen receptor positive (ER-positive) (Allred et al., 2004). Hormone therapy aims to stop the hormone oestrogen from stimulating the growth of breast cancer cells. Currently available hormone therapy medications work either by blocking the oestrogen hormone receptors

existing on the breast cancer cells or by suppressing the production of oestrogen in the body. Another approach to hormonal treatment involves the surgical removal of both ovaries (ovaries ablation), which stops oestrogen production completely. Patients with tumour cells positive to one or both receptors have a better prognosis and usually respond well to treatment. Ovaries ablation can also be achieved using chemical agents, in particular, drugs which are luteinising hormone-releasing hormone (LHRH) analogues, such as Goserelin or leuprolide. One of the most widely used standard drugs in hormone therapy is Tamoxifen, a drug which belongs to a class of chemicals known as selective oestrogen receptor modulators (SERMs), which resemble oestrogen in structure but do not perform its function within mammary cells (National Cancer Institute, 2014). SERMs compete with oestrogen molecules to bind to oestrogen receptors and thus intercept oestrogen from stimulating cancerous cell proliferation in the breast. Tamoxifen is often given to premenopausal patients with positive hormone receptors profile (hormone sensitive). Tamoxifen is suitable for use by premenopausal patients at all stages, mainly as an adjuvant therapy. It is prescribed to patients in the early stages of breast cancer and also to those with advanced and metastatic breast cancer, where cancer has spread to other parts of the body. Postmenopausal women are usually prescribed a different class of hormone therapy drugs known as aromatase inhibitors. Aromatase is an enzyme that turns small amounts of androgen into oestrogen in postmenopausal women. Aromatase inhibitors block the function of this enzyme and thus less oestrogen is produced in the body that would stimulate ER+ breast cancer cells. Aromatase inhibitor is prescribed only to postmenopausal women as it has little or no effect on premenopausal women where normal oestrogen levels continue to be produced by the ovaries.

1.3.4.4 Targeted Therapy

Advances in current knowledge of genetic and epigenetic alterations in cancer cells has led to the development of newer therapeutic agents which specifically target tumour markers; molecules which exist or are overexpressed only on cancerous cells and not normal cells and thus interfere with cancer progression. These drugs deliver a more effective treatment against cancer with side effects that are less severe or harmful to a patient. Targeted therapy is usually prescribed to patients who have breast cancers overexpressing the growth factor protein HER2 (HER2-positive breast tumour). HER2-positive tumours have a higher recurrence and metastatic rates than HER2-negative breast tumours, and around 20-25% of breast cancer patients have been found to be HER2-positive (Slamon et al., 2011; Slamon et al., 1987). Targeted therapy is also used in the treatment of triple-negative breast cancer (TNBC), which is insensitive to conventional treatment. Currently, targeted therapy uses two approaches; monoclonal antibodies to detect and target biomarkers which exist on the surface of a cancer cell, and small molecule targeting of biomarkers which exist inside a cancer cell where larger molecules, such as antibodies, are not able to penetrate. Targeted therapies with small molecules include gene expression modulators, apoptosis inducers, angiogenesis inhibitors, and signal transduction inhibitors, whilst monoclonal antibody therapy targeting HER2-positive tumours include drugs such as Trastuzumab (Herceptin) (Slamon et al., 2011) and Pertuzumab (Perjeta®). These are used together with chemotherapy as an adjuvant therapy and also for neoadjuvant therapy in the case of Pertuzumab. Another monoclonal antibody is Ado-trastuzumab, which is conjugated with an anti-cancer drug and is used to treat metastatic and recurring HER2-positive breast tumours. Small molecules used in targeted therapy include drugs such as Lapatinib, which is a tyrosine kinase inhibitor and may also be used in the treatment of HER2-positive breast cancer. Another small

molecule agent used in targeted therapy is PARP inhibitors which block DNA repair in cancer cells eventually causing their death, and is used for treating TNBC cases (National Cancer Institute, 2014).

1.4 Aims of the Investigation

The vital role of transcription factors in regulating many aspects of the physiology and function of PMNs is well established. Indeed, more transcription factors have been recognised in recent years to be involved in regulating differentiation, apoptosis, mobility, the production of cytokines, and the expression of surface receptors in PMNs. Understanding the expression and interactions of transcription factors in PMNs, as well as their influence on the immunological function of PMNs, continues to be an important area of research, and many questions remain to be answered. For example, does the expression of certain transcription factors in PMNs affect or even enhance their anti-tumoural role?

One of the more recently discovered transcription factor in PMNs is the transcription factor CTCFL. Investigating the existence and function of this transcription factor in different cell types has been the subject of a few studies but has produced contrasting results. Earlier studies suggested the exclusive expression of CTCFL in the testis and its function in *de novo* DNA methylation within the spermatocyte population (Klenova et al., 2002; Loukinov et al., 2002). Subsequently, CTCFL has been detected in several types of cancers including ovarian, prostate and breast cancer (D'Arcy et al., 2006; D'Arcy et al., 2008; Klenova et al., 2002). Interestingly, CTCFL was observed in PMNs collected from breast cancer patients and thus it has been suggested to be a potential marker for diagnosis/prognosis, as well as a therapy for breast cancer (D'Arcy et al., 2006). In contrast, a recent study proposed that CTCFL was widely expressed in normal soma as

well as cancer cells (Jones et al., 2011). Several questions need to be answered in order to understand the expression of the CTCFL transcription factor in PMNs. For example, as CTCFL can bind to the same DNA-binding domains as its ubiquitous paralogue CTCF, but exerts different cellular effects (Klenova et al., 2002; Phillips and Corces, 2009), it would be interesting to determine the different functions that CTCFL may exert in different cell types upon binding to these domains. This investigation focuses on the expression of CTCFL in PMNs, where it could be involved in certain activities in PMNs related to their differentiation, survival or apoptosis. CTCFL may also be regulating PMNs function in immunity, either directly or indirectly. Two hypotheses were proposed by D'Arcy et al. (2006) concerning the origin of CTCFL expression in the PMNs of breast cancer patients. The first hypothesis suggested that CTCFL was actively induced in PMNs by exogenous factors released from tumours cells and/or other cells during cancer (activated by signalling), whilst the second hypothesis suggested that CTCFL levels are elevated passively in PMNs due to the engulfment (phagocytosis) of the CTCFL-positive tumour cells by PMNs.

In summary, the aims of this project were as follows:

- To verify the levels of CTCFL expression in the PMNs of healthy individuals compared to levels in patients with breast cancer.
- To investigate the two hypotheses regarding the origin of CTCFL expression in PMNs.
- To investigate the levels and localisation of CTCFL expression in human PMNs during their differentiation.
- To assess the interaction of CTCFL with selected nuclear proteins involved in the differentiation of PMN and PML (promyelocytic leukaemia) nuclear bodies.

- To profile the function and immunophenotype of a PMN model which expresses CTCFL at high levels in comparison to the same model when CTCFL is down regulated due to differentiation.
- To manipulate CTCFL levels in a PMN model in order to be able to investigate the physiological and immunological alterations that may occur when CTCFL is overexpressed or when knocked-down in human PMNs.

Chapter 2 - **Materials and Methods**

2.1 Antibodies

A number of antibodies were used as probes to target specific epitopes expressed on various molecular structures (Table 2.1). These included antibodies specific to CTCFL, PML and the innate immune system recognition molecules, TLR2, TLR4 and CD14. The panel also included antibodies against the major histocompatibility class I, HLA-ABC, and class II, HLA-DR. Most primary antibodies were used in an indirect immunofluorescence staining protocol. Consequently, biotinylated or fluorophore-conjugated secondary antibodies were required to be used against the targeted primary antibodies (Table 2.2). These included goat FITC-conjugated anti-mouse against CTCFL and swine TRITC-conjugated anti-rabbit against human PML. Immunocytochemical (ICC) staining for CTCF required biotinylated IgM as the secondary antibody. In addition, experimental controls (Table 2.3) were used to minimise the cross-reactivity of non-specific staining. An IgM isotype control was used alongside ICC staining against CTCFL and β-actin to confirm equal sample loading during protein gel electrophoresis.

Table 2.1: Primary Antibodies
ICC – immunocyctochemical, IF – immunofluorescence, WB – Western blot, FCM – flow cytometry

Specificity	Clone	Raised in	Clonality	Isotype	Company/Lab	Assay	Titration
Human BORIS	4A7	Mouse	Monoclonal	IgM	Prof. Klenova	ICC/ IF	1:50
Human BORIS	4A7	Mouse	Monoclonal	IgM	Prof. Klenova	WB	1:500
Human PML	PML-97	Rabbit	Polyclonal	IgG	AbCam	IF	1:200
Human TLR2	TL2.1	Mouse	Monoclonal	IgG2a	Santa Cruz	FCM	100µg/ml
Human TLR4	76B357.1	Mouse	Monoclonal	IgG2b	Santa Cruz	FCM	200µg/ml
Human CD14	MEM-15	Mouse	Monoclonal	IgG1	ImmunoTools	FCM	100µg/ml
Human HLA-ABC	W6/32	Mouse	Monoclonal	IgG2a	ImmunoTools	FCM	25µg/ml
Human HLA-DR	HL-39	Mouse	Monoclonal	IgG3	ImmunoTools	FCM	50µg/ml

Table 2.2: Secondary Antibodies
ICC – immunocyctochemical, IF – immunofluorescence, WB – Western blot

Specificity	Presentation	Raised in	Clonality	Isotype	Source	Assay	Titration
Rabbit IgG	TRITC-Conjugated	Swine	Polyclonal	IgG	DAKO	IF	1:100
Mouse IgG	TRITC-Conjugated	Rabbit	Polyclonal	IgG	DAKO	IF	1:100
Mouse IgG	FITC-Conjugated	Goat	Polyclonal	IgG	Santa Cruz	IF	1:200
Mouse IgM	Biotinylated	Goat	Monoclonal	IgM	Vector labs	ICC	1:200
Mouse IgG	FITC-Conjugated	Rabbit	Polyclonal	IgG	Li-COR	WB	1:15000

Table 2.3: Control antibodies
ICC – immunocyctochemical, WB – Western blot

Specificity	Clone	Raised in	Clonality	Isotype	Source	Assay	Titration
Human IgM	PFR-03	Mouse	Monoclonal	IgM	ImmunoTools	ICC	1:100
Human β-actin	AC-15	Mouse	Monoclonal	IgG1	Sigma Aldrich	WB	1:15000

2.2 Patient Samples and Controls

2.2.1 Blood Sample Collection

Ethical approval was obtained from Colchester General Hospital for the collection of peripheral blood samples. Whole blood samples were obtained from cancer patients and healthy individuals with no familial history of breast malignancies. The samples were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes (Vacutainer®) and paired with a sample collected in serum-separation tubes (off-the-clot serum) for each donor and patient. EDTA samples were processed for granulocytes fraction isolation within 48 hours.

2.2.2 Isolation of Human Peripheral Granulocytes

Whole blood samples were fractionated via a buoyancy density method using Histopaque 1119-1 (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 7 ml of whole blood was layered over 5 ml of Histopaque 1119-1 and subjected to centrifugation at 2500 rpm for 40 minutes. Buffy coat was collected and washed three times with 10 ml of ice cold phosphate buffered saline (PBS) (Oxoid LTD, Hampshire, England), with the cells collected by centrifugation at 1500 rpm in a 15 ml falcon tube. The collected granulocytes were used immediately, as the viability of granulocytes has been shown to deteriorate drastically when frozen then thawed in optimisation studies for the culture of granulocytes.

2.2.3 Human Serum

Whole blood samples were paired with serum samples from the same patients or donors in clot-activator tubes (Vacutainer®). After clot formation the tubes were subjected to centrifugation at 2000 rpm for 5 minutes. Serum was collected and either used directly or

stored at -80°C in 15 ml falcon tubes. The sera from patients or donors were never pooled in any of the experiments performed in this investigation and each sample was used separately in the experiments.

2.3 Mammalian Cell Culture

2.3.1 Neutrophilic Granulocyte Cell Line Model

The NB4 cell line (DSMZ ACC-207) was used as a model for neutrophilic granulocytes. This cell line grows in suspension where the cells exhibit a round, polymorphic morphology. The NB4 cell line was established from a 23-year old woman with human acute promyelocytic leukaemia (APL = AML FAB M3) during her second relapse in 1989 (Lanotte et al., 1991). The cells harbour the t(15;17) (q22; q11-12) translocation in which the fusion oncoprotein PML-RAR-α is expressed (Melnick and Licht, 1999; Warrell et al., 1993). The NB4 cell line can be induced to differentiate into neutrophilic granulocytes using All-Trans-Retinoic Acid (ATRA) (Lanotte et al., 1991), (see Section 2.3.5).

2.3.2 Breast Cancer Cell Line Model

The CAMA1 cell line (ATCC No. HTB-21) served as a model for metastatic breast adenocarcinoma. CAMA1 is an adherent cell line which was established from the pleural effusion of a 51-year-old female patient with breast carcinoma (Fogh et al., 1977). The luminal epithelial-like cell line is human epidermal growth receptor 2 (Her2) negative, progesterone receptor / estrogen receptor (PR/ER) positive, and expresses has no mutations in *BRCA1* or *BRCA2* genes (Lacroix and Leclercq, 2004; Riaz et al., 2009; Saal et al., 2008). The CAMA-1 cell line has oncogenic mutations in PTEN and p53 genes, as well as an amplification of cyclin D1 gene (Lundgren et al., 2008; Saal et al., 2008;

Wasielewski et al., 2006). Also, the CAMA-1 cell line has a mutation in the E-cadherin gene which produces a non-functional protein (van Horssen et al., 2012).

2.3.3 Cell Line Passaging and Maintenance

2.3.3.1 Suspension Cells

NB4 cells were cultured in RPMI-1640 media (PAA Laboratories GmbH, Austria) complemented with 10% foetal bovine serum (FBS) (PAA Laboratories GmbH, Austria) and 50 µg/ml gentamycin (PAA Laboratories GmbH, Austria). NB4 cells were maintained in culture at a density of 2.5-3.0 × 10^6 cell/ml and at a sub-cultivation ratio of 1:3, which was performed every 37-48 hours.

2.3.3.2 Adherent Cells

For the adherent cell line CAMA1 which produced a confluent monolayer, an additional step of cell detachment for passaging was performed. After removing the culture media the cells were washed once with PBS and then incubated with 1-2 ml of Accutase™ enzyme (PAA Laboratories GmbH, Austria) for 5 minutes at 37°C. The detachment enzyme was then deactivated by adding 5 ml of fresh media. The sub-cultivation ratio of cells was 1:3–1:4 and the growth media volume was made up to 18 ml with fresh media. Cells were passaged 2-3 times per week. The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

The adherent HEK293T cell line, which is a human embryonic kidney cell line, was used as a positive control only in the specific optimisation experiments presented in Chapter 5, and these were maintained in the same manner as CAMA1 cell line.

2.3.4 Cryopreservation and Thawing of Cell Lines

Both NB4 and CAMA1 cell lines were kept at -80°C or in liquid nitrogen for prolonged storage. For NB4 cells, 1×10^6 cells were collected and subjected to centrifugation at 1000 rpm for 3 minutes, before discarding the supernatant and re-suspending the cells in 1 ml of freezing medium (10% Dimethylsulfoxide (DMSO) in FBS) in cryotubes. The CAMA1 cell line required an additional step to detach the cells using Accutase™, and the collected cells were re-suspended in 1 ml of freezing medium and placed into cryotubes. The cryotubes were immediately placed in an isopropanol-filled freezing jar at -80°C (for gradual freezing, -1°C per min), and after 24 hours the cells were transferred to be stored in liquid nitrogen. To thaw the cryopreserved cells the cryotubes were quickly defrosted at 37°C in a water bath. The cells were then added to 10 ml of fresh media in a drop-wise manner and subjected to centrifugation at 1000 rpm for 3 minutes. The supernatant was removed and the cells gently re-suspended in 12 ml of pre-warmed fresh complete RPMI media (PAA Laboratories GmbH, Austria) and transferred into a 75 ml T-flask. The cells were incubated at 37°C in a humidified 5% CO_2 incubator.

2.3.5 NB4 Cell Line Differentiation

NB4 cell differentiation was induced using *All-Trans* Retinoic Acid (ATRA) (Sigma-Aldrich, St. Louis, MO, USA), and the number of NB4 cells was kept to under 1.5×10^6 cells/ml. The ATRA stock solution was prepared in DMSO to a final concentration of 10 mM. ATRA doses were added cumulatively for 3 – 5 days depending on experiment requirements to the NB4 cell culture at a final concentration of 1 mM every 24 hours. Differentiation was observed in NB4 cells after 3 days of the cumulative addition of ATRA doses.

2.3.6 Culture of Polymorphonuclear Neutrophils with Human Serum

Experiments were carried out with primary PMNs as well as the PMN cell line model, NB4. For the culture of primary PMNs the PMN fraction and serum samples were collected from 6 healthy donors as well as 6 breast cancer patients and processed as described above. Each serum sample was diluted in RPMI media at a concentration of 50 % to maintain the viability of the PMNs in culture. Equal numbers of PMNs from each healthy donor were divided between two wells; in the first well the PMNs were cultured with serum from the same healthy donor as the PMNs, and in the second well the healthy donor's PMNs were cultured with serum from a breast cancer patient. The total volume of RPMI media supplemented with the 50 % of the human serum was 3 ml. The same experimental design was applied to PMNs collected from breast cancer patients, such that the PMNs of each patient were divided into two wells, and in one well the PMNs were cultured with serum collected from the same patient and in the other well with serum of a healthy donor. PMNs were incubated for 72 hours under standard culture conditions and 2.5×10^4 cells were collected from each well for the preparation of smears.

For the culture of the PMN cell line model using NB4 cells, serum from one healthy donor and one breast cancer patient were diluted to a 50 % concentration in RPMI media. NB4 cells were seeded at 1×10^6 cells/well in a 6-well plate, with 3 ml of RPMI media supplemented with serum from a donor or a patient. The cells were incubated at 37°C and slides and/or aliquots were prepared after 24 hours of culture.

2.3.7 Co-culture of Polymorphonuclear Neutrophils with Breast Cancer Cells

For the co-culture of primary PMNs with the breast cancer cell line CAMA-1, two experiments were performed. In the first, 2.5×10^5 CAMA1 cells were seeded into a 6-well plate with 2 ml of standard complete RPMI media (10 % FBS). After incubation for

24 hours, 2.5 x 10^5 of the healthy donor PMNs were resuspended in 2 ml of standard complete media and were added to a single well containing CAMA-1 cells. The co-cultured cells were then incubated for 48 hours.

In the second experiment, PMNs collected from a healthy donor were co-cultured with CAMA-1 in Transwell inserts as previously described but with some modification (Berggård et al., 2007). Transwell inserts are chambers where a microporous base has been fitted to the upper half of a well, thereby allowing the co-culture of two different cell types within the same culture environment (Figure 2.1). In total, 2.5 × 10^5 CAMA-1 cells/well were seeded into a 6-well plate with 2 ml of standard complete RPMI media 24 hours prior to co-culture with the PMNs After this initial incubation 0.4 µm pore-size-Transwell chambers (Nalge Nunc, Penfield, New York, USA) was inserted into the wells and 2.5 x 10^5 PMNs from a healthy donor were resuspended in 2 ml of 10 % FBS supplemented RPMI media and seeded into the chambers. The cells were incubated for 48 hours before being harvested for assessment of CTCFL levels.

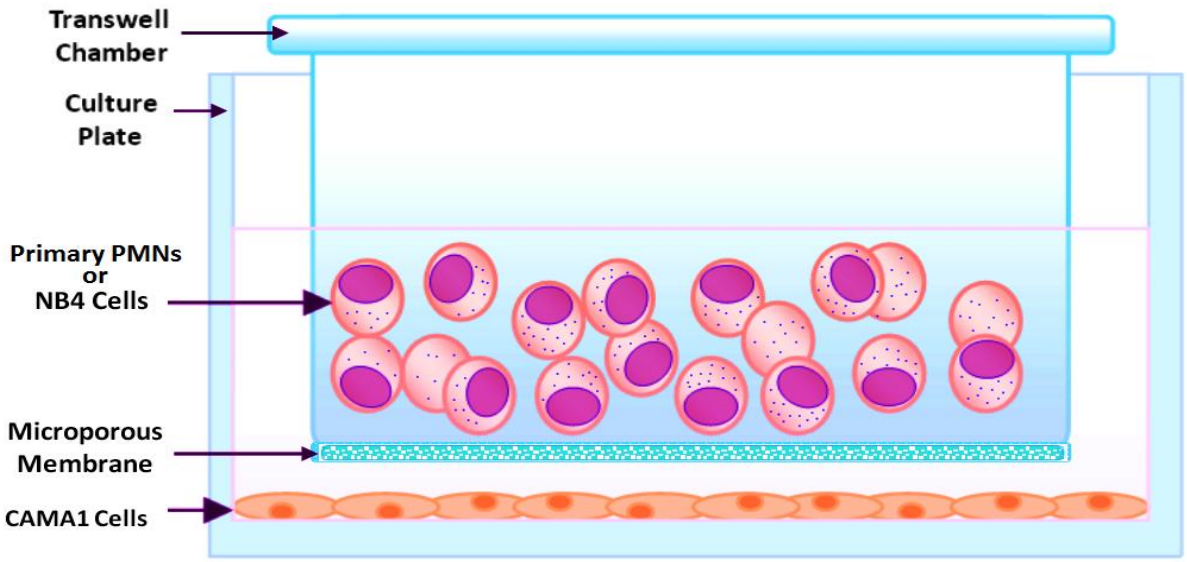

Figure 2.1: Co-culture of the breast cancer cell model (CAMA-1 cell line) with PMNs or NB4 cells using a Transwell chamber.

CAMA-1 cells were seeded into a 6-well plate at a density of 0.25×10^6 cells/ml and incubated for 24 hours. Transwell chambers were then placed into the wells containing the CAMA-1 cells, and an equal number of PMNS or NB4 cells added before incubating the two types of cells together for designated time periods.

For the co-culture of the NB4 cell line with the CAMA-1 cell line, 2.5×10^5 CAMA1 cells were seeded in each well of a 6-well plate with 3 ml of complete RPMI media. After 24 hours the Transwell chambers were placed into each well and 2.5×10^5 NB4 cells/ml were re-suspended in 6 ml of complete media and divided between the six Transwell chambers. The smallest size of pores in the Transwell chambers was selected to avoid NB4 cell migration. Both co-cultured NB4 and CAMA1 cells were incubated at 37°C for designated time intervals. The NB4 cells were collected from the chambers after 24 hours for further analysis.

2.4 Viability Tests

2.4.1 Trypan Blue Exclusion

Viability was assessed using a 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA. In total, 1 ml of NB4 cells were collected directly from a culture flask and were not washed prior to staining. The cells were stained by mixing equal volumes of the NB4 cell suspension and the trypan blue solution and incubated for a maximum of 2-3 minutes, before a 10 µl sample from the stained cell suspension was examined using a haemocytometer under a conventional light microscope. Cell death was determined by cell retention of the blue dye (blue-stained cells), as viable cell exclude the dye (unstained cells). The total number of all the cells present and the number of unstained cells were noted. The percentage of viable cells in the total population was then calculated as follows:

$$\text{Cell Viability (\%)} = (\text{number of viable cells} / \text{total cell count}) \times 100.$$

2.4.2 Apoptosis assay

This assay is based on the biological alteration of phosophatidyl serine (PS) during apoptosis, leading to its translocation to the outer cell membrane and exposure to the external environment, and the high affinity of the anticoagulant protein Annexin V to PS (Koopman et al., 1994). The distinction between viable cells from dead and apoptotic cells was determined using Alexa Fluor 488 Annexin V Dead cell apoptosis and a Propidium Iodide kit (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. Briefly, 1×10^6 cells were collected and washed with ice-cold PBS and fixation of the cells was performed using 70% ethanol at -20°C for 3 minutes. Before data acquisition the cells were allowed to rehydrate in PBS for 15 minutes and then washed twice with cold PBS. The cells were re-suspended in 100µl of 1× Annexin V binding buffer for each sample and the samples were stained with either 1 µl of 100µg/ml propidium iodide (PI) solution, 5 µl of Alexa fluor 488 or both, depending on the assay. The samples were incubated for 15 minutes at room temperature and then subjected to centrifugation at 1500 rpm, 4°C for 5 minutes. The cells were washed with 1 ml of PBS then re-suspended in 400µl of PBS. Data acquisition was performed immediately using a BD FASCaliber instrument or a BD FACSAria III Cell Sorter™ (Becton Dickinson Benelux N.V., Erembodegem-Aalst, Belgium) and analysed using FlowJo version 9.5.4 (Tree Star, Inc. California, USA).

2.5 Phagocytosis Assay

Phagocytosis is one of the earliest immunological defence mechanisms, whereby neutrophilic granulocytes internalise and ingest invading microorganisms. Consequently, phagocytosis is one of the main physiological characteristics to be assessed when profiling granulocytes and can be assayed through the incubation of granulocytes with

fluorescent particles, followed by flow cytometric analysis. The phagocytosis assay in this study was conducted using Fluoresbrite® Yellow Green Microspheres (Polysciences, Inc. Warrington, PA, USA). This assay is a modification of a formerly described protocol (Harvath and Terle, 1999). In order to elicit a response from the granulocytes, microsphere particles must first be opsonised with serum as this is necessary for particle engulfment by granulocytes. Equal volumes of Krebs' Ringers PBS (5.5mM D-glucose in 1 × PBS, pH 7.3) and FBS were mixed and 1×10^8 particles were added to the opsonisation solution. The particles were incubated at 37°C for 30 minutes with gentle rotation, before 10 µl of the opsonised particles were added to 100 µl of granulocytic cell suspension at a density of 1×10^7cells/ml in Krebs' Ringers PBS and incubated at 37°C for 30 minutes with gentle rotation. The reaction was quenched by washing the cells twice with 2 ml of ice cold PBS. Finally, the cells were re-suspended in 500µl of cold PBS, the samples kept at 4 °C and data acquisition was performed immediately by flow cytometry.

2.6 Nitric Oxide Production

2.6.1 Lipopolysaccharide Stimulation

NB4 cells were plated at a concentration of 1×10^5 cells/ml in a 96-well plate and the cells were incubated with fresh FCS-free RPMI media. The cells were stimulated into NO production using 100µg/ml of LPS from *Escherichia coli* strain 055:B5 (Sigma Aldrich, St. Louis, MA, USA) for 24 hours.

2.6.2 Greiss Assay

A 100 µl sample of the supernatant was removed and incubated with the 0.5% sulfanilamide, 0.05% (*N*-1-naphthyl) ethylenediamine dihydrochloride 2.5% H_3PO_4 in

97% H_2O for 30 min at room temperature in the dark. Absorbance was measured at 530 nm using a VERSAmax ELISA microplate reader spectrometer and calibrated using a standard curve constructed with sodium nitrite to yield the NO_2 concentrations.

2.7 Phase Contrast Microscopy

2.7.1 Slide Preparation

Cell smears of peripheral granulocytes or NB4 cells were prepared with approximately 2.5×10^4 cells in a Nunc eight-well chamber slide or a frosted glass slide. Smears were fixed with ice cold absolute methanol for 15 minutes at -20°C before being washed with 1 × PBS and stored at 4°C for no more than one week or -80°C for prolonged storage.

2.7.2 Cell Morphology

To investigate the cell morphology, 0.75 g of Leishman stain (Sigma-Aldrich cat# L6254) was diluted in 400 ml of absolute methanol and left to stand for 24 hours. The slides were dipped in the stain for 30 seconds and then incubated in 2 ml of Sorensen's phosphate buffer, pH 6.8, (25 ml of 0.076 M sodium phosphate monobasic solution mixed with 25 ml of 0.067 sodium phosphate dibasic solution) for 30 seconds.

2.7.3 Immunocytochemical Staining and Immunoreactive Scoring

ICC staining was used for the assessment of CTCFL expression in the granulocytes of breast cancer patients, healthy donors and NB4 cells. The expression of CTCFL was revealed as a brownish (DAB) coloration of the stained cells. The staining intensity conveyed the level of protein expression, as darker staining was due to higher expression of the protein and lighter staining denoted lower expression. The percentage of stained cells in the examined smear also reflected the level of CTCFL expression.

2.7.3.1 Blocking and Permeabilisation

For peroxidase blocking the slides were incubated in a Coplin jar with 1.6% H_2O_2 solution in methanol for 20 minutes at room temperature with gentle shaking. After washing twice with 1 × PBS the, resulting smears were permeabilised with 1 × PBS-0.2% Triton × 100 solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes at room temperature with delicate shaking. The slides were washed again twice with 1 × PBS before blocking with 2% heat inactivated horse serum, 0.05% Tween, 1% BSA in 1 × PBS for 1 hour at room temperature. Dehydration of smears was avoided by incubating the slides in humidified containers.

2.7.3.2 Primary and Secondary Antibody Probing

The blocked smears were incubated overnight with a primary antibody diluted in 1 × PBS-0.05% Tween-1% BSA at a 1:25 concentration. The primary antibody used was an anti-CTCFL affinity purified mouse monoclonal IgM, which had been raised against the N-terminal sequence of CTCFL. Following incubation with the primary antibody, the smears were washed twice with 1 × PBS. The smears were then incubated for 1 hour with a secondary antibody diluted 1:200 in 1 × PBS-0.05% Tween-1% BSA. The secondary antibody was affinity purified biotinylated anti-mouse IgM (Vector Laboratories, Burlingame, CA; USA), raised in goats.

2.7.3.3 Visualisation

The avidin-biotin complex (ABC) horseradish peroxidase reagent was prepared according to the manufacturer's instruction. Three drops of ABC were added to the smears and they were incubated for 1 hour. The slides were then washed as previously described. Peroxidase was visualised using a 3,3'-diaminobenzidine (DAB) substrate solution (Vector Laboratories; Burlingame, CA, USA), which was prepared as per the manufacturer's instructions immediately before application. The smears were incubated

with the substrate and gently agitated for 2 minutes before being washed with tap water for 3 minutes.

2.7.3.4 Counterstaining

The smears were counterstained using VECTOR® Haematoxylin QS (Vector Laboratories, Burlingame, CA; USA) for 10 seconds and then washed under running tap water. To dehydrate the smears they were dipped for 1-2 minutes in 95% ethanol followed by 1-2 minutes each in 100% ethanol, then 100% xylene. The slides were air-dried and mounted with VECTAMOUNT® mounting medium (Vector Laboratories, Burlingame, CA; USA). Immunoscoring was carried out by counting the ICC stained cells under an Olympus BX-41 inverting microscope (× 60).

2.7.3.5 Immunoreactive Scoring

The immunoreactive score (IRS) system is a clinical semi-quantitative method to assess protein expression levels in ICC stained cells via microscopic examination (Beck et al., 1995). This is accomplished by firstly grading the percentage of stained cells from 1 to 4, where 1 reflects that less than 10% of cells are stained and 4 indicates that more than 80% of cells are stained (Table 2.4). The staining intensity was also graded using values ranging from 0-3 (Table 2.5). Finally, the IRS can be calculated by multiplying both the percentage and the intensity grades, yielding a score which between 0-12. The corresponding level of protein expression from the resulting IRS is as described in Table 2.6.

Table 2.4: Grading system given to the percentage of stained cells (positive cells) examined under a light-inverted microscope

Positive Cells (%)	Grade
<10%	1
11-50%	2
51-80%	3
>80%	4

Table 2.5: Grading system corresponding to stain intensity examined in three fields examined under an inverted light microscope

Staining Intensity	Grade
No detectable	0
Mild	1
Moderate	2
Strong	3

Table 2.6: Values of calculated IRS and the corresponding designated level of protein expression

Immunoreactive Score	Protein Expression
0-4	Low expression
5-8	Moderate expression
9-12	High expression

2.8 Fluorescent and Confocal Microscopy

2.8.1 Slide Preparation

Slides were prepared as for the smear preparations (see Section 2.5.1).

2.8.2 Immunofluorescent Staining

In the assessment of the differentiation of NB4 cells, methanol-fixed slides were stained with anti-PML mouse monoclonal antibody using indirect immunofluorescent staining. The slides were permeabilised with 0.25% Triton-X in PBS for 20 minutes, followed by three washes with 1 × PBS for 10 minutes. The NB4 cell smears were blocked with blocking solution (2% heat-inactivated serum 0.05% Tween-1%BSA in 1 × PBS) for 1 hour and the smears were incubated with primary antibodies at the optimum dilution (see Table 2.1) in 1 × PBS with 0.05% Tween-1%BSA for 2 hours. This was followed by three further washes in 1 × PBS for 10 minutes. The secondary antibody was diluted in the same dilution buffer as the primary antibody at the optimal concentration (see Table

2.2), and the smears were incubated with the secondary antibody for 1 hour. Finally, the smears were washed three times in 1 × PBS for 10 minutes. In order to counterstain the nuclear material, the smears were stained with 5µg 4'6-diamidino-2-phenylinodole dilactate (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes, washed, air dried and then mounted with VECTASHEILD® Permanent Mounting Medium (Vector Laboratories, Burlingame). Imaging of the immunofluorescent stained smears was performed using a Nikon ECLIPSE Ti confocal microscope (Nikon UK Ltd., Surrey, UK) and the images were analysed using NIS-Elements 3.2 software.

2.9 Western Blot Analysis

2.9.1 Quantification of the Total Protein Concentration

A Bradford assay was performed to determine the total protein concentration in whole cell lysate samples prepared from NB4 and CAMA1 cells, as well as peripheral human granulocytes. The CooAssay Standard Protein Kit (Inter Chim, Montlucon Cedex, France) was used for the Bradford assay according to manufacturer's instruction. Briefly, 50 µl of each sample/control was added to a 96-well plate and 250 µl of the Coomassie reagent was added. Optical absorbance was detected within 15 minutes at a wavelength of 959 nm using a VERSAmax ELISA microplate reader.

2.9.2 Sample Preparation

In total, 1×10^6 cells were collected and washed with ice-cold 1 × PBS and then subjected to centrifugation at 2000 rpm for 5 minutes. Cell lysates were prepared by treating the resulting cell pellet with 100 µl of lysis buffer (0.1M tris/HCl pH 6.8, 7M Urea, 10% β-mercaptoethanol, 4% SDS and phenol red dye) and vortexing at full speed for 5 minutes (Klenova *et al.* 1993). The proteins were denaturated by heating the

samples at 95°C for 5 minutes and then immediately subjecting them to centrifugation at maximum speed for 5 seconds. The supernatant was collected in a clean tube and the pellet discarded. If not immediately loaded the samples were kept at -80°C for prolonged storage.

2.9.3 Polyacrylamide Gel Electrophoresis

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protocol used was a modification of Laemmli (1970). A 4% acrylamide stacking gel [4% polyacrylamide solution, 0.1M tris/HCl, pH6.8, 0.1% SDS, 0.02% (v/v) TEMED (Sigma-Aldrich, St. Louis, MO, USA) and 0.05% ammonium persulfate (APS)] was loaded on top of a 10% acrylamide resolving gel [10% polyacrylamide, 0.1M tris/HCl, pH 8.9, 0.1% SDS, 0.02% (v/v) TEMED and 0.05% APS] in a gel slab apparatus. In total, 20 µl of whole cell lysate was loaded into the polymerised gel and electrophoresis was performed in electrophoresis buffer (0.025 tris/HCl, 0.192M glycine, 0.1% SDS) using a PROTEAN® II electrophoresis system (Bio-Rad, Hertfordshire, UK). The first 30 minutes of electrophoresis was carried out at 40mA/50V and after the loaded samples had travelled through the stacking gel the electrical current was raised to 40mA/125V for 2 hours. All SDS-PAGE gels were run with a protein standard marker for molecular weights ranging between 60 kDa to 175 kDa (New England Biolabs, USA).

2.9.4 Protein Transfer via Semi-Dry Immunoblotting

Resolved proteins were transferred using a Pierce® Fast Semi-Dry Transfer blotter (Thermos Scientific, Rockford; USA) on a polyvinylidene fluoride (PVDF) membrane which had previously been soaked in absolute methanol. The PVDF membrane, together with the gel and blotting paper were equilibrated in 1 × Pierce fast semi-dry transfer buffer for 10 minutes. Transfer was performed at 500 mA/25 V for 22 minutes and

following the transfer the membrane was washed in 20% methanol for 5 minutes and then twice in distilled water for 2 minutes.

2.9.5 Blocking, Probing and Signal Visualisation

The membrane was blocked for 1 hour in blocking buffer (1% fat free milk powder, 0.1% Tween-20 in 1 × PBS) and then washed three times with washing buffer (0.1% Tween-20 in 1 × PBS) for 7 minutes. The membrane was then incubated overnight at 4°C with the optimum concentration of the primary antibody (Table 2.1) diluted in 1% milk in 1 × PBS. The membrane was then washed three times with washing buffer for 7 minutes before T incubating the membrane with a fluorescently-labelled secondary antibody (green or red) for 1 hour. Finally, the membrane was washed again three times with washing buffer for 7 minutes. The gel was scanned using an Odyssey® Infrared Imaging System (Li-cor Biosciences, Lincoln, NE, USA).

2.10 Flow Cytometric Analysis of Immune System Receptor Expression

2.10.1 Sample Preparation, Staining and Fixation

For sample preparation and antibody staining, 0.5×10^6 cells were collected. The cells were subjected to centrifugation at 1500 rpm for 2 minutes and the supernatant removed. The cells to be assessed for intracellular expression of immune system receptors were permeabilised for 20 minutes using 0.025% Tween-20 in PBS. The permeabilisation step was omitted for cells being assessed for extracellular expression of immune system receptors. The cells were washed for 1 minute with 1 ml of wash/block buffer (0.1% BSA in PBS) and subjected to centrifugation for 2 minutes to remove the supernatant. The cells were then re-suspended in 40µl of wash/block buffer, and 4 µl of primary antibody was added to the cell suspension to give a final concentration of 1:10 before incubating at

room temperature for 1 hour with slow rotation. The cells were washed and re-suspended in 50 µl of block/wash buffer and 1 µl of secondary antibody was added before incubation for 30 minutes. Finally, the cells were washed and re-suspended in 350-400 µl of wash/block buffer in preparation for data acquisition. Cell fixation was performed post-staining using 1 × BD CellFIX™ (Becton Dickinson Benelux N.V., Erembodegem-Aalst, Belgium) according to the manufacturer's instructions.

2.10.2 Flow Cytometry Data Acquisition and Analysis

For the assessment of innate and adaptive immune receptors present on neutrophils as well as intracellular expression, data were acquired from samples using a BD FASCaliber™ Instrument and a BD FACSAria III Cell Sorter™ (Becton Dickinson Benelux N.V., Erembodegem-Aalst, Belgium), and data analysis was performed using FlowJo version 9.5.4 (Tree Star, Inc. CA, USA). For transfection optimisation, the acquired data were analysed by Flowing software version 2.5.1(Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland).

2.11 Ectopic Transient Transfection in the NB4 cell line

2.11.1 DNA Plasmids

Enhanced green fluorescent protein (EGFP) plasmid (pEGFP), pEGFP-CTCFL and CTCFL-pCMV6 constructs were kindly provided by Professor Elena Klenova.

2.11.2 Transformation and Sub-Culturing of Competent Cells

Both pEGFP and CTCFL-EGFP were transformed into DH5α competent *E. coli* cells (Invitrogen, Carlsbad CA, USA) [genotype F– Φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1]. In total, 2 µl of DNA was added to the competent cells. The cells were incubated on ice for 30 minutes

before heat shocking them for 20 seconds in a 37°C water bath and then incubating them for 2 minutes on ice. Subsequently, 200 µl of the transformed cells were plated onto pre-warmed Luria Betani (LB) agar plates (10 g NaCl, 10 g tryptone, 5 g yeast, pH 7.0, 15 g Bacto agar, 50 µg/ml kanamycin) and incubated overnight at 37°C.

For small-scale plasmid DNA isolation, sub-culturing was performed by inoculating 5 ml of LB broth (10 g NaCl, 10 g tryptone, 5 g yeast, pH 7.0, 50 µg/ml kanamycin) with a single colony from a transformed cells, and then incubated overnight at 37°C with vigorous shaking. For large-scale plasmid DNA isolation, the 5 ml bacterial culture was added to 250 ml of LB broth and again incubated over night at 37°C with vigorous shaking.

2.11.3 Small-Scale Extraction of Plasmid DNA for DNA Analysis

A Qiaprep Spin Miniprep kit (QIAGEN, Hilden, Germany) was used for small-scale extraction of plasmid DNA. In total, 1.5 ml of the 5 ml overnight bacterial culture was transferred to a sterile centrifuge tube and subjected to centrifugation at 8500 rpm for 2 minutes. The bacteria pellet was re-suspended in 250 µl of P1 buffer (50mM tris-HCl pH8.0, 10 mM EDTA, 100 µg/ml RNase A) and vortexed before adding 250 µl of P2 buffer (200mM NaOH, 1%SDS) to the tube and inverting six times. Finally, 350 µl of N3 buffer was added and gently mixed until the suspension became cloudy. The suspension was subjected to centrifugation at 13000 rpm for 10 minutes and the supernatant transferred to a Qiagen Mini Spin column, placed into a 2ml collection tube and subjected to centrifugation at 13000 rpm for 60 seconds. The flow-through was discarded and 500 µl of PE Buffer (washing buffer) was added to the column and the centrifugation step repeated. The flow-through was again discarded and the empty column was spun for an additional 60 seconds to remove any residual washing buffer. For DNA elution, 50 µl of

elution buffer (10 mM Tris-HCl pH 8.5) was added to the centre of the column which was placed into a new sterile centrifuge tube. The column was left to stand for 1 minute then subjected to centrifugation at 13000 rpm for 60 seconds. The flow-through from this step contained the purified plasmid DNA. The concentration and purity of the extracted plasmid DNA was determined using a NanoDrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA was stored at -20°C.

2.11.4 Restriction Enzyme Digestion of Plasmid DNA

Plasmid DNA was digested with the restriction enzyme *Xho*I (Fermentas, Germany) for 30 minutes at 37°C (0.5 µg plasmid DNA, 2 µl buffer, 1 µl *Xho*I restriction enzyme, final sample volume of 20 µl made up by adding ddH_2O). The complete digestion of the plasmid was confirmed by agarose gel electrophoresis.

2.11.5 Agarose Gel Electrophoresis

To 10 µl of the digested plasmid DNA, 5 µl of DNA loading buffer (50% glycerol, 1 × TAE buffer (40 mM tris, 20 mM acetate, 1 mM EDTA, pH 8.0) and 0.25% bromphenol blue) was added. The DNA was then loaded onto a 1% agarose gel (1× TAE, 1 g agarose, 0.05 µg/ml ethidium bromide) and together with a 1-10 kb DNA ladder (New England BioLab Ltd. Hitchin, Herts., UK). Electrophoresis was performed for 30 minutes at 80 V and the gel was scanned under UV illumination using an AlphaImager® EP imaging system (Alpha Innotech San Leandro, CA, USA).

2.11.6 Extraction and Purification of Plasmid DNA

Plasmid DNA was purified for transfection using a MaxiPrep plasmid purification kit (QIAGEN, Germany). Briefly, transformed bacteria (250 ml) were pelleted in a cooled centrifuge (4°C) at 6000 rpm for 15 minutes. The supernatant was discarded and the

bacterial pellet was re-suspended in 10 ml of cold P1 buffer (50 mM tris-HCL, pH 8.0, 10 mM EDTA, 100µg/ml RNase) and votexed vigorously in a sterile 50 ml tube. Next, 10 ml of P2 buffer (200mM NaOH, 1% SDS) was added and the tube gently inverted 6 times before being left at room temperature for 10 minutes. This was followed by adding P3 Buffer (3M potassium acetate pH5.5) and again inverting gently 6 times before leaving at room temperature for 5 minutes. The resultant cell lysate was filtered using a Qiafilter cartridge into a sterile 50 ml tube, and 2.5ml of ER buffer was added to the filtered lysate, the tube was inverted 10 times and incubated on ice for 30 minutes. A Qiagen-tip 500 ion-exchange column was equilibrated by adding 10ml of QBT buffer (Qiagen) which was allowed to flow through under gravity. The filtered lysate was added to the Qiagen-tip 500 column and allowed to flow through the resin under gravity. The column was washed twice with 15ml QC buffer and the DNA was eluted after the second wash with 15ml of the same buffer.

DNA precipitation was accomplished by adding 10.5 ml of isopropanol at room temperature, mixing and then subjecting the tube to centrifugation at 11000 rpm at 4°C for 30 minutes. The supernatant was discarded and the DNA pellet washed with 5 ml of endotoxin-free 70% ethanol and subjected to centrifugation at 11000 rpm at 4°C for 20 minutes. The supernatant was again discarded and the DNA pellet was air-dried before re-suspending in 60 µl of TE buffer. The re-suspended DNA was left at 4°C overnight to ensure total re-suspension of the plasmid DNA. The DNA concentration and purity were measured using a NanoDrop spectrometer (Thermo Firsher Scientific, Waltham, MA, USA) and the DNA was stored at -20 °C.

2.11.7 Non-Liposomal Transfection

A commercial non-liposomal, multicomponent transfection reagent was trialled for the transfection of NB4 cells. The X-treme GENE® HD (Roche Diagnostics GmbH, Germany) reagent was used according to the manufacturer's instructions. Briefly, 0.5×10^5 of NB4 cells were washed and re-seeded with 200 µl of Opti:MEM® I reduced serum media (Gibco, NY, USA) per well in a 48-well plate. Next, 1 µg of EGFP was diluted in a 100 µl total volume of Opti:MEM media (final concentration is 0.01 µg/ µl) for each sample and gently mixed. To determine the optimum concentration of reagent to DNA for high yield transfection, the cells were transfected using 1:1, 2:1, 3:1 and 4:1 ratios of reagent (µl) to plasmid DNA (µg). Accordingly, 1 µl, 2 µl, 3 µl and 4 µl of the transfection reagent were added directly to the 100 µl of diluted DNA for each corresponding ratio, and the samples were incubated for 15 minutes at room temperature. For a 48-well plate, only 30 µl of the prepared reagent/DNA complex was added to the cells for each sample in a drop-wise manner. The plate was gently swirled to ensure even distribution and the cells were incubated under standard culture conditions for 48 hours. Transfection was assessed after 24 and 48 hours using flow cytometry.

2.11.8 Cationic Liposome Transfection

TurboFect (Thermo Fisher Scientific, MA, USA) is a cationic polymer in water solution for the transfection of mammalian cells, including primary and hard-to-transfect cells. The reagent was used according to the manufacturer's instructions to investigate transfecting NB4 cells. In total, 0.5×10^6 NB4 cells were seeded into 2 ml of standard RPMI culture media 24 hours prior to transfection in a 24-well plate. It was imperative that the cells were in log phase at the time of transfection. For transfection, the cells were washed and resuspended in 1 ml of RPMI culture media free from serum and antibiotics.

Two sets of DNA dilutions were prepared for the trial, 1 µg and 2 µg of pEGFP in serum-free and antibiotic-free RPMI media, and 2 µl of TurboFect was added to the diluted DNA samples while vortexing. The final sample volume was 100 µl for each well and the DNA-TurboFect mix was incubated at room temperature for 15 minutes. The DNA-TurboFect mix was added to each well in a drop-wise manner and the plate was gently swirled to ensure even distribution. The cells were incubated under standard culture conditions and transfection efficiency was assessed after 24 and 48 hours using flow cytometry.

2.11.9 Electroporation of Plasmid DNA

NB4 cells were transfected according to the previously published method of Rizzo et al. (1998). Briefly, 1.2×10^7 NB4 cells were collected by centrifugation at 1000 rpm for 5 minutes, the supernatant was removed and the cells resuspended in 800 µl of electroporation (EP) buffer (21 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4 and 6 mM glucose). The cells were incubated on ice for 10 minutes, before adding 1 µg or 2 µg of plasmid DNA to the NB4 cells. Electroporation was carried out at 1.6 kV and 25µF with a Gene-Pulser (Bio-Rad Laboratories, Hercules, CA, USA) and the cells then incubated on ice for 10 minutes. The cells were plated into a 6-well plate at a density of 0.5×10^6 cells/ml in complete media (with no antibiotics) and incubated under standard culture conditions for 48 hours. Transfection efficiency was assessed 24 and 48 hours post-transfection by fluorescent microscopy or flow cytometry.

2.11.10 Nucleofection of Plasmid DNA

Nucleofection technology is an improved electroporation technology with cell line specific pre-programed parameters. The Amaxa™ Cell Line Optimization 4D-Nucleofector™ X Kit (Lonza, Cologne, Germany) was used for optimising the

transfection of NB4 cells according to the manufacturer's recommendations. To determine the best Nucleofector program and solution for the transfection of NB4 cells (primary optimisation), 16 different Amaxa Nucleofector programs were undertaken in combination with three 4-D Nucleofector X Solutions: SE, SG and SF. The reactions were carried out in 16-well Nucleocuvette™ strips. Aliquots of the supplemented Nucleofector solutions SE, SG and SF were prepared by adding 57.6 µl of the supplement to 262.4 µl of each Nucleofector solution (ratio of solution to supplement was 4.5:1 and the total volume of each aliquot was 320 µl). Three solutions were tested, therefore, three aliquots were prepared, one for each solution. For each aliquot, 8×10^6 NB4 cells (0.5×10^6 cells per reaction) were collected via centrifugation at $90 \times g$ for 10 minutes. All supernatant media was completely removed and the cells were resuspended in aliqoutes before 12.8 µg of plasmid DNA (CTCFL-EGFP) was added and gently mixed (0.8 µl per reaction). Next, 20 µl of each of the three prepared aliquots were transferred into the wells of three 16-well Nucleocuvette strips and gently tapping to remove any air bubbles. The Nucleofection programs were setup on an Amaxa Nucleofector instrument and the Nucleocuvette strips were placed in the device. After run completion, the Nucleocuvette strips were removed from the device and were incubated for 10 minutes at room temperature. The cells in each well were resuspended by adding 80 µl of pre-warmed RPMI media and gently pipetting up and down a couple of times before incubating at room temperature for a further 5 minutes. The total amount in each well (100 µl) was transferred to a 12-well culture plate and topped up with 300 µl pre-warmed media. The cells were incubated under standard culture conditions and transfection was assessed after 24 hours via a fluorescent microscope. Once the optimum program and reagent for transfecting the NB4 cells had been determined, the same Nucleofection procedure was carried out, except that 2×10^6 NB4 cells were resuspended in 100 µl supplemented

Nucleofector solution (Nucleofector single cuvettes with 4 μg of plasmid DNA). The cells were resuspended in 10 ml pre-warmed media for each Nucleofector cuvette and plated in 6-well plates or small flasks.

2.12 Knock-down of Endogenous CTCFL in NB4 Cells

2.12.1 Small Interfering RNA

In order to investigate the extent of CTCFL involvement in the transcriptional regulation of granulocytes, including cell differentiation, immunological functions, and apoptosis, endogenous CTCFL was targeted for knock-down using ON-TARGET plus SMARTpool Human CTCFL small interfering RNA (siRNA, Dharmacon, Lafayette, CA, USA). The sense strand nucleotide sequences of the pooled siRNAs targeting CTCFL are presented in Table 2.7. The red transfection indicator siGLO (Dharmacon) was used in trials for transfection optimisation and siGENOME non-targeting siRNA (Dhrmacon) was used as a negative control.

Table 2.7: CTCFL sequences targeted by siRNA

ON-TARGET-plus SMARTpool CTCFL-siRNA CAT. No.	Target sequence
J-003819-05	GGAAAUACCACGAUGCAAA
J-003819-06	GCUGUGGAGUUGCAGGAUA
J-003819-07	GUUCACCUCUUCUAGAAUG
J-003819-08	CAUCCCGACUGUUUACAAA

2.12.2 Cationic-Lipid Transfection of siRNA

Commercial reagents were trialled for transfecting NB4 cells with siRNA. Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's recommendations. For optimisation, 0.5×10^5 NB4 cells were seeded per

well in a 48-well plate with 200 µl of Opti:MEM media before transfection. Three different final concentrations of siRNA (1 nM, 25 nM and 50 nM) were tested in combination with three Lipofectamine RNAiMAX concentrations (0.2 µl, 0.4 µl and 0.6 µl). Three aliquots were prepared for each siRNA concentration to be trialled with the three different Lipofectamine concentrations. For each transfection (well), siRNA was diluted to the desired concentration in 20 µl of Opti:MEM I media with gentle mixing, and Lipofectamine was also diluted to the desired concentration in 20 µl of Opti:MEM I. The siRNA/Lipofectamine transfection complex was prepared by mixing the diluted siRNA with the diluted Lipofectamine (40 µl total volume of siRNA/Lipofectamine transfection complex), then incubating the transfection complex for 15 minutes at room temperature. Next, the transfection complex was added to the plated cells in a drop-wise manner. The plate was gently swirled and incubated under standard culture conditions and transfection was assessed after 24 hours and 48 hours using a fluorescent microscope or flow cytometer.

Another reagent trialled for transfection was DharmaFECT™ (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK), which was used according to the manufacturer's recommendations. The DharmaFECT reagent is supplied as four different formulations, DharmaFECT 1, 2, 3 and 4, for transfection optimisation. Briefly, 0.1×10^6 cells were plated per well in a 96-well plate with antibiotic-free RPMI media, an hour before transfection. Three different concentrations of siRNA were tested in combination with three different concentrations of reagent for each DharmaFECT reagent (1, 2, 3 and 4), as described in Table 2.8. The siRNA/DharmaFECT complex was prepared by mixing each diluted siRNA aliquot with the designated volume of diluted DharmaFECT and then incubating at room temperature for 15 minutes. The siRNA/DharmaFECT complexes were then added to each well in a drop-wise manner and the plate was swirled gently. The

cells are incubated under standard culture conditions and transfection was assessed using a flow cytometer.

Table 2.8: siRNA and DharmaFECT dilutions per well in a 96-well plate
* indicates the number of the DharmaFECT reagent (1, 2, 3 or 4), as the same preparation was prepared for all four reagents, making 36 assays in total.

	Prepared siRNA dilution (per well)			**DharmaFECT* dilution (ul/well)**	
Well No.	**Concentration of siRNA (nM)**	**siRNA volume (µl)**	**Serum-free media (µl)**	**DharmaFECT* volume (µl)**	**Serum-free media (µl)**
1	25	0.5	9.5	0.2	9.8
2	25	0.5	9.5	0.4	9.6
3	25	0.5	9.5	0.8	9.2
4	50	1.0	9.0	0.2	9.8
5	50	1.0	9.0	0.4	9.6
6	50	1.0	9.0	0.8	9.2
7	100	2.0	8.0	0.2	9.8
8	100	2.0	8.0	0.4	9.6
9	100	2.0	8.0	0.8	9.2

2.12.3 Electroporation of siRNA

NB4 cells were transfected according to the previously described protocol by Tartey and Takeuchi (2014). Briefly, 3.5×10^6 NB4 cells were collected and re-suspended in 0.5 ml of Opti-MEM with either 25 nM, 50 nM or 100nM concentration of siRNA before being electroporated at 260V, 1050µF with a Gene-Pulser (Bio-Rad Laboratories, Hercules). Afterwards, the cells were plated in a 6-well plate at 0.5×10^6 cells/ml in complete media with no antibiotics. Transfection was assessed after 24 and 48 hours by flow cytometry or fluorescent microscopy.

2.12.4 Nucleofection of siRNA

The same protocol for the optimisation of plasmid DNA Nucleofection in Nucleocuvette strips was employed. As described above, 20 µl of supplemented Nucleofector solution

and 0.5×10^6 NB4 cells were plated in a 48-well plate, with 0.4 μg/μl of siRNA used in the optimisation. After optimisation, Nucleofection was performed with 12.5 μl siRNA (final concentration 50 μM) in a total volume of 100 μl of supplemented solution. The cells were incubated under standard culture conditions and transfection was assessed as for plasmid Nucleofection.

2.13 Evaluation of Transfection Efficiency

A fluorescent microscope were used to examine the transfection efficiency 24 hours post-transfection. Data for transfection efficiency was collected after 48 hours post-transfection using flow cytometric analysis, except for electroporation of EGFP plasmid, where data were collected 24 and 48 hour post-transfection. For fluorescent microscope evaluation of transfection efficiency, only cells exhibiting strong fluorescence were considered positive. Briefly, 20 % of the plated cells were collected and slides prepared as described above, and the assessment of transfection efficiency was determined by calculating the mean percentage of positive cells detected in three fields of view. In the cytometeric analysis, cells were collected and washed twice with PBS, fixed with BD as described earlier, then resuspended in cold PBS. Data were collected from 10,000 events for each sample. A green fluorescence signal (FL1) for EGFP was detected with a 530 nm band pass filter and a red fluorescence signal (FL3) for siGLO siRNA was detected with a 630 nm band pass filter. The assessment of Nucleofection efficiency was carried out 24 hours post-transfection using a fluorescent microscope only. Levels of CTCFL protein over-expression or knock down were assessed via Western blot analysis.

2.14 Statistical Analysis

Statistical analysis was carried out using an unpaired student's *t* test. Values were considered significant when the probability was below the 5 % confidence level ($P \leq 0.05$).

Chapter 3 - **Profiling of Polymorphonuclear Neutrophil Model during Differentiation**

3.1 Introduction

CTCFL was detected in the PMNs of breast cancer patients when assessed by ICC staining (D'Arcy et al., 2006). In comparison, the PMNs of healthy individuals and also patients of inflammation or trauma showed insignificant levels of CTCFL. These findings raise the question if CTCFL is involved in the transcriptional regulation of maturation, differentiation, self-renewal and apoptosis. Furthermore, the alteration of CTCFL levels in this population would lead to certain alteration(s) in their physiological and functional characteristics. Several promyelocytic leukemia cell lines have demonstrated significant levels of CTCFL expression when assessed by Western blot and PCR techniques (Renaud et al., 2007). The advantage of using myeloid leukemia cell lines in studying the expression of cancer testis antigens (CTAs), such as CTCFL, is the possibility of inducing the cell line into differentiation to the desired myeloid linage (i.e. macrophages or granulocytes). Thus, the levels of CTCFL expression and the co-occurring physiological and immunological alteration could be investigated during the granulocytic differentiation of these leukemia cell lines. NB4 cell line is an acute promyelocytic leukemia (APL) cell line that harbors the t(15, 17) chromosomal translocation which causes the fusion of the PML gene with the retinoic acid receptor (RAR) gene, and results in the production of the PML-RARα fusion protein (Bernardi and Pandolfi, 2007; Lanotte et al., 1991). PML is a protein implicated in many vital cellular processes, including transcription, self-renewal, apoptosis and differentiation (Bernardi et al., 2008; Lallemand-Breitenbach, 2010). The expression of the aberrant fusion proteins PML-RARα and RARα-PML in NB4 cells disrupts the differentiation and programmed cell

death (i.e. apoptosis) pathways in these cells and lead to their infinite propagation (Grignani et al., 1993; Lallemand-Breitenbach, 2010). However, NB4 could be induced into neutrophilic differentiation by introducing *All-trans*-retinoic acid (ATRA) to the culture (Rossi et al., 2010). ATRA is an anti-cancer drug that was used for the treatment of promyelocytic leukemia patients and has been shown to modulate differentiation and subsequently apoptosis in these cells (Altucci et al., 2001; Lennartsson et al., 2006).

3.2 Experimental Approach

The main objective in this panel of experiments is to establish an immuno- and phenotypical profile as well as a CTCFL levels profile for NB4 cells when induced into neutrophilic differentiation. To induce neutrophilic differentiation in NB4 cells, the differentiating agent *All- Trans* Retinoic Acid (ATRA) was used. To select optimum concentration of ATRA for the neutrophilic differentiation of NB4 cells, three different concentrations of ATRA (1 μM, 2 μM or 5 μM) were introduced to NB4 cells in culture every 24 hours in a cumulative manner over 6 days. For each trialed concentration, 3 x 10^6 of NB4 cells were seeded in a small flask with 10 ml of complete media. Volumes from an ATRA stock solution (10 mM) were added to the culture every 24 hours as described in Table 3.1. NB4 cells were assessed for morphology, propagation in culture (growth curve) and cells viability every 24 hours before adding the next dose of ATRA. The selection of optimum of ATRA concentration was based on induction of differentiation in NB4 cells with minimal necrotic effect to cells.

Table 3.1: ATRA volumes added to culture every 24 hours over 6 days for the optimisation of NB4 cells differentiation.

Flask Number **	ATRA concentration (μl per ml media)	ATRA volume (μl) added to culture	Final ATRA concentration in culture
1	1	10	1 μM
2	2	20	2 μM
3	5	50	5 μM

** $3x10^6$ NB4 cells seeded in 10 ml complete media per small flask

Several experiments were carried out after selecting the optimum ATRA dose concentration for NB4 cells differentiation. Firstly, the physiological characteristics of ATRA-induced NB4 cells were investigated at each stage of the differentiation. For this, the ATRA-induced NB4 cells were profiled for (i) cellular morphology (ii) survival and apoptosis (iii) phagocytic activity and (iv) production of Nitric Oxide (NO). Secondly, immunophenotyping of the differentiating NB4 cells was performed. The panel of innate immune system receptors investigated here are TLR-2, TLR-4 and CD14. The adaptive immune system receptors profiled were molecules involved in antigen presentation, class I and II MHC molecules (HLA-ABC and HLA-DR respectively). Thirdly, the levels of CTCFL expression in the differentiating NB4 cells, which concur with the physiological and immunological features profiled above, were also investigated. Lastly, the association of CTCFL expression with granulocytic differentiation was investigated with spatial protein-protein proximity or overlap determination (i.e. co-localisation). The co-localisation study was performed using confocal imaging of fluorescent-labeled PML and CTCFL proteins at each stage of NB4 cells differentiation. The manner in which PML nuclear bodies (NBs) localise in the nucleus is an established biomarker that could be used in acute non-lymphoid leukemias (ANLLs) diagnosis and thus as a differentiation marker in promyelocytic leukemia (Falini et al., 1997).

3.3 Results

3.3.1 Phenotyping of ATRA-induced NB4 cells

3.3.1.1 Optimisation of ATRA concentration for the differentiation of the NB4 cell line

NB4 cells were assessed every 24 hours for cell count after the addition of the first ATRA dose. Cell count and viability of differentiating NB4 cells were assessed using trypan blue. The morphology for differentiating NB4 cells was assessed using Wright's stain. A

hallmark of differentiation in promyelocytic leukemia cells is decreased cellular size, condensation of nuclear material and increased cytoplasm to nuclear material ratio. All tested ATRA concentrations have induced distinguishable neutrophilic differentiation after 72 hours (3 days) from adding the first doses of ATRA. Also, as cells undergo differentiation they stop multiplying and growth of cells in culture enters stationary phase. The propagation of NB4 cells continued to decline in culture to reach static numbers beyond 72 hours in culture for all tested ATRA concentrations. Total cell death was observed after 120 hours of culture in all assessed ATRA concentrations. Comparing the viability of NB4 cells exposed to different concentrations of ATRA, higher number of dead NB4 cells were observed at 5 μM of ATRA than NB4 cells exposed to the other two concentrations after 72 hours (Figure 3.1). Eventually, the optimum concentration of ATRA selected for the differentiation of NB4 cells was at 1 μM final concentration in culture every 24 hours for 4 to 5 days.

3.3.1.2 Cell morphology

NB4 cells were seeded in complete RPMI at $3x10^5$ cells per ml. Differentiation of NB4 cells was induced by adding 1μl of ATRA to each 1 ml of culture every 24 hours for 4 days. In order to examine the morphological alterations occurring to the nuclei of NB4 cells as differentiation progressed, 0.5 $x10^5$ cells were collected from the flask every 24 hours and smears were prepared for Wright's staining. The collection of cells for smears was done before the addition of the next ATRA dose to the flask. The morphology of NB4 cells treated with ATRA was compared to the morphology of untreated NB4 cells (figure 3.2). NB4 cells demonstrated nuclear material distinction after the third day of ATRA addition to culture. The undifferentiated NB4 cells exhibit the morphological characteristics of promyelocytes, which include enlarged cellular size when compared to mature neutrophils and undefined nucleus. ATRA-induced NB4 cells showed signs of

differentiation to myelocytes and metamyelocytes after 48 hours of adding ATRA doses. Some band or segmented neutrophilic differentiation was observed after 3 After 72 hours (3 days) of adding ATRA doses to NB4 cells.

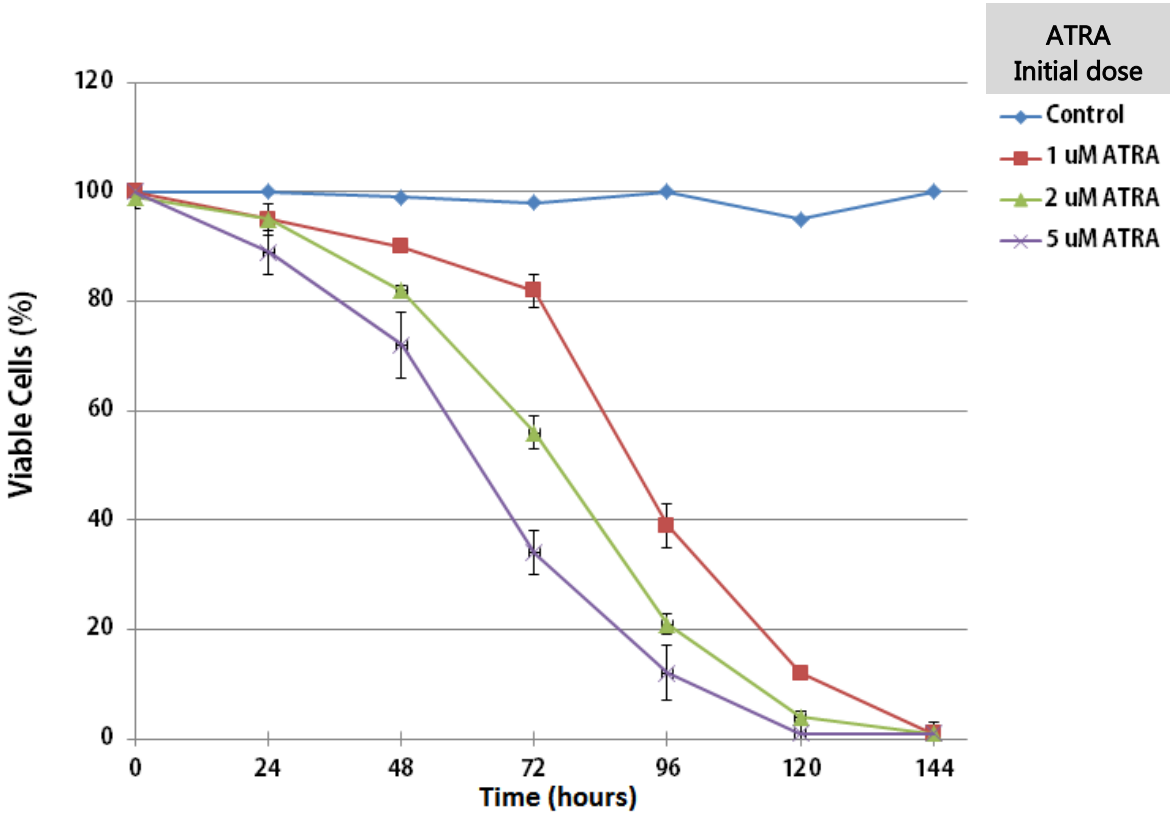

Figure 3.1: Viability assessment of NB4 cells subjected to different initial concentrations of ATRA for 144 hours (6 day)

Three different concentrations of ATRA were tested to optimise doses to add to culture media every 24 hours for NB4 cells differentiation; 1µl per ml of media (initial dose 1 µM represented with red line), 2 µl per ml (2 µM initial dose represented with green line) and 5 µl per ml (initial dose 5µM represented with purple line). As a control, the viability of NB4 cells treated with ATRA was compared to the viability in NB4 cells not treated with ATRA (blue line). Experimental repetition was carried out three times for each tested concentration (***n*=3**). Error bars are based on calculated standard deviation.

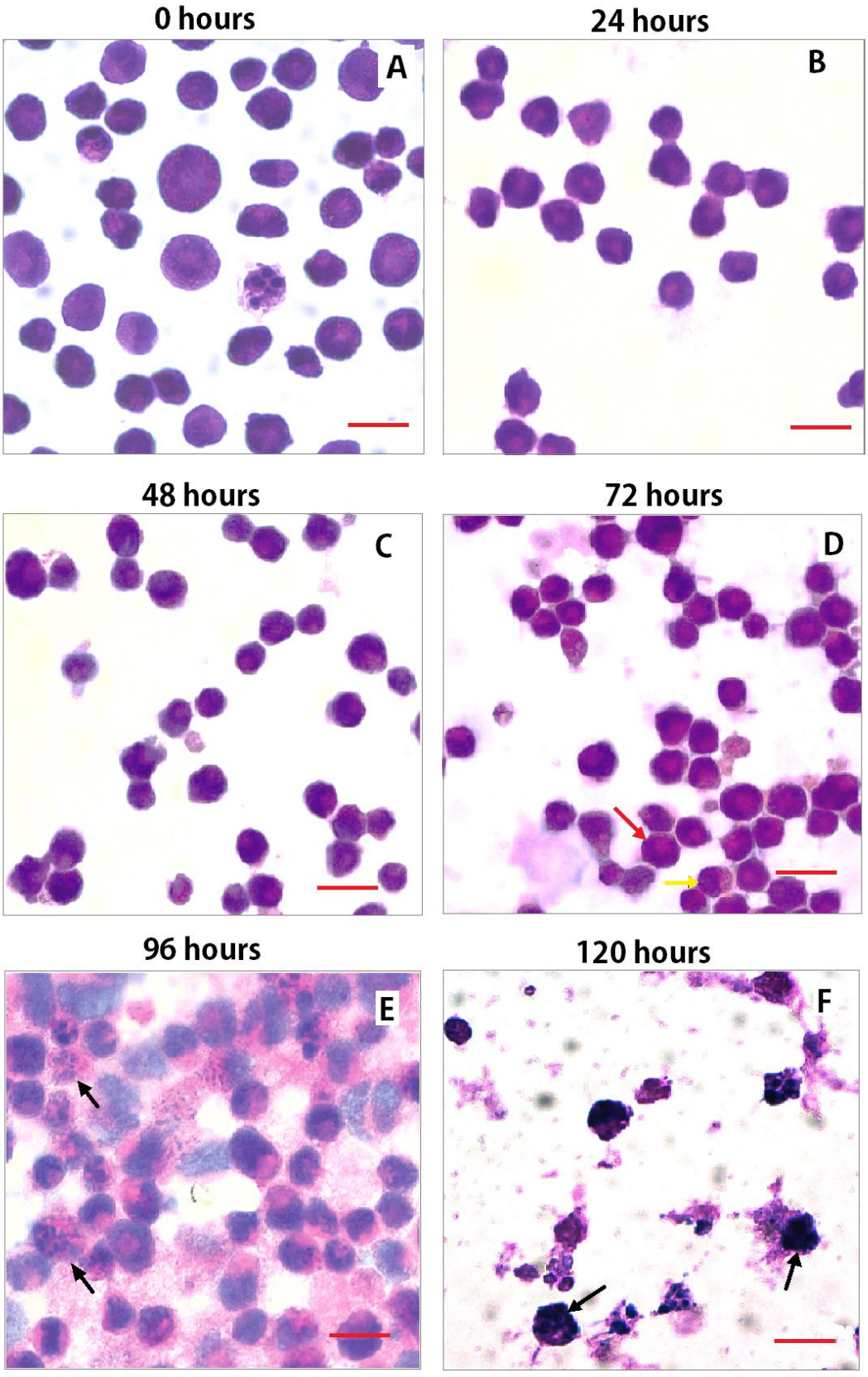

Figure 3.2: Morphological examination of differentiating NB4 cells over 120 hours (5 days) using Wright's stain.

After the introduction of the initial dose, smears of ATRA-induced NB4 cells were prepared every 24 hour prior to the introduction of the next ATRA dose (1μM of ATRA is added in culture every 24 hour in cumulative manner). The morphology of ATRA-induced NB4 cells was compared to the morphology NB4 cell untreated with ATRA (0 hours) (A). Decrease in cellular size was noticed after 24 hours (B) and 48 hours (C) of introducing ATRA doses. After 72 hours of ATRA introduction to culture, NB4 cells showed a considerable degree of nuclear material condensation (deep violet staining) and distinction from cytoplasmic material (bright pink staining) (D). Metamyelocytyes (yellow arrow) and band cells (red arrows) were also observed at this stage. After 96 hour (4 days) of introducing ATRA to NB4 cells (E) apoptotic cells were noticeable with blotched nuclear material and shrinkage of cells. NB4 cells manifested total degradation of nuclear material and cell fragmentation after 5 days of introducing ATRA (F). Apoptotic cells are indicated with black arrows. Images were taken at x 100 magnification. Red bar = 20 μm.

3.3.1.3 Viability assessment and apoptosis assay

Growth arrest was observed after 48 hour from introducing the first dose of ATRA to NB4 cells. Trypan blue exclusion was used to assess the percentage of viable cells in NB4 cells treated with ATA for 5 days. NB4 cells showed more than 95% viability before ATRA-induction (Figure 3.3). NB4 cells were able to survive after the addition of ATRA doses to culture for up to 4 days. The viability of ARA- induced NB4 cells declined dramatically by the 4th day, where less than 20% of cells were viable.

The onset of apoptosis in ATRA-induced NB4 cells was assessed with Annexin V- Alexa Flour 448 and Propidium Iodide (PI) staining of cells. Data acquisition and analysis for apoptosis assay was performed using flow cytometric analysis (Figure 3.3). Apoptotic cells exhibit positive staining for AnnexinV-Alexa Fluor, where its signal is detected on the green channel (FL1-H). Dead cells exhibit positive staining for PI and its signal is detected on the red channel (FL3-H). The assessed NB4 cells population was firstly gated on the forward (FSC-H) and side (SCC-H) scatter plot of undifferentiated and unstained NB4 cells. Other controls were processed for instrument setup and data acquisition, which included staining NB4 cells (untreated with ATRA) with Annexin V-Alexa Fluor 488 only or with Propidium Iodided (PI) only. This makes it possible to correct overlapping signal between green and red channels. Flow cytomoetric analysis of ATRA-induced NB4 cells showed an increase in the green signal of Annexin V Alexa fluor 488 after 24 hours of the administration of ATRA as well as red signal for PI staining (Figure 3.3) which reflects signal for apoptotic cells and necrotic cells respectively. However, the majority of detected events showed negative signal for Annexin V-Alexa fluor 488 and PI staining, which indicates that ATRA-induced NB4 cells to be predominantly viable over the 72 hours of adding ATRA doses to culture. By the 4th day necrotic events appeared much higher in ATRA-induced NB4 cells than viable or apoptotic cells.

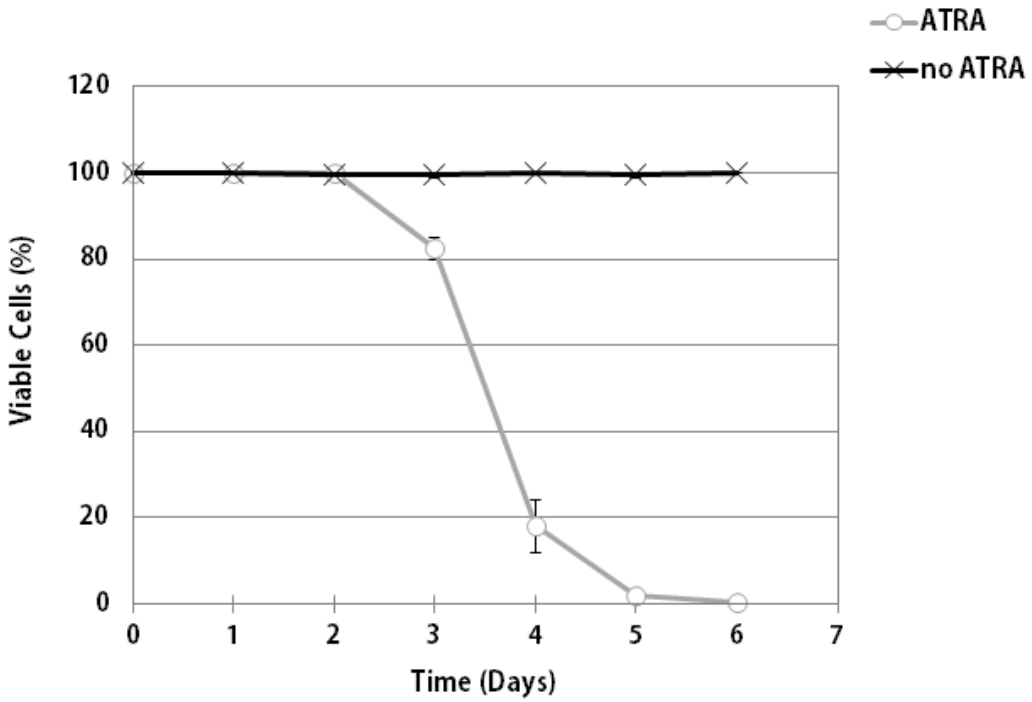

Figure 3.3: Viability assessment by trypan blue dye exclusion in differentiating NB4 cells.
Cell count demonstrated a decline in growth reaching static numbers by the third day of administrating ATRA. More than 90% of NB4 cells maintained viability for the first 72 hours of introducing 1μM initial dose of ATRA to culture. However, the percentage of viable cells declined dramatically to less than 40% by the fourth day of adding ATRA to culture and going under total death by the 5th day.

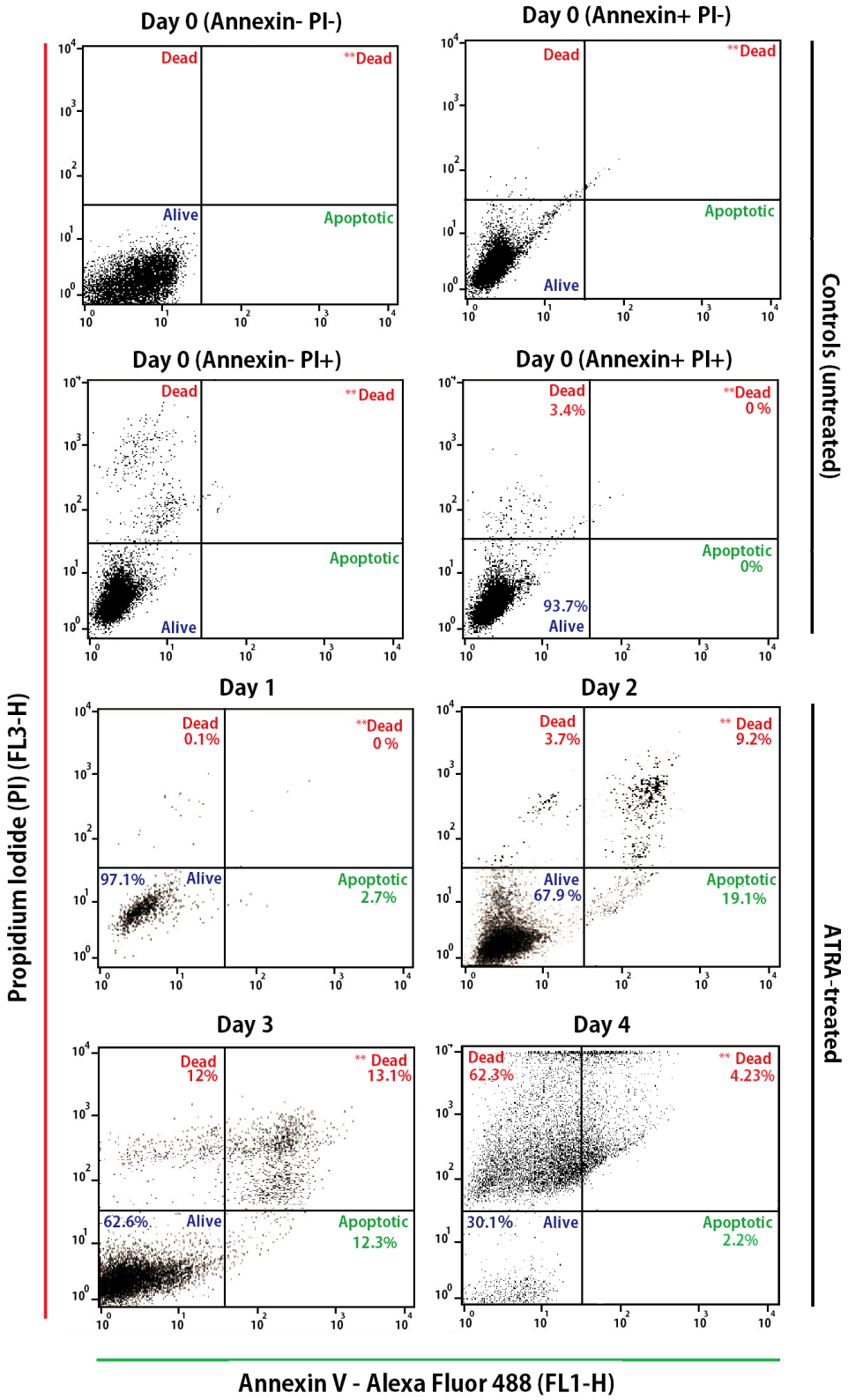

Figure 3.4: Flow cytometric analysis for apoptosis in NB4 cells over 4 days of cumulative introducing ATRA doses in culture.

The first four dot plots represent controls performed for instrument set up with NB4 cells which were not treated with ATRA and were either stained with AnnexinV only (AnnexinV+ PI-), PI only AnnexinV – PI+), or unstained (AnnexinV- PI-). Dead cells show a positive signal to Propidium Iodide (PI) staining while apoptotic cells show positive signal for Alexa fluor 488 - Annexin V staining. Positive PI staining signal was detected on the red channel (FL3-H), while Alexa fluor was detected by the green channel (FL1-H). Events detected in the bottom left region of the dot plot reflect negative Alexa fluor and PI staining, indicating viable cells (designated as alive in blue font). Events detected in the bottom right region reflect positive staining for Alexa flour, which indicates early apoptotic cells. Events detected in the top right and left regions of the dot plot reflects dead cells (designated as dead in red font) or late apoptotic cells (designated as **dead in red font) with positive staining to PI.

Phagocytic activity

For the assessment of phagocytic activity in differentiating PMNs, NB4 cells were treated with 1 µM ATRA every 24 hours for 3 days. Yellow green (YG) fluorescent latex microbeads were incubated with ATRA-induced NB4 cells for 30 minutes at 37°C. These were compared to non-induced NB4 cells incubated with the microbeads also for 30 minutes at the same temperature. Both samples were washed with ice cold 1 x PBS buffer after incubation for the remove of free microbeads which was not engulfed by NB4 cells. For the experiment set up, negative controls were performed which included undifferentiated NB4 cells without incubation with microspheres and also undifferentiated NB4 cells incubated with the fluorescent microspheres for 30 minutes but at 4°C. Data acquired by flow cytometry showed an increased signal in the FL1 channel for the NB4 cells induced with ATRA (figure 3-b), when compared to YG fluorescent signal of non-differentiating NB4 cells (figure 3-a). The increased intensity of the fluorescent signal of differentiated NB4 cells substantiates the increase in their engulfment capacity as they advance into differentiation.

3.3.1.4 Generation of nitric oxide (NO)

The capacity of ATRA- induced NB4 cells for nitric oxide production was investigated using the Griess assay. The assay measures a nonvolatile byproduct of the generated nitric oxide radicals, nitrite (NO_2^-) concentration in culture. NB4 cells were induced into neutrophilic differentiation using ATRA. To perform the Griess assay for all samples at the same time in this experiment, 1x 10^5 NB4 cells were seeded in one well in a 96-well plate and 1µM ATRA was added. After 24 hours, a new well was seeded with the same number of NB4 cells and a 1µM ATRA was added to the newly seeded well, and also to the previously seeded well. A third well was seeded after another 24 hours with the same number of cells and 1 µM was added to each well. The three wells were incubated for

another final 24 hours. ATRA-induced NB4 cells with media in each well were collected in tubes and were incubated for one hour with 100 μg/ml of LPS. After incubation with LPS, cells were centrifuged and 50 μl of the supernatant was assayed for NO concentrations. This was calculated from absorbance measurement against a reference curve. Analyzed data showed an exponential increase in the nitrite concentrations produced by differentiated NB4 cells over 72 hours (3 days) of adding ATRA doses (figure 3.6).

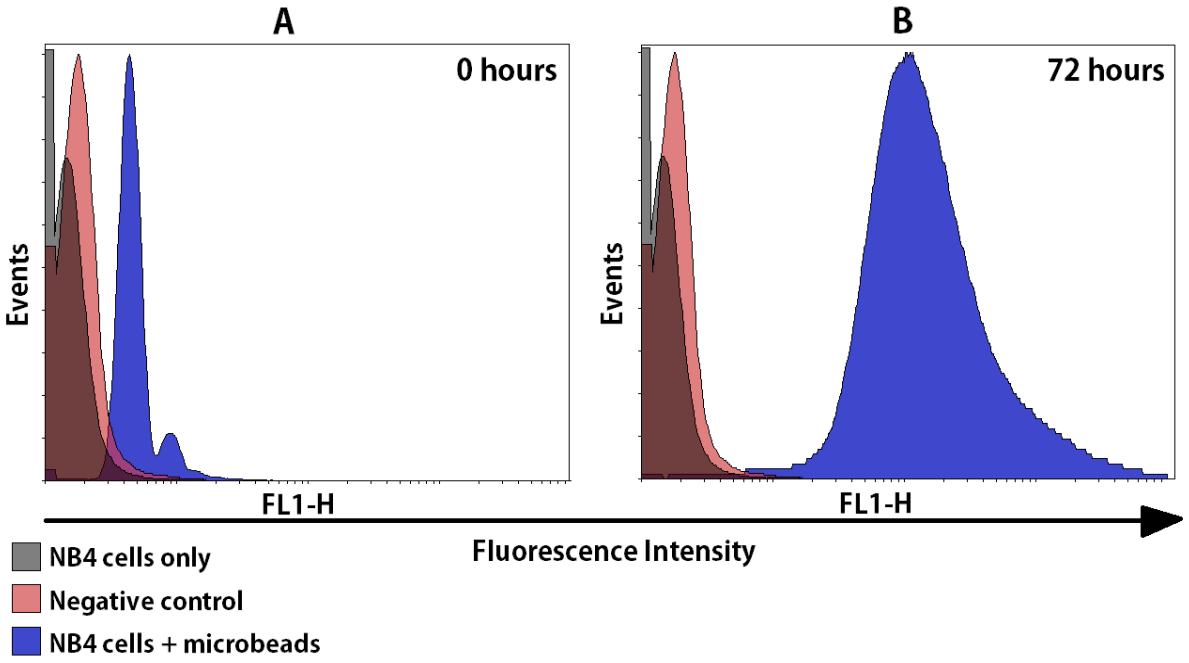

Figure 3.5: Phagocytic activity in NB4 cells after 72 hours of introducing ATRA.
NB4 cells were induced into differentiation by adding 1 µl of ATRA per 1 ml of culture every 24 hours. Flow cytometric evaluation of phagocytic activity was performed using fluorescent microbeads. Grey histogram represents ATRA-induced NB4 for 72 hours not incubated with florescent microbeads (NB4 cells only). Red histogram represents ATRA-induced NB4 cells incubated with microbeads for 30 minutes at 4°C (negative control). Blue histograms reflect NB4 cells incubated with microbeads at 37 °C for 30 minutes. Blue histogram in overlay (A) shows phagocytic activity of NB4 cells when untreated with ATRA (0 hours), while blue histogram in overlay (B) shows phagocytic activity of NB4 cells treated with ATRA doses for three days. The shift of histogram towards the right reflect an increase in the intensity of the YG (yellow green) fluorescent signal.

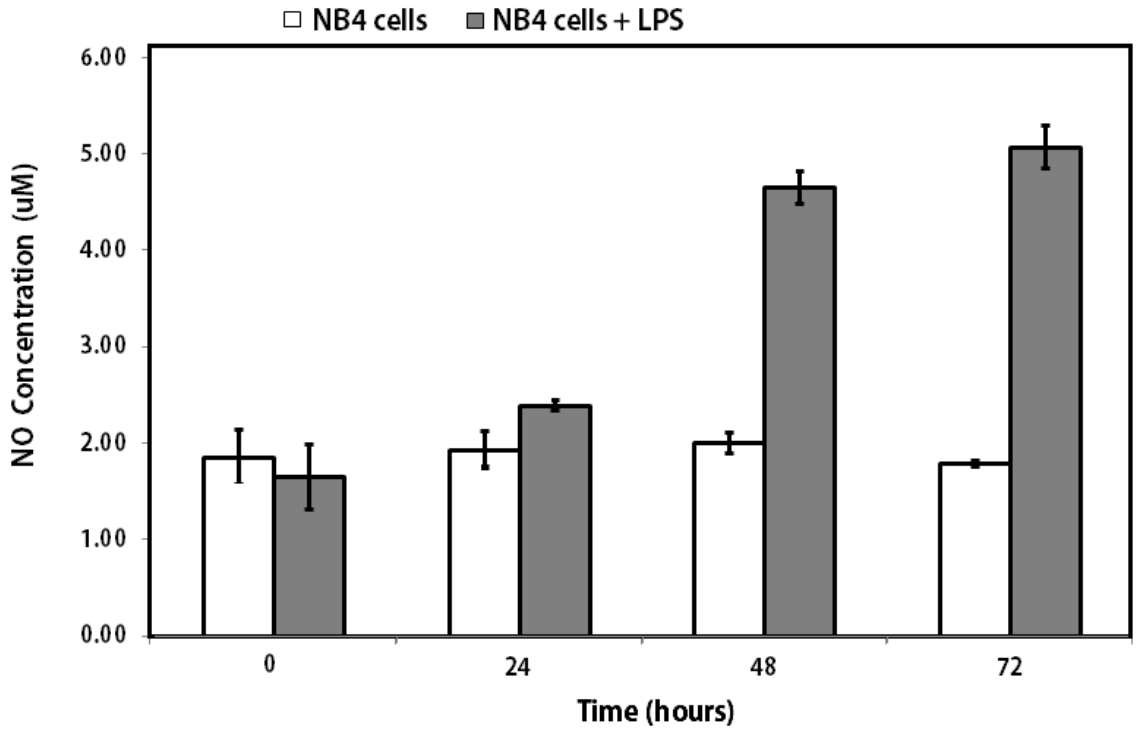

Figure 3.6: Nitric oxide generation in ATRA- induced NB4 cells.
Griess assay was used to assess nitric oxide (NO) production in ATRA-induced NB4. The assay measures the non-volatile by-product nitrite (NO_2^-). Samples for assay were measured in spectrometer using a 520 nm filter. NB4 cells were induced into differentiation using ATRA doses over 72 hours (3 days) in a cumulative manner. Grey columns represent NO_2^- levels produced by NB4 cells when stimulated using 100 µg/ml LPS, while white columns represent unstimulated NB4 cells. Data represented here were calculated based on 2 separate experimental repetitions (n=2).

3.3.2 Immunotyping of ATRA-induced NB4 cells

The receptors investigated in this experiment were TLR2, TLR4 and CD14, as these receptors are the first to make the distinction of apoptotic particles released due to microbial invasion, and also HLA-ABC & HLA-DR, which are major histocompatibility (MHC) molecules involved in antigen presentation and the modulation of the adaptive immunity. The levels of the mentioned receptors were assessed on the surface of NB4 cells before the introduction of ATRA, then were assessed every 24 hours on these cells after the introduction of the first dose of ATRA for the next 72 hours. In a parallel experiment, intracellular levels of TLR2, TLR4, CD14, HLA-ABC and HLA-DR were all assessed in permeabilized NB4 cells before the introduction ATRA, and every 24 hours after the introduction of the first ATRA dose over a 72 hours period. The levels of intracellular or surface receptors were investigated using flow cytometric analysis. Geometric mean values of acquired histograms were processed using Microsoft excel software (Figure 3.7). The flow cytometry data revealed insignificant increase in the levels of the investigated surface receptors on NB4 cells when treated with ATRA over 72 hours, with the exception of HLA-ABC, which showed a significant exponential increase in it levels on NB4 cells when treated with ATRA (Figure 3.7-A). For the intracellular levels of the investigated receptors, TLR 2 were at its highest levels after 72 hours of ATRA-introduction, while TLR4 showed the most significant increase after 48 hours of ATRA treatment, to declining again after 72 hours of ATRA treatment. In contrast to HLA-ABC levels on the surface of the ATRA-induced cells, the receptor showed a gradual decline in its intracellular levels over the 72 hours period of treatment (Figure 3.7-B).

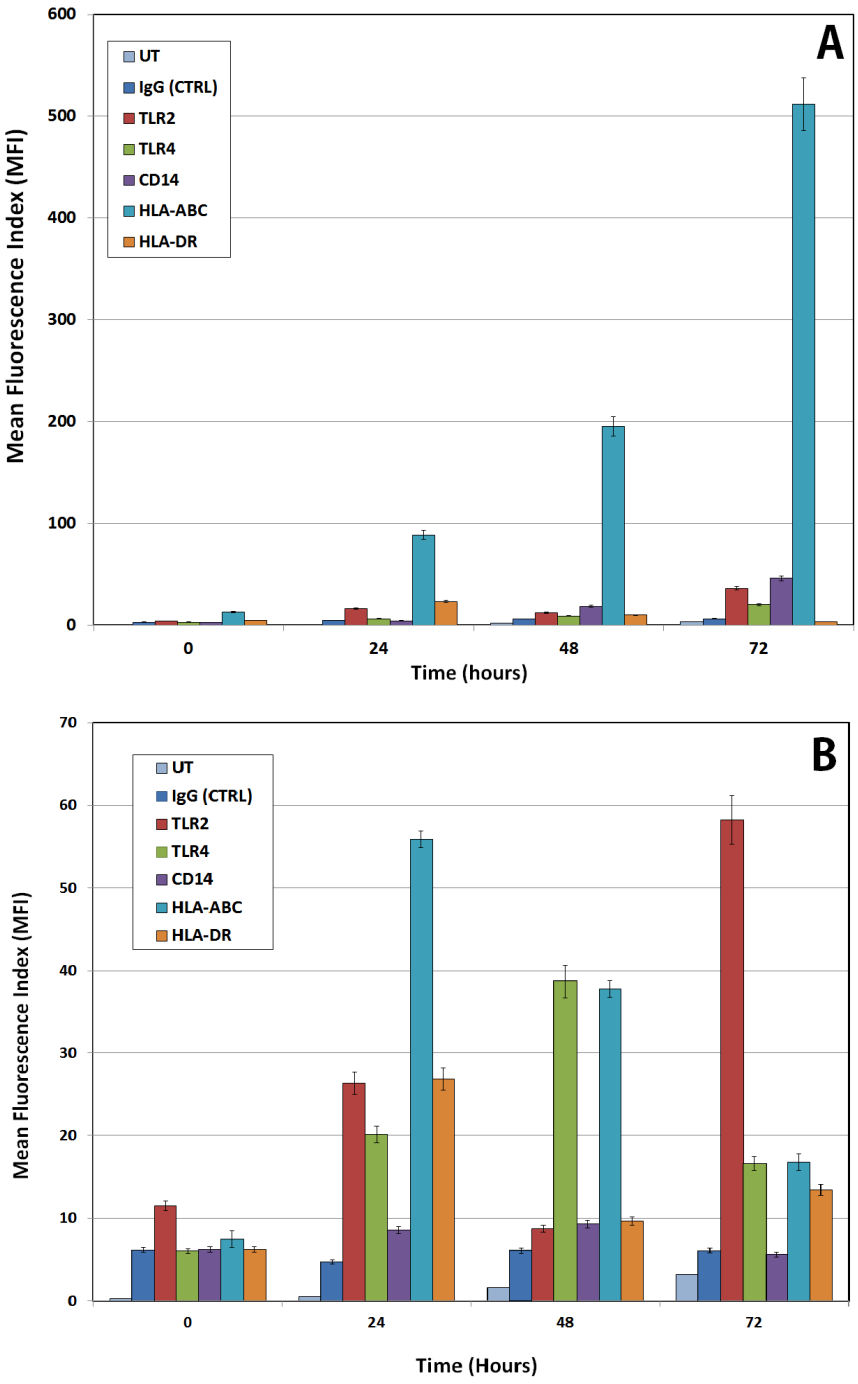

Figure 3.7: Expression of immune system receptors in NB4 cells during induction of differentiation with ATRA.

NB4 cells were induced to neutrophilic differentiation using ATRA doses every 24 hours for 72 hour (3 days) Data of intracellular and surface immune system receptors was acquired using flow cytometer and data were analysed (A) Expression of surface TLR2, TLR4, CD14, HLA-ABC & HLA-DR over 72 hours of introducing ATRA doses to culture every 24 hours. (B) Intracellular expression of TLR2, TLR4, CD14, HLA-ABC & HLA-DR during 72 hours of introducing ATRA doses to culture every 24 hours.

3.3.3 Assessment of CTCFL expression in ATRA-induced NB4 cells

3.3.3.1 Western blot

The expression of CTCFL in the ATRA- induced NB4 cells was assessed by Western blot. Every 24 hours before the administration of ATRA, $3x10^6$ cells were collected in duplicate and processed for whole cell lysates. Total protein quantification in the whole cell lysate samples was performed using a Bradford Assay. The membrane containing the transferred proteins was probed with primary antibody of CTCFL at 1:500 dilutions. Anti-mouse FITC-conjugated antibody was used as a secondary antibody at a 1:15000 dilutions for protein detection. The protein was visualized with Li-COR infrared imaging system (Figure 3.8). Immunoblotting assessment showed CTCFL to be expressed in NB4 cell line. The first 24 and 48 hours showed unchanged levels of CTCFL. The assay however suggested CTCFL to disappear in NB4 cells after three days (72 hours) of treatment with ATRA (Figure 3.8). The membrane also showed nonspecific bands around 58 kDa, 30kDa and 17 kDa. A closer observation of detected protein revealed a double band around 83 kDa. Probing the stripped membrane with anti-actin antibody confirmed the loading of equal volumes of total protein from each sample.

3.3.3.2 Immunoreactive score (IRS) for immunocytochemical staining

ICC staining was used for the evaluation of CTCFL levels during NB4 cells differentiation through 72 hours of ATRA-induction in culture. Double smears were prepared every 24 hours from each granulocyte population where one was stained with the primary and the secondary antibodies, while the second smear was stained with secondary antibody only as a staining control. The slides were assessed using phase contrast microscopic examination. Before ATRA-induction, NB4 cells showed a high immunoreactive score (IRS = 12) for CTCFL. This suggested a high levels of CTCFL in the undifferentiated NB4 cells. The immunoscores calculated for CTCFL levels in

ATRA-induced NB4 cells through the next 72 hours appeared to decline rapidly (IRS=4 after 72 hours). This reflects a decrease in the levels of CTCFL when induced into differentiation with ATRA (Figure 3.9).

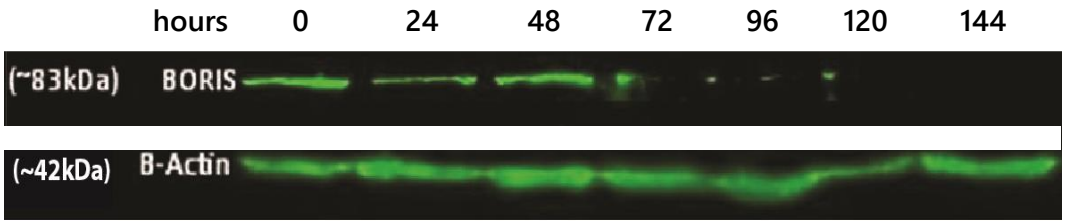

Figure 3.8: Western blot analysis for the levels of CTCFL (BORIS) expression in ATRA-induced NB4 cells.

1µl of ATRA was added to each 1 ml of media in culture every 24 hours for 6 days (144 hours). Whole cell lysates were subjected to Western blot analysis. CTCFL levels assessed in the ATRA-induced NB4 cells (24 hours to 144 hours) were compared to CTCFL levels in non-treated cells (0 hours). CTCFL was detected at the molecular weight ~83 kDa with Mouse anti-CTCFL (IgM) using 1:500 dilution. The membrane stripped and re-probed for β-actin as a control to confirm equal volumes of sample loading.

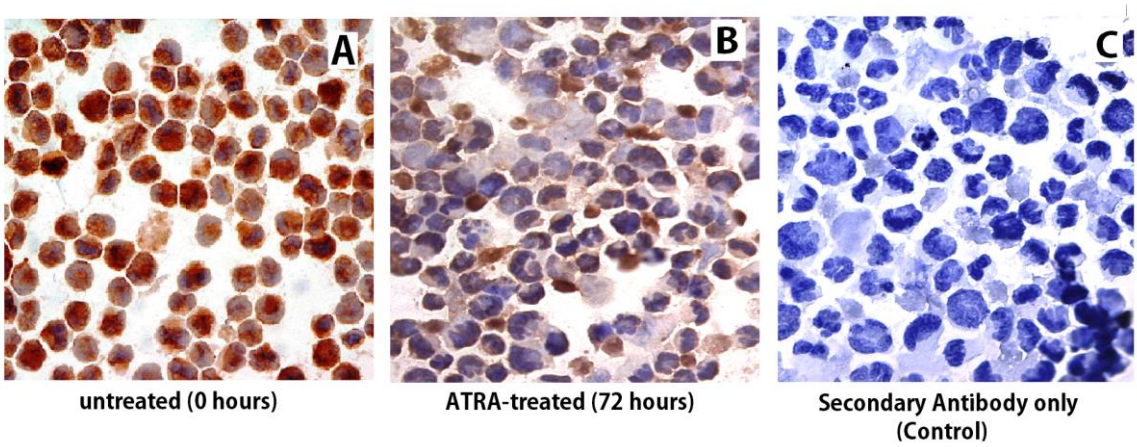

Figure 3.9: Immunocytochemical staining (ICC) for CTCFL expression in differentiated NB4 cells after 3 days of introducing ATRA doses to culture.
Untreated NB4 cells were incubated with primary mouse anti-CTCFL overnight at 1:100 dilution (**A**). The same staining preparation was performed for smears of ATRA-induced NB4 cells (**B**). Comparing ATRA-induced NB4 cells to untreated cells it was evident that CTCFL expression declined from an Immunoreactive score (IRS) of 8 (high expression) to a score of 4 (low expression) after 73 hours of introducing ATRA into culture. NB4 cells were also incubated with HRP secondary goat anti-mouse antibody only as a negative control for ICC staining (**C**).

3.3.4 CTCFL co-localisation with PML protein during differentiation

The verification of NB4 cell differentiation was attained by probing for PML oncoprotein using Immunofluorescent (IF) Staining. A diagnostic characteristic of acute promyelocytic leukemia (APL) cells is the expression of PML protein in an abnormal state where it is fused with the retinoic acid receptor (RAR-α) protein, resulting in the fusion protein PML-RARα. This event is caused by reciprocal chromosomal translocation which is manifested as satellite microgranules in the cytoplasm (5 – 20 speckles). The differentiation of granulocytes should show degradation of this fusion protein and a re-localization of the PML protein into concentrated nuclear speckles, ranging between 3- 5 in number. The NB4 cell line is derived from APL, therefore expresses PML-RARα. Our results show the characteristic microgranules of the fusion protein PML-RARα as a green signal (alexa fluor 488) in non-differentiated NB4 cells (Figure 3.10). The degradation of microgranules and gradual re-localisation of the PML protein was observed in the first 72 hours of administering ATRA doses to NB4 cells. In parallel, wild type PML detected as individual speckles after 72 hours of ATRA administration. More than 50% of the differentiated NB4 cells showed signs of apoptosis by the 4^{th} day of ATRA induction, including blebbing and fragmentation of nuclear material.

Co-localisation analysis of PML proteins with CTCFL in NB4 cells was assessed using multiple-label fluorescent staining using alexa fluor 488 (green signal) for PML and alexa 555 (red signal) for CTCFL (Figure 3.10). For this study, images of several planes in each slide were produced for data analysis. The obtained images had to be processed for deconvolution, background correction and later statistical calculation of potential regions using NIS-elements© software and JACoP plug-in with ImageJ© software. Statistical calculation of co-localization events were produced by over laying red and green channels of acquired images and Pearson's Correlation Co-efficient (PCC

or *Rr*). The values of PCC (*Rr*) range from -1 to 1, where 1 represents complete positive correlation, -1 represents negative correlation, and zero represents no correlation. In this sense, mid-range coefficient existing between -0.1 to 0.5 indicate absence of colocalisation events, while values falling between 0.5 and 1.0 suggest partial or full localization in the examined image region of interest (ROI) (Bolte and Cordelieres, 2006; Zinchuk and Grossenbacher-Zinchuk, 2008). The first visual examination of processed images suggested possible co-localisation events between CTCFL and PML, appearing mainly as yellow regions in 48 hours (day 2) and 72 hour (day 3) ATRA- induced NB4 cells, and was mainly a cytoplasmic concurrence (Figure 3.10). This possible colocalisation also reappeared in in 5 days ATRA-induced NB4 cells, which were undergoing apoptosis. However, statistical calculation of *Rr* values for days 2 and 3 in induced NB4 cells were 0.4205 and 0.3091 respectively. These values reflect possible co-localisation between PML and CTCFL protein in NB4 cells, before ATRA induction and after. The calculation of the Mander's overlap coefficient (*R*) exhibited values between o.6 and 0.7 before and during differentiation, which indicates partial colocalisation of both proteins.

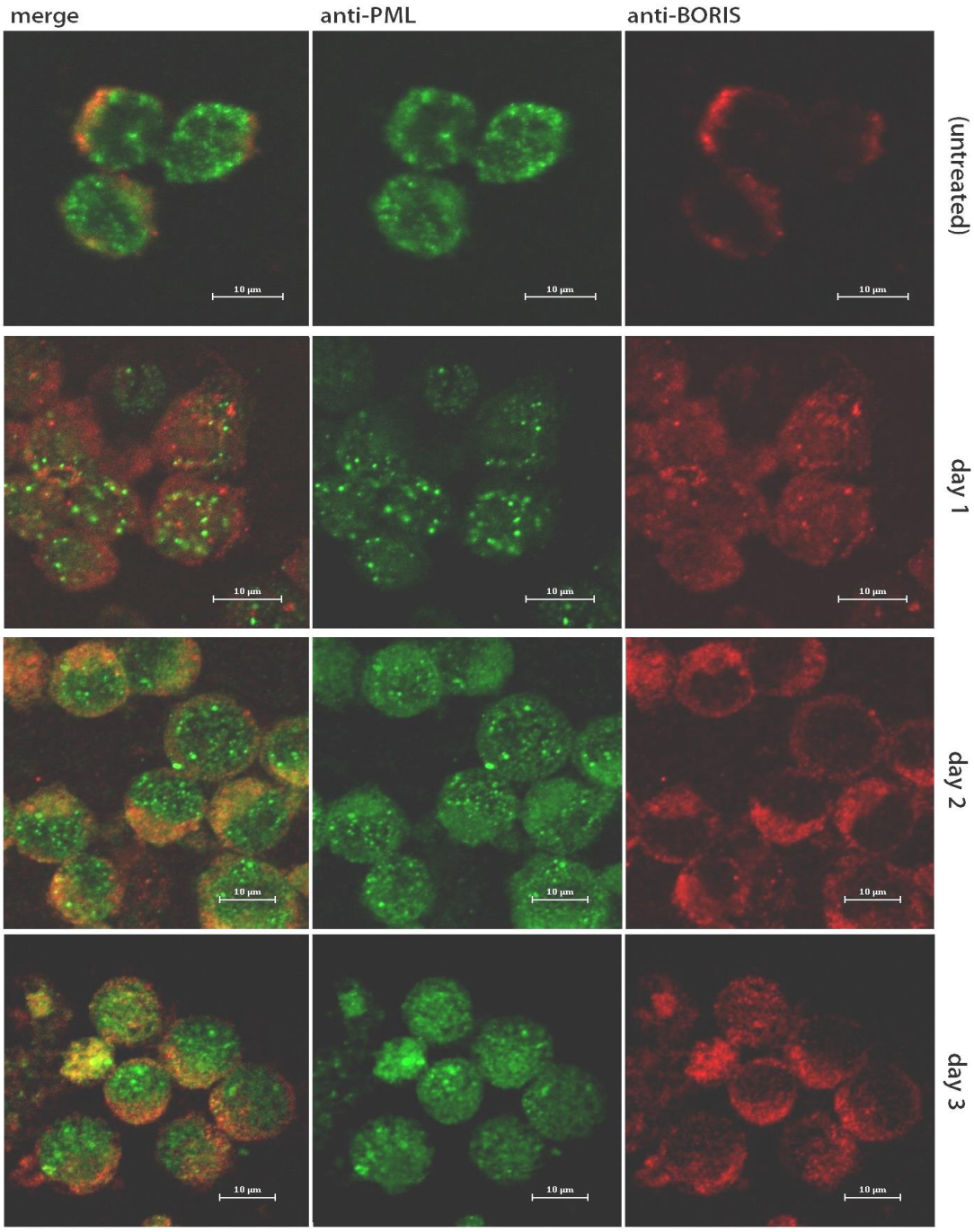

Figure 3.10: Localization of PML protein and CTCFL in ATRA- induced NB4 cells using immunofluorescence staining.

Anti-PML was labeled with Alexa fluor 488 (green signal) and anti-CTCFL was labled with Alexa fluor 555 (red signal). A typical microgranular pattern of the fusion protein PML-RARα was detected in non-differentiated NB4 cells (control) as well as ATRA-induced cells in day 1 and day 2. By day 3, degradation of microgranules and PML protein relocalization were evident as distinct speckles were formed. By day 4 and day 5 more than half of the induced cell population was undergoing apoptosis. A complete co-localisation of CTCFL with PML protein was not detected in untreated cells or in any stage of NB4 cells differentiation.

3.4 Discussion

We aimed to investigate a possible correlation of CTCFL with the function and physical characteristics of granulocytes. For this reason, a phenotypical and immunological profiling was to be established for the PML model cell line, NB4 cells, during their differentiation. The phenotyping of NB4 cells included the assessment of their morphology, their viability throughout the different stages of differentiation and the onset of apoptosis during differentiation. Phagocytosis and subsequent NO generation are major functional characteristics of PMNs which were included in the phenotyping of ATRA- induced NB4 cells. Promyelocytic leukemia (PML) Nuclear bodies (NB) were also investigated to serve a double proposes; as a confirmatory marker of differentiation and more importantly, investigating the possibility of CTCFL association in the differentiation and/or maturation of granulocytes. On the other hand, immunotypical profiling dictates the assessment of expression levels of both surface and intracellular molecules, known for their association with the immunological function of granulocyte (i.e. phagocytosis and microbial killing). These molecules would include clusters of differentiation (CD) and pathogen pattern recognition (PPR) molecules. Here, we have chosen three of the main receptors involved in granulocytes recognition and internalization of pathogens; TLR2, TLR4 and CD14. In addition, HLA-ABC (MHC class I) and HLA-DR (MHC class II), which are associated with antigen presentation and modulation of adaptive immunity. In parallel, profiling of CTCFL levels in granulocytes during their differentiation was also investigated by immunocytochemistry and Western blot in ATRA-induced NB4 cells. The addition 1 μM of ATRA every 24 hours for 3 days was the optimum conditions established after several optimizations with 1 μM, 2 μM or 5μM of ATRA over 6 days. Morphological study confirmed the maturation of NB4 cells after 3 days of ATRA- induction into culture. This was evident in the concentration of

nuclear material and its distinction from cytoplasm. Furthermore, maturation was evident with the decrease in NB4 cell size and the appearance of the typical metamyelocytes morphology (band granulocytes) and even few segmented granulocytes. These findings concurred with the viability test and the apoptosis assay which also showed cellular viability up until the third day of differentiation. However, the onset of apoptosis appeared to be after only 24 hours of introducing ATRA to culture, though, NB4 cells sustained viability with over 80% of the population in the first 72 hours. Cell necrosis was marked morphologically after 96 hours (4 days) of ATRA administration. On a functional level, the differentiation of NB4 cells also increased phagocytic activity as well as the generation of nitric oxide. NO production results correlate with our phagocytosis assay findings and confirm a further non-impaired killing capacity of differentiated NB4 cells. In this investigation we intended to profile NB4 cells expression of their immunological receptors before and after differentiation where these findings should be correlated later with CTCFL expression. Profiling of ATRA- induced NB4 cells immunotype demonstrated a minor elevation but insignificant in the expression of TLR2 and CD14 on the surface of treated cells. The most marked elevation is in the exponential expression levels of the MHC class I surface molecules (HLA-ABC) during the first 72 hours of ATRA administration, while its intracellular levels appeared to be in its highest levels after 24 hours of ATRA administration to cells, followed by an exponential decline over the next 48 and 72 hours. This suggests ATRA to enhance the expression of HLA-ABC on the surface of NB4 cells. It is know that HLA class I serves as a cell surface recognition molecule during the interactions of neutrophils with the endothelium, fibroblasts, other inflammatory cells, and pathogens (Mantegazza et al., 2013). The expression levels of the second class of MHC molecules, HLA-DR, remained unaltered in the differentiated NB4 cells. The class II MHC molecules are mainly

involved in antigen presentation, and while PMNs could present antigens under specific stimulation (Abdallah et al., 2011), PMNs are considered non-professional antigen presenting cells as they do not express class II MHC molecules readily. The levels of the other assessed receptors (TLR2, TLR4 and CD14) on the surface of NB4 cells did not change before and after treatment with ATRA and were considered insignificant. In parallel to immunophenotype profiling of differentiating NB4 cells, profiling of CTCFL levels in ATRA-induced cells were substantiated with two methods; Western blot and immunocytochemical staining. Both techniques used an IgM antibody for the detection. Previously, CTCFL was reported not to exist in peripheral granulocytes of healthy individuals (D'Arcy et al., 2006). Another study also reported CTCFL expressed in several lymphoid leukemia cell lines and also the myeloid leukemia cell line HL-60, though at different levels (Renaud et al., 2007). However CTCFL was never investigated in the NB4 cell line. At the same time, CTCFL paralogue CTCF was reported to be expressed at low levels in the NB4 cell line. Data acquired with both Western blot and immunostaining detected CTCFL to be at an elevated levels in undifferentiated NB4. These levels seemed to decline continuously over the next 72 hours of introducing the first dose of ATRA. The protein levels disappeared completely after 96 hours (4 days) of cell treatment. These finding suggest that CTCLF levels decline with the progress of neutrophilic differentiation but does not disappear in the cell until the onset of cell death after 96 hours. Immunofluorescent (IF) staining was employed to confirm cellular differentiation on morphological bases with a key differentiation marker, promyelocytic leukemia nuclear bodies (PML-NBs). Also, preliminary study of CTCFL colocalisation with PML-NB was also investigated in the same IF preparations of non-induced and ATRA-induced NB4 cells. For this, each protein was stained with a different fluorophore in the cells. One of the main characteristics of APL is the expression of the fusion protein

PML-RARα, which appears as numerous microgranules in the nucleus of the cell. On the other hand, the wild-type PML protein in differentiated myeloid cells appear as concentrated, less distributed speckles. Our data showed that NB4 cells do express these numerous microgranules in their non-differentiated state. After 72 hours of introducing ATRA to culture, NB4 differentiation was evident as the introduction of ATRA led to the degradation of the fusion protein PML-RARα and the re-localization of PML protein as concentrated speckles. In the co-localisation study we aimed to examine the possible involvement of CTCFL in neutrophilic differentiation. In vivo, the interaction of two proteins in the nucleus bring them close spatial proximity and thus, the two proteins would appear colocalised or overlapping under microscopic examination. Although a mere coexistence of the two proteins in a close physical proximity is not in itself a solid proof of functional association, it can however provide valuable clues clarifying if there is any common structural characteristics or functional interactions. Here, colocalisation was marked by the partial or complete overlap of fluorescent signals of the two protein at the same region of interest (ROI). The spatial distribution of the two proteins was examined in a three-dimensional images acquired by confocal microscope before and during the differentiation of NB4 cells. The assessment of co-localisation by cell imaging is a qualitative analysis rather than a quantitative one. For the quantification of the colocalisation event we relied on intensity correlation coefficient based (ICCB) analysis as Pearson's correlation coefficient (PCC) and Mander's coefficient (*R*) of ROI then global colocalisation signals in the image. The calculated Mander's coefficients indicated a partial colocalisation of the two proteins during the differentiation of the NB4 cells. Unlike complete colocalisation, it is not possible to have a clear conclusion if the two proteins have indeed positive correlation or if the two proteins just randomly overlap in

the case of partial colocalisation. To overcome this, the acquired images should be subjected to an object-based analysis.

Chapter 4 - **The Effect of Breast Malignancy on the Expression of CTCFL and Immunological Receptors in Polymorphonuclear Neutrophils**

4.1 Introduction

A previous study demonstrated that CTCFL was elevated at different levels within primary breast cancer cells and also in 16 different malignant breast carcinoma cell lines (D'Arcy et al., 2008), whilst present at much lower levels in non-malignant breast cell lines, if expressed at all. The same study also highlighted a higher frequency of CTCFL activation in metastatic cell lines and primary tumours than in primary lesions. CTCFL has also been detected in the peripheral PMNs of breast cancer patients (D'Arcy et al., 2006), where significant amounts of CTCFL were found to be expressed in the PMNs of 88.5% of the tested breast cancer patients. The studied group included patients with all types of breast tumours, including ductal carcinoma in situ (DCIS). According to D'Arcy et al. (2006), the increase in CTCFL levels in the PMNs of breast cancer patients was detected even in very small-sized breast tumours (>10 mm) and levels were found to correlate strongly with tumour size. In contrast, CTCFL was not detected in PMNs collected from healthy donors or patients with non-cancerous diseases, such as inflammation or trauma.

However, Jones et al. (2011) suggested that CTCFL expression is not exclusive to breast carcinomas and may exist in other somatic cells. Prior to this study the expression and function of CTCFL in different somatic cells had not been investigated as vigorously as its paralogue, CTCF. CTCFL expression has yet to be investigated further in different cell types and at different stages during a cell's life cycle. Knowing the pattern of CTCFL

expression at the different stages of development for a specific cell type, and the interactions of CTCFL with other transcription factors at different developmental stages could explain the function of CTCFL in different cell types. Furthermore, it could provide insight as to whether CTCFL has a role in the transition of a cell into a malignant state.

D'Arcy et al. (2006) stated that the origin of CTCFL expression in the PMNs of breast cancer patients had not been investigated, although the authors suggested two possible sources for the origin. The first was the release of chemical signals into the blood stream of a patient (i.e. blood serum) which activate or up-regulate CTCFL expression, while the second potential origin of CTCFL expression may be due to the phagocytosis of tumour cells by PMNs. In the latter scenario, CTCFL would be passively accumulated rather than up-regulated within PMNs. Furthermore, it is unknown whether this aberrant activation has any contribution to the advancement of breast cancer or metastasis.

The ability of PMNs to recognise malignant cells which possess altered genetic profiles is well established (Colombo et al., 1992; Di Carlo et al., 2001). However, PMNs are driven towards either a pro-tumoural or an anti-tumoural role depending on the microenvironment that is created due to the cancer state (Bolte and Cordelieres, 2006). In many cases, despite the inherent function in initiating an immunological response against tumour cells (anti-tumoural), PMNs eventually contribute to the spread of a tumour (pro-tumoural) by promoting angiogenesis and tumour cell metastasis (De Larco et al., 2004).

CTCFL is suggested to be a transcription factor which is involved in cells survival and self-renewal, and is capable of genome-wide alterations in gene expression through altering DNA methylation patterns (epigenetic programming) of gene promoters or by direct regulation of its DNA targets (Monk et al., 2008). Thus the alteration of CTCFL expression in PMNs could alter the expression of genes controlling the survival,

inflammatory, and more importantly their immunological responses of PMNs against malignant cells.

This chapter investigates the origin of CTCFL altered expression in the PMNs of breast cancer patients and attempts to determine if the expression of CTCFL is actively up-regulated through chemical signals released into the blood serum of breast cancer patients or is simply present in PMNs due to phagocytosis. Initially, levels of CTCFL in PMNs from healthy individuals and breast cancer patients were assessed. In addition, the immunophenotype of CTCFL-expressing PMNs (i.e. the cell line, NB4) when exposed to serum from breast cancer patients was compared to their immunophenotype when exposed to serum from healthy individuals.

4.2 Experimental Approach

The aim of this chapter is to investigate the origin of CTCFL expression and the effect that breast cancer may have on the level of CTCFL expression in PMNs. CTCFL expression has been suggested to be altered as a result of the release of chemical signals (e.g. cytokines) into the blood stream of breast cancer patients or to accumulate in PMNs following phagocytosis of tumour cells. To investigate this observation several experiments were designed. Firstly, the levels of CTCFL in the PMNs of healthy donors and breast cancer patients were assessed via ICC staining. The mean IRS of the ICC stained smear was calculated based on the grading of positively stained cells and the intensity of the staining as described by Chen et al. (2012). Mouse anti-CTCFL antibody was used as the primary antibody and goat anti-mouse as the secondary conjugated antibody. Staining for CTCFL was visualised using a DAB substrate, and haematoxylin was used for cellular counterstaining of the smears. An IgM isotype control (mouse anti-IgM) was performed on a duplicate smear from one patient and one donor during each

staining experiment in order to detect non-specific staining. Negative controls were performed for each donor or patient smear, whereby the primary antibody was omitted to confirm non-specific binding was not present. The PMNs from 27 patients and 10 healthy donors were collected to determine if the IRS values for CTCFL levels in the two investigated populations were statistically significantly different using the student's *t*-test. *P*-values of ≤ 0.05 were used to indicate if there was a significant difference between the IRS values for CTCFL expression in the two populations.

In the next set of experiments, the existence of components within serum that could influence the expression of CTCFL in human PMNs was investigated. This was achieved through culturing PMNs collected from healthy donors with serum from breast cancer patients and assessing CTCFL levels via ICC staining. The plasma of donors and patients were not considered in these experiments, as plasma contains a wide array of coagulation factors that cause agglutination of the cultured cells and so sera was used instead. PMNs and serum samples were collected from six healthy donors and six breast cancer patients; the samples were not pooled. PMNs from a healthy donor were cultured with serum from a breast cancer patient, and as a comparative control, the PMNs of the healthy donor where cultured with their own serum. Similarly, PMNs from a breast cancer patient were cultured with serum from a healthy donor, and also with serum from the breast cancer patient. Based on the results of earlier trials conducted within this laboratory, PMNs were cultured with 50 % human serum in RPMI media in order to sustain cell viability. Isotype controls and negative controls for ICC staining were as described above. CTCFL levels in CTCFL-expressing PMNs and the NB4 cell line were also assessed via Western blot analysis.

To examine whether CTCFL in PMNs is due to phagocytosis of cancer cells, peripheral PMNs from two healthy donors were co-cultured with the metastatic adenocarcinoma cell

line, CAMA-1, and CTCFL levels were assessed via ICC staining. Transwell chambers were not employed when co-culturing the PMNs and CAMA-1 cell line, as it is easy to distinguish between these two cell types due to morphological differences. The selection of the CAMA-1 cell line for co-culture experiments was based on an assessment of CTCFL expression levels in adenocarcinoma cells lines, where CAMA-1 was reported as possessing the highest CTCFL levels (D'Arcy et al., 2008). CTCFL levels were also assessed in the NB4 cell line when co-cultured with CAMA-1 via Western blot analysis, and in these experiments the NB4 cells were contained in a Transwell chamber. Separating the two cell types by a microporous membrane allowed both cell lines to share the same media and thus allow chemicals and signals to be exchanged, whilst ensuring that the two populations did not mix and could be assessed separately through Western blot analysis.

Finally, the surface receptors involved in innate immunity and adaptive immunity were assessed for their presence on the surface of NB4 cells, which is a CTCFL-expressing PMN cell line, when incubated with serum from breast cancer patients or healthy donors. The immunological markers assessed were the main functional receptors involved in the detection and elimination of pathogenic antigens, such as CD14, TLR2 and TLR4, and also the receptors involved in antigen presentation to adaptive immunity cells, namely HLA-ABC (class I MHC) and HLA-DR (class II MHC) molecules.

4.3 Results

4.3.1 Comparison of CTCFL Levels in Polymorphonuclear Neutrophils from Breast Cancer Patients and Healthy Donors

The peripheral granulocyte fraction was obtained from 27 female patients diagnosed with breast cancer. IICC staining of the granulocyte fraction was performed using an anti-

CTCFL antibody and smears were assessed for CTCFL levels of expression by calculating the IRS. As a negative control for the staining protocol a duplicate smear was prepared for each sample whereby the primary antibody was omitted from the staining protocol. An IgM isotype control was performed for every staining experiment for each patient and donor (Figure 4.1).

CTCFL protein was detected in the majority of the breast cancer patients (Table 4.1). According to the IRS values for the CTCFL levels in the granulocytes of the assessed cohort, 52 % of breast cancer patients (14 patients) demonstrated high levels of CTCFL expression, while 30 % (8 patients) had moderate levels of CTCFL expression, and 18 % (5 patients) revealed a low level of CTCFL expression; the mean IRS value for CTCFL expression in this cohort was 8 ± 3 (Table 4.1). The expression of CTCFL in the granulocytes of the breast cancer patients was compared to the expression of CTCFL in the granulocytes of 10 healthy donors (2 males and 8 females), who had never been diagnosed with any type of cancer and did not have a familial history of cancer. The ICC analysis revealed that 8 out of the 10 healthy donors had low levels of CTCFL expression in their granulocytes (Table 4.2), and the mean CTCFL IRS value for this cohort was 2.9 ± 1.5. Using the student's *t*-test, the difference in IRS values between the two assessed groups (breast cancer patients and healthy donors) was determined to be statistically significant, with a P value ≤ 0.0001 (Figure 4.2). These findings suggest that CTCFL is expressed at low levels in the PMNs of healthy donors, while the PMNs of breast cancer patients expressed much higher levels of CTCFL.

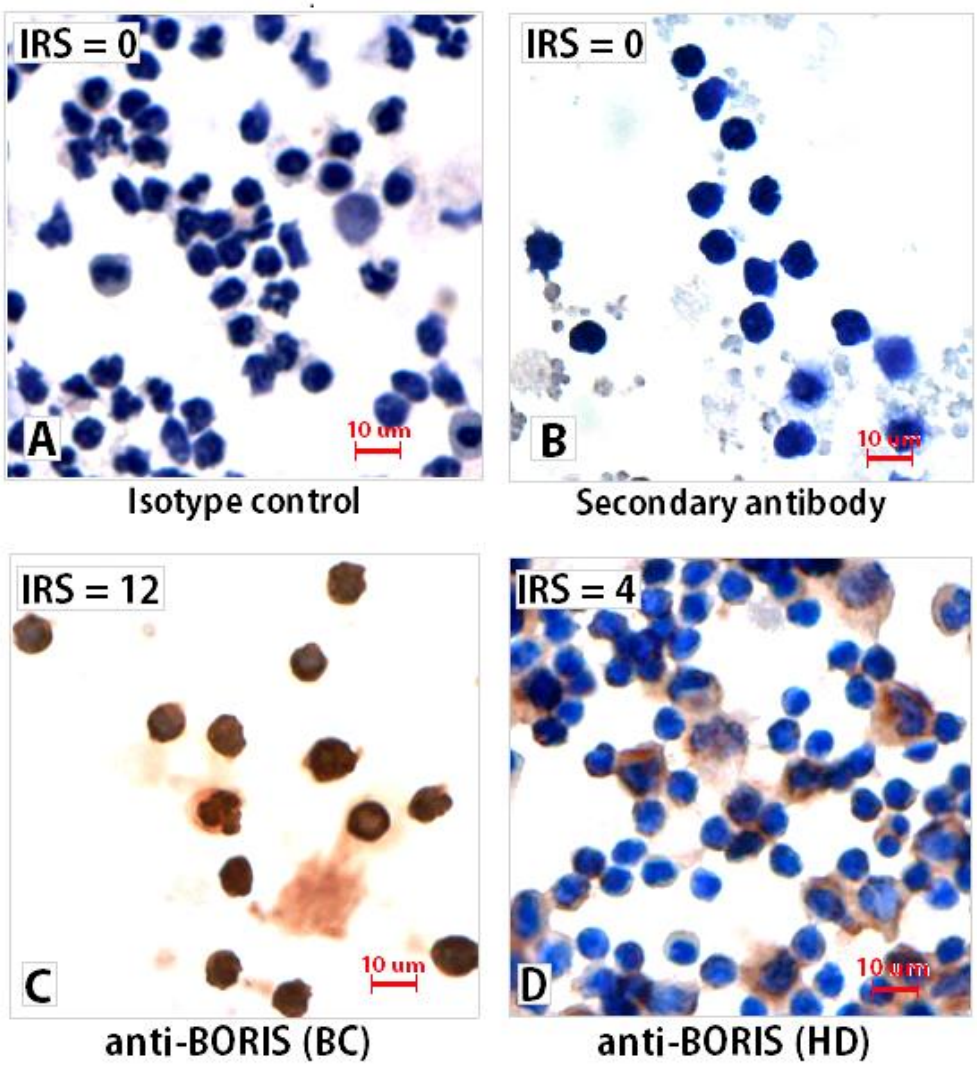

Figure 4.1: ICC staining of peripheral human PMNs and corresponding IRS values for CTCFL expression

(A) Peripheral human PMNs from breast cancer patients were incubated with an IgM antibody as an isotype control. **(B)** As a negative control for each ICC staining experiment, a smear from one breast cancer patient and one healthy donor were incubated with only the secondary antibody only (goat anti-mouse IgM), and the primary antibody was omitted. **(C** and **D)** Smears prepared from breast cancer patients (BC) and healthy donors (HD) were incubated with an anti-CTCFL antibody (primary) and a biotinylated goat anti-mouse IgM (secondary) antibody and staining was visualised using DAB (brown) with haematoxylin used as a counter stain (blue). The brown staining reflects the presence of CTCFL protein in the PMNs of patients and donors. The IRS calculation depends on the grades for intensity and percentage of positive cells stained brown. Images were acquired using a phase contrast light microscope at 100× magnification.

Table 4.1: Assessment of CTCFL levels in peripheral granulocytes obtained from 27 patients diagnosed with breast cancer

ICC staining of granulocyte smears was performed using an anti-CTCFL antibody and the percentage of CTCFL-positive cells and staining intensity graded as previously described in section 2.7.3.5. The IRS for CTCFL expression in the granulocytes was calculated by multiplying the CTCFL-positive percentage grade with the staining intensity grade.

Patient ID	Percentage of CTCFL-positive cells grade	Staining intensity grade	IRS
BC1	4	3	12
BC2	3	3	9
BC3	3	2	6
BC4	4	2	8
BC5	4	3	12
BC6	3	2	6
BC7	3	2	6
BC8	2	2	4
BC9	3	1	3
BC10	3	2	6
BC11	3	3	9
BC12	4	1	4
BC13	4	3	12
BC14	3	3	9
BC15	4	2	8
BC16	4	1	4
BC17	4	3	12
BC18	4	3	12
BC19	4	2	8
BC20	4	3	12
BC21	2	3	6
BC22	4	1	4
BC23	4	3	12
BC24	4	3	12
BC25	4	3	12
BC26	4	3	12
BC27	4	3	12

Table 4.2: Assessment of CTCFL levels in peripheral granulocytes obtained from 10 healthy donors

ICC staining of granulocytes smears was performed using an anti-CTCFL antibody and the percentage of CTCFL-positive cells and staining intensity graded as previously described 2.7.3.5. The IRS for CTCFL expression in the granulocytes was calculated by multiplying the CTCFL-positive percentage grade with the staining intensity grade.

Donor ID	Percentage of CTCFL-positive cells grade	Staining intensity grade	IRS
HD1	2	2	4
HD2	2	2	4
HD3	1	2	2
HD4	2	2	4
HD5	1	1	1
HD6	0	0	0
HD7	1	1	1
HD8	4	1	4
HD9	1	3	3
HD10	0	0	0

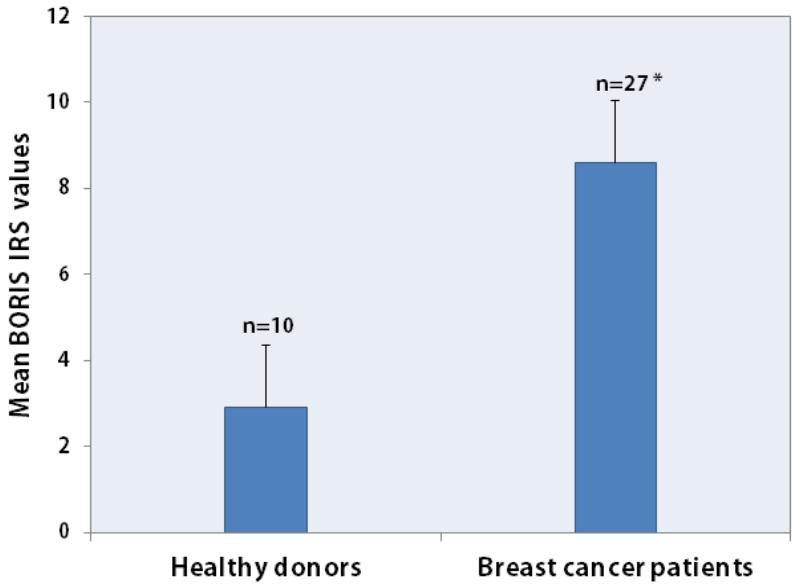

Figure 4.2: Comparison between CTCFL levels represented as IRS values in the granulocytes of healthy donors and breast cancer patients

Key: *n* indicates the number of samples in the assessed cohort and * indicates a statistically significant result with a *P* value ≤ 0.05.

4.3.2 The Effect of Serum from Breast Cancer Patients on the Expression of CTCFL Levels in Peripheral Human Polymorphonuclear Neutrophils

4.3.2.1 Immunocytochemistry Analysis

To investigate if the source of CTCFL elevation in the PMNs of breast cancer patients was due to factors that exist within the serum of a cancer patient, PMNs and serum samples from six healthy donors and six breast cancer patients were collected. The PMNs were cultured with human serum for 72 hours and in order to maintain cell viability during culture, a 1:1 ratio of serum to media was employed. The PMN sample obtained from each healthy donor was divided into two, and cultured with either serum from the same donor as an autologous control or serum from a breast cancer patient. Similarly, PMNs from a breast cancer patient were cultured with serum from the same patient as an autologous control or with serum from a healthy donor. Smears were prepared for all PMNs after 24 hours, 48 hours and 72 hours in order to observe any changes in CTCFL levels during culture. Smears were also prepared from the PMNs directly after fractionation and before culturing (0 hours). ICC staining was carried out using a mouse anti-CTCFL as the primary antibody and biotinylated goat anti-mouse IgM as the secondary antibody. A negative control was performed with every staining session using only the secondary antibody to confirm staining specificity. The ICC staining for the cultured PMNs of a breast cancer patient and the PMNs of a healthy donor is shown in Figure 4.3 as an example. Based on the intensity of the staining and the percentage of positive cells, IRS values were calculated (Figure 4.4). The IRS values reflect the level of CTCFL expression in the PMNs of the assessed individuals. As detected earlier in section 4.3.1, the immunoreactive scoring of smear prepared of PMNs before culture (0 hours) showed the PMNs of healthy donors to have low levels of CTCFL, while breast cancer patients have high levels of CTCFL. The PMNs of the same patients and donors appeared

to sustain the same levels of CTCFL after culture with autologous serum over 24 and 48 hours. The levels in the PMNs cultured with autologous serums have declined or even disappeared after 72 hours in culture. Thus, Figure 4.4 display data collected after 0, 24, and 48 hours only. Comparing CTCFL IRS values for healthy donor PMNs before culture (0 hours) and following culture with breast cancer patient serum, suggested that the IRS values appeared to slightly increase after 48 hours (IRS = 4 in 2 donors, IRS = 3 in 1 donor, IRS = 2 in 1 donor and IRS = 1 in 2 donors). However, this slight increase was considered to be insignificant, as an IRS value between 0 and 4 reflects low level protein expression. Consequently, CTCFL was found to be expressed at low levels in healthy donor PMNs before and after culture with breast cancer patient serum. In contrast, the IRS values of breast cancer patient PMNs appeared to noticeably decline after 48 hours when cultured with healthy donor serum, yielding a mean IRS value of 2. This indicates a decline in CTCFL levels in the PMNs of breast cancer patients when cultured with serum from a healthy donor. The expression of CTCFL protein in the PMNs of breast cancer patients and healthy donors appeared to eventually be 'lost' after 72 hours of culture, regardless of the source of serum they were cultured in.

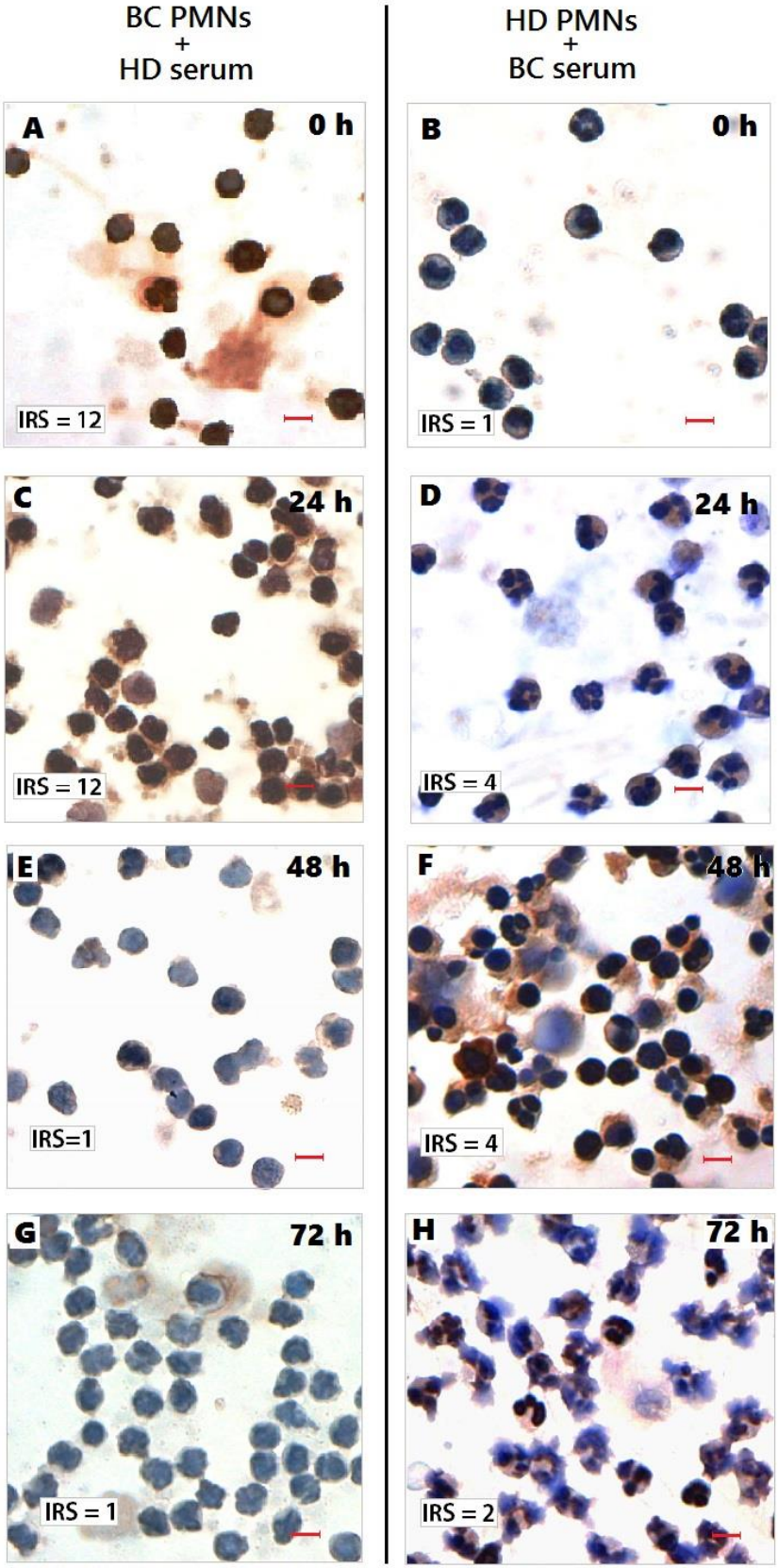

Figure 4.3: ICC staining example showing CTCFL levels in the PMNs of a breast cancer patient cultured with the serum from a healthy donor over 72 hours (left images), in comparision to the PMNs of a healthy donor when cultured with a the serum of a breast cancer patient over the same period of time (right images).

Peripheral PMNs from one breast cancer patient were cultured with the serum of one healthy donor (BC PMNs + HD serum), whilst PMNs from one healthy donor were cultured with serum from breast cancer patients (HD PMNs + BC serum). 1:1 ratio of serum to RPMI culture media was used. ICC staining for CTCFL was performed using a mouse anti-CTCFL as a primary antibody and biotinylated goat anti-mouse IgM as a secondary antibody. Staining was visualised using DAB (brown staining) and haematoxylin was used for cellular counterstaining (blue staining). The left column shows ICC staining of PMNs from one breast cancer patient **(A)** before culture (0 hours) and **(C)** after 24 hours, **(E)** 48 hours, and **(G)** 72 hours culture with serum from a healthy donor. The right column shows ICC staining of PMNs from a healthy donor **(B)** before culture (0 hours) and **(D)** after 24 hours, **(F)** 48 hours and **(H)** 72 hours culture with serum from a breast cancer patient. All images were taken using a phase contrast microscope at 100× magnification, bar = 10 μm. Key: HD = healthy donor, BC = breast cancer patient

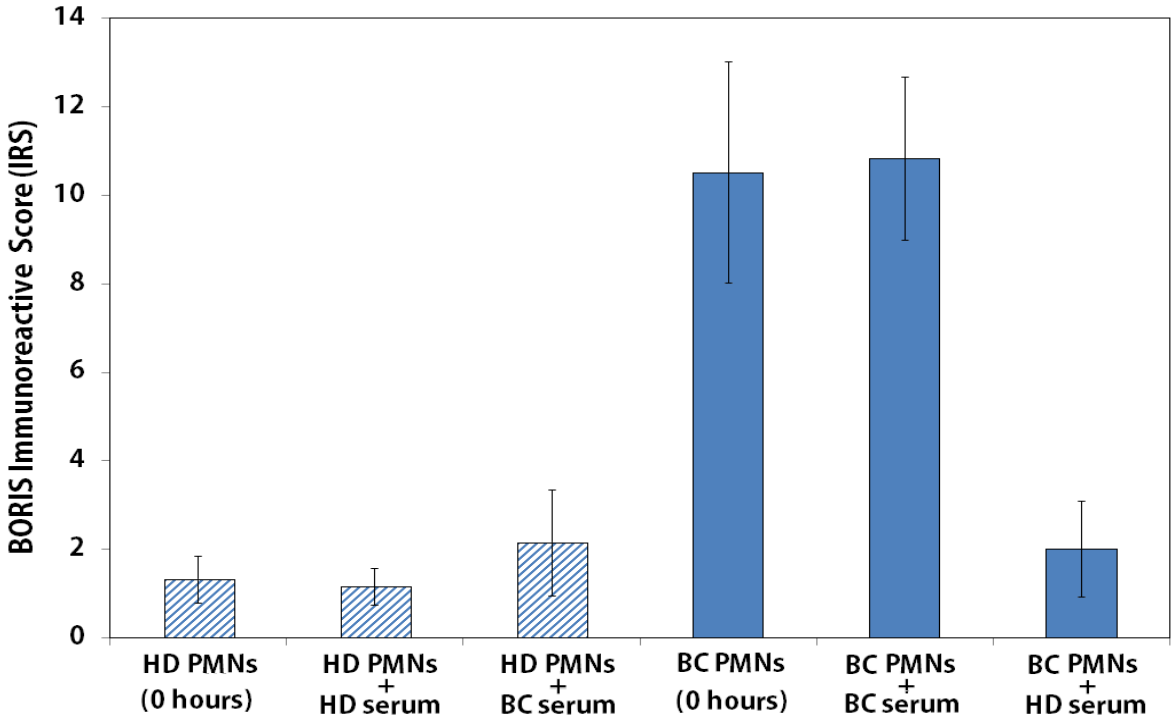

Figure 4.4: Mean IRS values for CTCFL expression in peripheral PMNs after culture with human serum for 48 hours.

PMNs and sera were collected from six breast cancer patients and six healthy donors (*n* = 6). PMNs collected from each healthy donor were cultured with serum from the same donor (HD PMNs + HD serum) as an autologous control or the serum of one breast cancer patient (HD PMNs + BC serum). PMNs collected from a breast cancer patient were cultured with serum from the same patient (BC PMNs + BC serum) as an autologous control or serum from one healthy donor (BC PMNs + HD serum). The mean CTCFL IRS values for the cultured PMNs (breast cancer patients and healthy donors) were compared to the mean IRS values of PMNs before culture (no culture). Diagonally striped columns represent the mean CTCFL IRS values for healthy donor PMNs, while the solid blue columns represent the mean CTCFL IRS values for breast cancer patient PMNs. Key: HD = healthy donor, BC = breast cancer patient.

4.3.2.2 Western Blot Analysis

A further experiment was performed in order to study the effect of serum from breast cancer patients on the expression of CTCFL in PMNs. An equal number of NB4 cells were cultured for 24 hours in RPMI media supplemented with 50 % serum from either a healthy donor serum or a breast cancer patient. As a control, NB4 cells were also cultured in RPMI media supplemented with 50 % FBS. After 24 hours, whole cell lysates were prepared from the cultured NB4 cells and were subjected to Western blot analysis (Figure 4.5). Probing for the CTCFL protein was accomplished using a mouse anti-CTCFL (primary) antibody and FITC-conjugated anti-mouse IgG (secondary) antibody. CTCFL was detected at the expected molecular weight (~83 kDa) in all three samples, although the signal strength for CTCFL in NB4 cells cultured with serum from a healthy donor was slightly lower than the signal obtained for NB4 cells cultured with serum from a breast cancer patient, although NB4 cells exhibited significant CTCFL expression when cultured with serum from a healthy donor. Interestingly, NB4 cells demonstrated a higher level of CTCFL expression when cultured with media supplemented with FBS than when cultured with human serum.

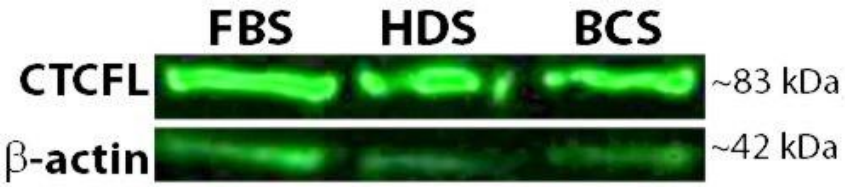

Figure 4.5: Western blot analysis of CTCFL levels in NB4 cells when cultured with serum from a breast cancer patient or a healthy donor

NB4 cells were cultured in RPMI media supplemented with 50 % serum from either a breast cancer patient (BCS) or a healthy donor (HDS). CTCFL levels in the NB4 cells cultured with human sera were compared to CTCFL levels in NB4 cells cultured with 50 % FBS-supplemented RPMI media. Whole cell lysates were prepared after 24 hours of culture and β-actin was used as a loading control. A mouse anti-CTCFL antibody was used at a concentration of 1:500, while the antibody for detecting β-actin was used at a concentration of 1:15000. The probed proteins were visualised using a FITC-conjugated anti-mouse IgG.

4.3.3 The Effect of Adenocarcinoma Cells on CTCFL levels in Polymorphonuclear Neutrophils

4.3.3.1 Immunocytochemistry Analysis

To investigate the effect of direct contact with adenocarcinoma cells on the expression levels of CTCFL in PMNs, PMNs from two healthy donors were co-cultured with the CAMA-1 adenocarcinoma cell line for 48 hours. Cells were incubated in standard complete media (10 % FBS supplemented RPMI) and no human serum was used. ICC staining was performed on smears prepared of co-cultured and CAMA-1 cells only using an mouse anti-CTCFL (primary) antibody and a biotinylated goat anti-mouse IgM (secondary) antibody. The staining of the smears was visualised using DAB substrate and counterstained using haematoxylin (Figure 4.6). PMNs from both donors showed a low level of positive staining prior to co-culture with CAMA-1 cells (IRS values for CTCFL = 0 and 2), corresponding to a low level of CTCFL expression in both healthy donors. CAMA-1cells express high levels of CTCFL, which was confirmed via ICC staining (IRS = 12). After 48 hours of co-culture, the IRS values for CTCFL expression increased slightly to 4 for both donors. This value is still considered to represent low level expression of CTCFL and thus this increase was not a significant elevation in CTCFL levels. Interestingly, the intensity of CTCFL staining for CAMA-1 appeared to be less in co-culture, as the IRS value calculated was 8, reflecting a moderate expression level of CTCFL.

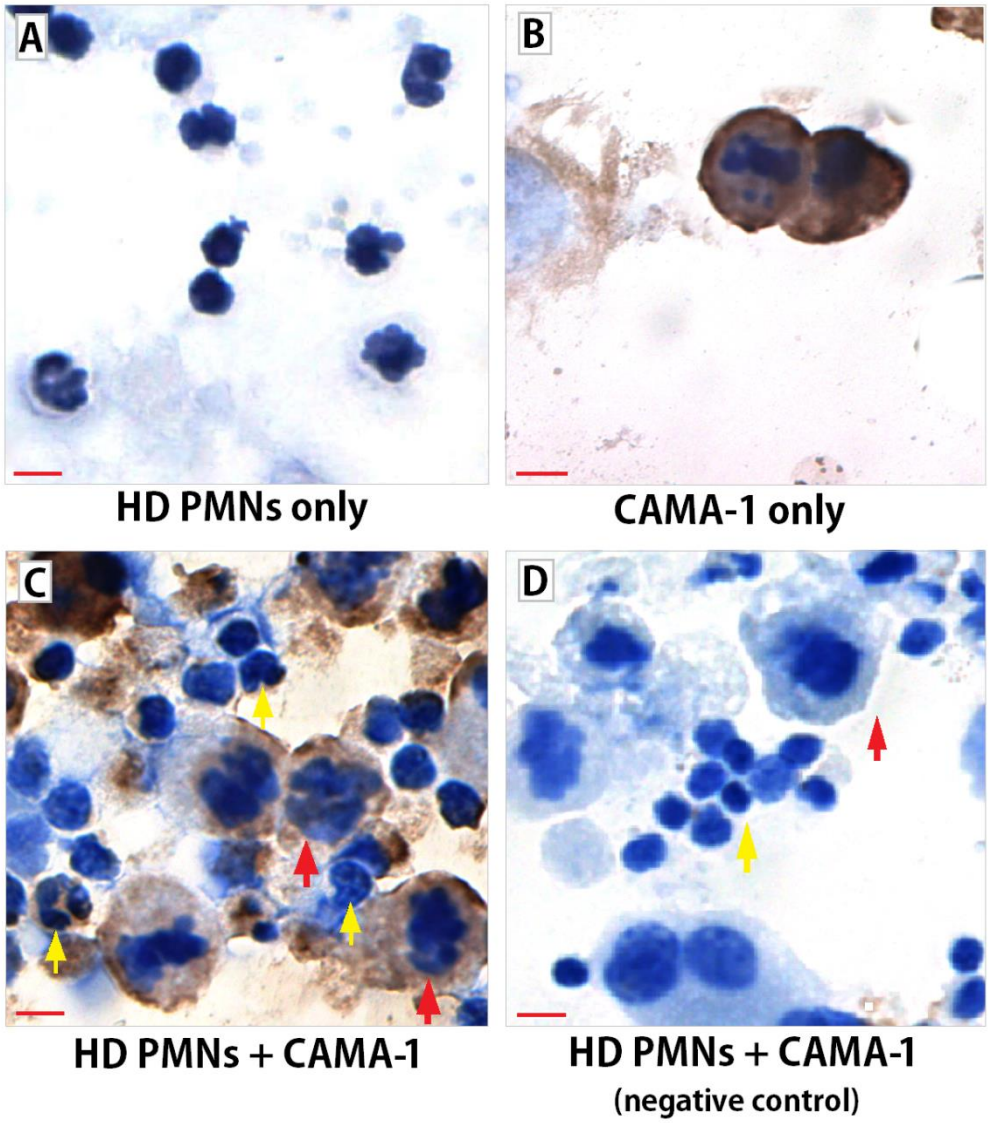

Figure 4.6: ICC staining of co-cultured peripheral PMNs from a healthy donor with CAMA-1 cells

Before co-culturing commenced, a smear was prepared from (**A**) the PMNs from the healthy donor, and (**B**) the CAMA-1 cells. ICC staining of the smears was performed using mouse anti-CTCFL (primary) antibody and biotinylated goat anti-mouse IgM (secondary) antibody. A further smear (**C)** was prepared after 48 hours of co-culture with the breast cancer cell line, CAMA-1, together with a negative control (**D**) where the primary antibody was omitted. CAMA-1 cells were distinguished from PMNs according to size. The brown staining indicates CTCFL expression in the co-cultured cells, whilst haematoxylin was used as a counterstain (blue stain). The red arrows indicate CAMA-1 cells and the yellow arrows donor PMNs. All images were taken using a phase contrast microscope at 100× magnification, bar = 10 μm. Key: HD = healthy donor.

4.3.3.2 Western Blot Analysis

Further experiments were undertaken to investigate the level of CTCFL in human granulocytes when exposed to a metastatic breast cancer cell line via Western blot analysis. In the first experiment, CAMA-1 cells were seeded in a 6- well plate and after 24 hours a Transwell chamber was placed on top of the well seeded with CAMA-1 cells. The chamber was filled with standard complete culture media (10 % FBS in RPMI) and seeded with NB4 cells. Equal numbers of NB4 and CAMA-1 cells were co-cultured and after 48 hours of co-culture the NB4 cells were collected from the Transwell chamber and whole cell lysates prepared for Western blot analysis. The level of CTCFL expressed in the co-cultured NB4 cells was compared to CTCFL levels in NB4 cells grown independently (Figure 4.7). Using an anti-CTCFL antibody, CTCFL was detected at the expected molecular weight (~83 kDa). The analysis revealed that NB4 cells maintained the same levels of CTCFL after 48 hours of co-culture with CAMA-1 cells.

In the second experiment, Western blot analysis was performed on PMNs from a healthy donor. These PMNs were co-cultured with CAMA-1 cells in the same manner described above for NB4 cells and whole cell lysates were prepared for the co-cultured PMNs after 48 hours incubation (Figure 4.8). CTCFL levels in the co-cultured PMNs from the healthy donor were compared to CTCFL levels in donor PMNs not co-cultured with CAMA-1. The analysis revealed that CTCFL was expressed at very low levels in the peripheral PMNs from the healthy donor before culture; however, CTCFL levels were elevated following co-culture with the breast cancer cell line, compared to non-cultured PMNs from the same donor.

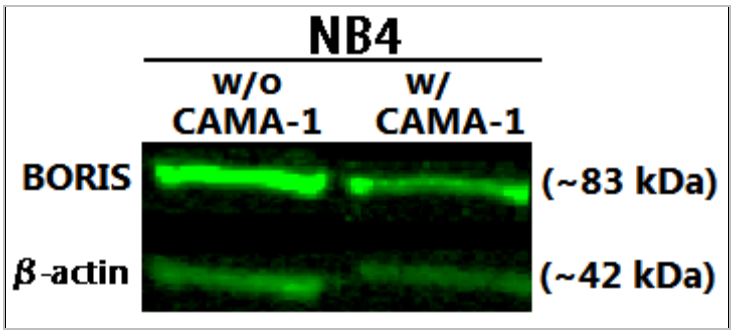

Figure 4.7: Western blot analysis of CTCFL levels in the human granulocytic cell line NB4 when co-cultured with the adenocarcinoma cell line CAMA-1 for 24 hours

The left lane represents CTCFL levels in NB4 cells cultured in standard growth media (10% FBS in RPMI) without co-culture with CAMA-1 cells (w/o CAMA-1), whilst the right lane shows NB4 cells after 24 hours of co-cultured with CAMA-1cells (w/CAMA-1) in standard growth media and a Transwell chamber. Using an anti-CTCFL antibody, bands were detected at around 83 kDa, which correspond to the CTCFL protein. β-actin was used as a loading control and was detected at around 42 kDa using an anti-β–actin antibody. Key: w/o CAMA-1 = without CAMA-1, w/ CAMA-1 = with CAMA-1.

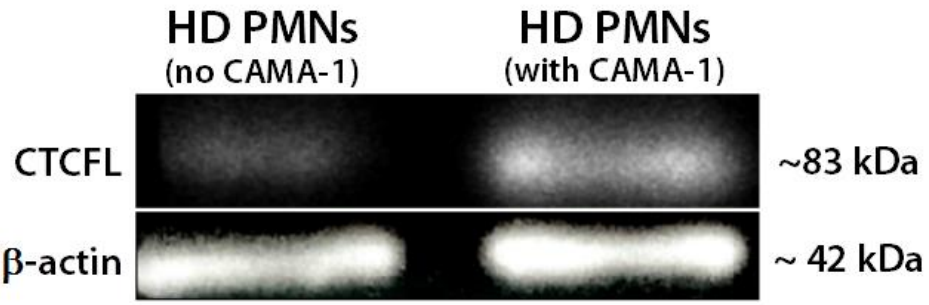

Figure 4.8: Western blot analysis of CTCFL expression in human PMNs when co-cultured with the adenocarcinoma cell line CAMA-1 for 48 hours

The left lane represents CTCFL expression in healthy donor PMNs before culture, whilst the right lane shows CTCFL expression in healthy PMNs after 48 hours of co-culture with CAMA-1 cells in standard growth media and a Transwell chamber. Using an anti-CTCFL antibody, bands were detected at around 83 kDa, which correspond to the CTCFL protein. β-actin was used as a loading control and was detected at around 42 kDa using an anti-β–actin antibody.

4.3.4 Flow Cytometric Analysis of Immune System Receptors Present on Polymorphonuclear Neutrophils Following Culture with Serum from a Breast Cancer Patient

This experiment was performed to assess the expression patterns of immune system receptors on CTCFL-expressing PMNs (i.e. NB4 cells) when exposed to serum from a breast cancer patient compared to when exposed to serum from a healthy donor. Equal numbers of NB4 cells were cultured in 50 % serum collected from either a healthy donor or a breast cancer patient. The experiment was repeated with serum from two additional healthy donors and two further breast cancer patients (n=3). Flow cytometric analysis of immune surface receptors expression was performed after 24 hours of incubation with the serum. The NB4 cells were assayed for surface receptors using immunostaining via the corresponding antibodies. In order to detect any auto-fluorescence, NB4 cells cultured with serum from different individuals were not subjected to any immunostaining to yield an 'untreated' control. An IgG isotype control was also employed as a negative control. The investigated innate immune system receptors were CD14, TLR2 and TLR4, and the adaptive immune system receptors included HLA-ABC and HLA-DR. The levels of all the investigated receptors were compared to the levels in NB4 cells cultured in standard complete media (RPMI supplemented with 10 % FBS), i.e. without any human serum present (Figure 4.9). The NB4 cells showed a higher level of expression for the investigated surface receptors when exposed to serum from a breast cancer patient than serum from a healthy donor. An exception to this finding was the expression of CD14, which was significantly higher in NB4 cells cultured with healthy donor serum than in NB4 cells cultured serum from a breast cancer patient. For all the investigated surface receptors the highest levels of expression were recorded for NB4 cells cultured in standard FBS-supplemented media.

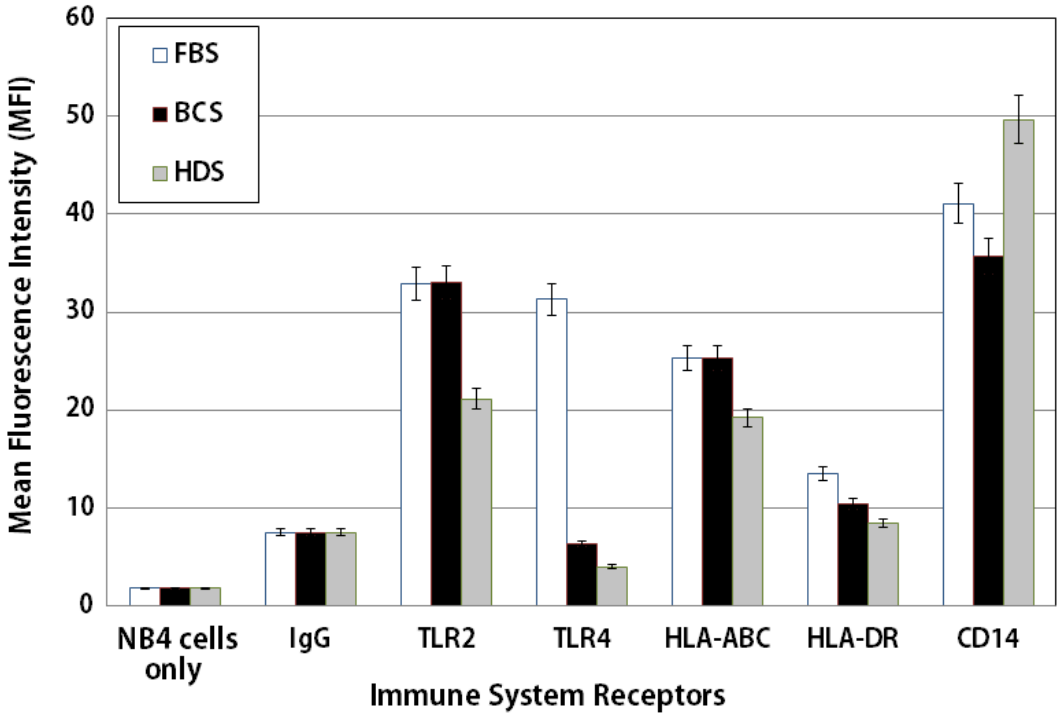

Figure 4.9: Evaluation of the expression of immune system receptors on the surface of NB4 cells after 24 hours of culture with human serum

Flow cytometric analysis was used to assess the expression of surface receptors on NB4 cells. The white columns represent mean fluorescence intensity (MFI) values for the expression of the investigated receptors on NB4 cells cultured in standard growth media supplemented with FBS and are considered here as a comparison control for CTCFL levels before culture with human serum. The black columns represent the MFI values for the expression of receptors on NB4 cells cultured with serum from a breast cancer patient. The grey columns represent MFI values for receptors expressed on NB4 cells cultured with healthy donor serum. The experiment was repeated with serum from three healthy donors and three breast cancer patients (*n=3*). IgG isotype staining of NB4 cells was performed as a negative control for cells cultured with the different serum types, whilst unstained NB cells alone were used to determine the population. Key: FBS – cultured with foetal bovine serum, BCS – cultured with serum from a breast cancer patient, HDS – cultured with serum from a healthy donor.

4.4 Discussion

This chapter aimed to investigate the effect of breast cancer on the expression of CTCFL in human PMNs. In an earlier report by D'Arcy et al. (2006), it was suggested that CTCFL was not expressed in the peripheral PMNs of healthy donors, and was only detected in the PMNs of breast cancer patients, or at low levels in healthy donors with a familial history of cancer. Furthermore, the level of CTCFL in the PMNs of breast cancer patients was suggested to correlate with breast tumour size. Investigations into the immunogenicity of CTCFL have revealed its capacity to elicit IgG antibodies in patients with various cancers (Ghochikyan et al., 2007). These findings have led to the proposal of CTCFL as a tumour marker, with the potential to be used in the diagnosis/prognosis of breast cancer, as well as in immunotherapy. D'Arcy et al. proposed two hypotheses on the origin of CTCFL in the PMNs of breast cancer patients but did not investigate them. The first hypothesis suggested that CTCFL expression was actively influenced by chemical signals released into the blood serum of cancer patients, which activate CTCFL expression or up-regulate endogenous expression. The second hypothesis suggested that CTCFL is passively accumulated in the PMNs of breast cancer patients. Due to the anti-tumoural role of PMNs this accumulation could be due to phagocytosis of metastasised tumour cells present in the blood stream or at a tumour site. CTCFL could also be accumulated in the PMNs of breast cancer patients via the uptake of CTCFL molecules, which may be released into the serum of these patients. D'Arcy et al. (2006) also noted that CTCFL was not detected in the blood plasma of breast cancer patients. However, anti-CTCFL antibodies were detected in the sera of breast cancer patients recently (Lobanenkov et al., 2010).

The first experiment in this chapter was designed to verify the levels of CTCFL in human PMNs. To achieve this, CTCFL levels were assessed in the PMN fractions of 27 patients who were diagnosed with breast cancer, and who were not known to be undergoing chemotherapy. ICC staining and IRS values were used here to assess the levels of CTCFL as in D'Arcy et al. (2006). However, a mouse anti-CTCFL IgM antibody was employed for ICC staining in this investigation instead of a chicken anti-CTCFL antibody as used by D'Arcy et al. (2006). The levels of CTCFL in breast cancer patients were compared to levels in PMNs from 10 healthy donors and were detected at a moderate to high level in the assessed PMNs from breast cancer patients (52 %). In contrast to the previous report, this study detected CTCFL in 8 out of the 10 donors at low levels with ICC staining. The positive staining detected in the PMNs from healthy donors could potentially be non-specific binding of the secondary antibody (goat anti-mouse IgM); however, negative controls, as well as isotype controls, were employed during the ICC experiments, and non-specific staining (false positive) was not observed in these controls. Thus, the reason for this contrasting result in CTCFL levels in this study compared to the earlier report may be due to the mouse anti-CTCFL antibody having a higher sensitivity, which enabled the detection of CTCFL at lower levels than was achievable using the chicken anti-CTCFL antibody. These findings also suggest that the expression of CTCFL in the PMNs of breast cancer patients is upregulated and not activated, as findings show that CTCFL is already expressed at low levels in individuals free from cancer, and it increases in the PMNs of breast cancer patients. Yet, these suggestion should be confirmed in the future using polymerase chain reaction (PCR) experiments.

The second experiment investigated the effect of serum from breast cancer patients and healthy donors on CTCFL levels in peripheral human PMNs. This was achieved by assessing CTCFL levels in PMNs from healthy donors when cultured with serum from

breast cancer patients. In parallel, PMNs from breast cancer patients were cultured with serum from healthy donors for the same period of time. ICC staining of PMNs from healthy donors when cultured with breast cancer patient serum revealed low expression of CTCFL, whereas PMNs from breast cancer patients demonstrated a steep decline in CTCFL levels when cultured with serum from healthy donors after 48 hours. The CTCFL levels in PMNs from all the healthy donors did not alter and remained at low levels for 48 hours when cultured with serum from the same donor, whilst PMNs from breast cancer patients retained high CTCFL levels when cultured with serum from the same patient. The decline in CTCFL levels in PMNs from breast cancer patients could not be due to apoptosis or cell death, as high levels of CTCFL were retained when cultured with serum from the same patient. These findings may suggest that chemical signals existing in the serum of breast cancer patients, which can influence CTCFL expression, do not exist in the serum of healthy donors. CTCFL is known to localise to both the nucleus and the cytoplasm and the staining observed for many of the healthy donor PMNs appeared to shift from a cytoplasmic to nuclear location when examined under the microscope, which may suggest the active expression of CTCFL in these PMNs. The passive accumulation of CTCFL protein in the PMNs of breast cancer patients, whether through phagocytosis or uptake of CTCFL molecules present in serum, is unlikely, as levels of nuclear CTCFL would remain unchanged in this case while levels of cytoplasmic CTCFL would increase. This should be investigated further in the future by assessing CTCFL mRNA levels using RT-PCR and could also be assessed with Western blot analysis of cytoplasmic cell lysate compared to nuclear cell lysate.

Levels of CTCFL were investigated in CTCFL-expressing PMNs (i.e. the NB4 cell line) when exposed to serum from breast cancer patients and healthy donors using Western blot analysis. The analysis showed that NB4 cells express significant levels of CTCFL

which did not decline when cultured with serum from healthy donors for 24 hours. Based on this finding, as well as findings from breast cancer patient PMNs cultured with healthy donor serum, it is proposed that the serum of healthy donors does not contain any elements that are capable of influencing CTCFL levels or actively down-regulate CTCFL expression. NB4 cells showed no increase in CTCFL levels when cultured with serum from breast cancer patients.

As serum from breast cancer patients did not lead to an elevation in CTCFL levels in PMNs from healthy donors, as well as in NB4 cells, it appears even more likely that chemical signals do not affect CTCFL levels, rather it is possible that CTCFL expression may occur in response to specific cellular events within PMNs, such as survival and induction of apoptosis.

Chapter 5 - **Manipulating CTCFL Levels in the NB4 Cell Line**

5.1 Introduction

It is now recognised that transcription factors are not only involved in regulating the production, linage commitment and differentiation of PMNs (Bjerregaard et al., 2003; Theilgaard-Mönch et al., 2005), but are also involved in regulating several aspects of PMN immunological function upon stimulation (Beauvillain et al., 2007; Girard et al., 1997; Wasielewski et al., 2006). CTCFL is a transcription factor that initially was discovered as being expressed exclusively during male germ cell development. However, more recently CTCFL has been detected in other cell types, including ovaries and embryonic stem cells (Monk et al., 2008), as well as many primary tumours and cancer cell lines (Klenova et al., 2002). The function of CTCFL in different cell types is currently unclear but it appears to be a methylation-independent protein that activates other genes (Hong et al., 2005; Nguyen et al., 2008). CTCFL has also been detected in PMNs of breast cancer patients, (D'Arcy et al., 2006), although the consequences of its expression on the physiology and function of PMNs has not been investigated to date. CTCFL involvement in regulating the physiology and function of PMNs is thought to be through the alteration of DNA methylation patterns (i.e. epigenetically) for specific genes or by direct regulation of its DNA targets. In previous experiments, the expression of CTCFL has been detected to coincide with specific changes in a human PMN cell model. For example, CTCFL levels declined in a CTCFL-expressing acute promeylocytic leukaemia (APL) cell line when induced into differentiation (section 3.3.3). This suggested a possible relationship between CTCFL expression and the differentiation of PMNs, and also that CTCFL may support an anti-apoptotic role in this leukemic cell line.

However, further experiments are required to confirm if CTCFL is actively involved in regulating the differentiation of human PMNs.

To determine the effect of CTCFL expression on different aspects of PMN differentiation, survival and function, it is important to be able to manipulate CTCFL levels in the investigated cells. This can be achieved through transfection, where ectopic nucleic material (DNA or RNA) is introduced into a cell using biological (e.g. virus mediated transfection), chemical (e.g. cationic liposomal transfection) or physical methods (e.g. electroporation or laser-based transfection). A transfected gene has the ability to up-regulate (overexpress) or down-regulate (i.e. knock down) the expression of the investigated protein. An ideal transfection method should result in a high number of cells accepting the introduced nucleic acid with minimum toxic cellular effects and should be easy to reproduce in the selected cells. Transiently transfected nuclear material remains only temporarily within a cell, as such material is not incorporated into the genome of a cell, and consequently is lost during cell division (Mantegazza et al., 2013).

5.2 Experimental Approach

The objective in this part of the investigation was to manipulate CTCFL expression levels in the PMN model by significantly increasing production (overexpression) or decreasing the existing levels (knock down). Manipulating CTCFL levels was a crucial step in order to study the effects that this may have on the immunological phenotype and physiological characters of PMNs (e.g. surface receptor expression or PMN phagocytic activity). CTCFL over-expression in the PMN model was achieved by transfection of the NB4 cell line with a vector containing the whole CTCFL cDNA sequence, CTCFL-pCMV6. In order to decrease the existing expression levels of CTCFL protein in NB4 cells siRNA was utilised, as this can be designed to target the protein of interest (i.e. CTCFL protein)

and then degrade it in the transfected cells. Identifying the optimum method for transfecting the NB4 cell line was a major step forward in achieving the objective of this part of the study.

As these cells grow only in suspension under normal culture conditions, NB4 cells are inherently resistant to DNA uptake when using many of the transfection methods available, and are therefore considered 'hard-to-transfect' cells. However, a few earlier studies (Rizzo et al., 1998; Tartey and Takeuchi, 2014), as well as some commercial methods have demonstrated a degree of success in transfecting NB4 cells or other hard-to-transfect cell lines. Several chemical, as well as non-chemical methods were trialled in order to determine the optimal approach for transfecting NB4 cells. In addition, it was important to optimise the transfection conditions (e.g. DNA or siRNA concentration to volume of transfection reagent) to ensure high yields of DNA or siRNA delivery into cells with minimum toxic effects to the transfected cells.

Trials and the optimisation of DNA delivery to NB4 cells for CTCFL over-expression experiments were mainly conducted using pEGFP, which is a reporter plasmid, or the CTCFL-EGFP, which is a reporter plasmid construct. Consequently, pEGFP and CTCFL-EGFP were amplified in *E.coli* competent cells then extracted and purified using a MaxiPrep kit as described in Section 2.11.6. A sufficient volume of the CTCFL-pCMV6 construct was provided for the actual CTCFL overexpression experiment. As for the trials and optimisation of siRNA delivery in NB4 cells, the siGLO red transfection indicator was employed. ON-TARGET plus SMARTpool Human CTCFL was used in the actual knock-down CTCFL experiments.

The transfection efficiency of all the trials and optimisations were assessed using a fluorescent microscope or flow cytometer. For the acquisition of flow cytometric data, ten

thousand events were collected for each sample. The selection of the examined population and exclusion of cellular debris was achieved through gating using the scatter plot of non-transfected NB4 cells (untreated). Transfection efficiency was determined as the percentage of events within the gated population that exhibited a strong level of fluorescence, excluding cells with signals adjacent to untreated NB4 cells and negative controls. The MFI of the signal was calculated from the logarithmic scale histogram using Cell Quest software and further data processing was performed using Flowing 2.5.1 software. Viability of the cells was always examined using the simple trypan blue exclusion test directly prior to the transfection efficiency assessment.

5.3 Results

5.3.1 Extraction and Purification of Plasmid DNA

For the optimisation of the transfection of NB4 cells, larger amounts of both EGFP and CTCFL-EGFP were prepared through transformation of *E. coli* competent cells and later extraction and purification with a MaxiPrep kit. The plasmid DNA yield was diluted to a total volume of 60 μl in TE buffer and the concentration and purity of the plasmid DNA were measured using a nano-photo-spectrometer device (i.e. NanoDrop). The purity of the DNA sample was determined by two absorbance ratios; A260/280 and A260/230. The A260/280 absorbance ratio determines the protein contamination present in the nucleic acid sample, while the A260/230 absorbance ratio indicates the level of organic contaminants, such as salts or aromatic compounds that are used in the process of plasmid DNA extraction. The A260/280 and A260/230 ratios values should be somewhere around 1.8 and 2.0, respectively, with lower values indicating a significant amount of contamination present in the DNA sample. Both the extracted plasmids proved to be pure samples in the NanoDrop analysis (Table 5.1). To verify that the purified plasmids were

pEGFP and CTCFL-EGFP, a small amount of each was digested with the restriction enzyme *Xho*I and the products analysed via 1% agarose gel electrophoresis. The results showed that both EGFP and CTCFL-EGFP were digested by the restriction enzyme as expected, resulting in two fragments of ~ 6.0 kb, 5.0 kb for EGFP and three fragments of ~6.0 kb, 5.0 kb and 2.5 kb for CTCFL-EGFP (Figure 5.1).

Table 5.1: NanoDrop analysis of the purity of the extracted pEGFP and CTCFL-EGFP plasmids

The purity of the DNA samples was determined using the A260/280 and A260/230 ratios. Values should be somewhere around 1.8 and 2.0, respectively.

Plasmid DNA	A260	A280	260/280	260/230
pEGFP	250.59	135.14	1.85	2.20
CTCFL-EGFP	183.29	99.00	1.85	2.27

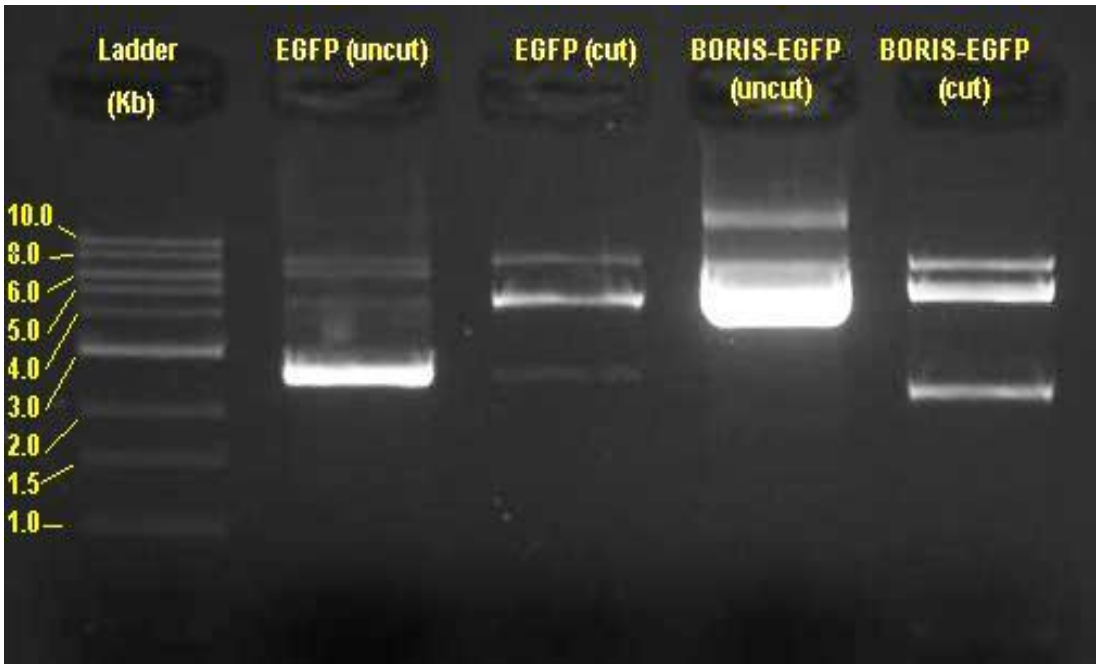

Figure 5.1: Agarose gel electrophoresis for the verification of the extracted EGFP and CTCFL-EGFP plasmids

Plasmids were extracted and purified using a MaxiPrep kit before being digested with the restriction enzyme *Xho*I and separating the products on a 1% agarose gel. The first lane contains a DNA ladder (size 1-10 Kb), the second lane shows undigested pEGFP, the third lane represents pEGFP digested with *Xho*I which has resulted in two fragments of 8 Kb and 5 Kb. Lane 4 contains uncut CTCFL-EGFP and lane 5 CTCFL-EGFP digested with *Xho*I which has resulted in three fragments of 8kb, 5kb and 3.5kb.

5.3.2 Optimisation Studies for Plasmid DNA Transfection in NB4 cells

Chemically-based transfection reagents X-treme GENE HD and TurboFect were trialled for the transfection of the NB4 cell line using pEGFP. For the X-treme GENE HD reagent, four different reagent to DNA ratios (Xtreme : pEGFP) were trialled; 1:1, 2:1, 3:1 and 4:1. NB4 cells were examined 24 hours post-transfection for the EGFP signal using a fluorescent microscope, and 48 hours post-transfection the NB4 cells re-evaluated for the EGFP signal using a fluorescent microscope before histogram data for transfection efficiency were acquired using a flow cytometer (Figure 5.2). The transfection efficiency was determined as the percentage of events detected with a positive signal for EGFP in the examined population. A number of controls were performed for the flow cytometric data acquisition and analysis. Negative controls included non-treated NB4 cells grown under normal conditions and not subjected to the transfection protocol (Figure 5.2-a and -b). In addition, NB4 cells were incubated with the transfection reagent only and no pEGFP was added (Figure 5.2-c) and NB4 cells subjected to plasmid DNA (pEGFP) without the addition of the transfection reagent (Figure 5.2-d) were also employed as negative controls. Fluorescent microscopy showed a very low positive EGFP signal for the examined NB4 cells 24 hours post-transfection, while the flow cytometric evaluation of NB4 cells showed no increase in EGFP-positive events 48 hours post-transfection (Figure 5.2 from e to h). This data suggests that NB4 cells did not take up the pEGFP plasmid when transfected using X-treme GENE HD reagent.

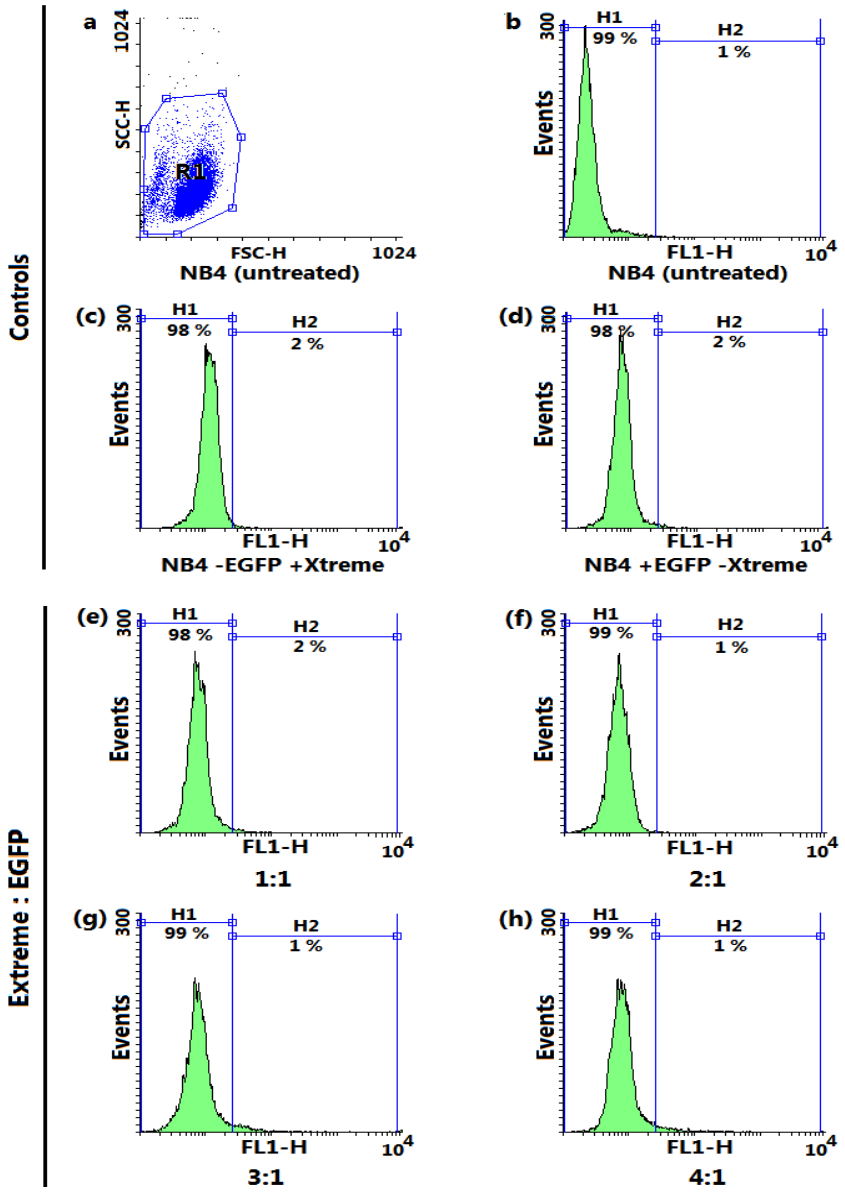

Figure 5.2: Flow cytometric evaluation of pEGFP transfection efficiency 48 hours post-transfection in NB4 cells using the transfection reagent Xtreme GENE HD

NB4 cells were transfected with 1µg of pEGFP plasmid and the green signal for EGFP expression in NB4 cells was detected via the FL1-H channel. Region H1 reflects the percentage of events (cells) negative for the EGFP signal, while region H2 reflects the percentage of events with a positive signal for EGFP. **(a)** NB4 cells were isolated from other artefacts by setting the gate region R1 on the scatter plot of non-transfected NB4 cells. Controls for the acquisition of flow cytometer data included: **(b)** non-transfected NB4 cells (NB4 untreated), **(c)** NB4 cells incubated only with Xtreme GENE HD transfection reagent without pEGFP (NB4–GFP+Xtreme), and **(d)** NB4 cells incubated with pEGFP only (NB4+EGFP–Xtreme). Four ratios of Xteme GENE HD reagent to pEGFP (Extreme : pEGFP) were used in optimising the transfection of NB4 cells: **(e)** 1:1, **(f)** 2:1, **(g)** 3:1, and **(h)** 4:1. Only 2 % or less of the transfected NB4 cells demonstrated a positive EGFP signal.

For the TurboFect reagent, 2 µl of the reagent was trialled with two pEGFP concentrations for transfection of NB4 cells; 1 µg or 2 µg. The cells were inspected for a positive EGFP signal 24 hours post-transfection using a fluorescent microscope and 48 hours post-transfection were subjected to flow cytometric evaluation (Figure 5.3). Negative controls included untreated NB4 cells (Figure 5.3-a), NB4 cells incubated with pEGFP only (Figure 5.3-b), and NB4 cells incubated with transfection reagent only (Figure 5.3-c). The human embryonic kidney cell line, HEK 293, which is routinely grown in our laboratory were used as a positive control (Figure 5.3-d). HEK 293 cells were subjected to the same TurboFect transfection protocol using 1µg of pEGFP and 2 µl of TurboFect reagent. While the HEK 293 cells showed a strong signal (70 % of cells were EGFP positive) when examined by fluorescent microscopy 24 hours post-transfection, EGFP-positive NB4 cells were not detected. Data acquired using a flow cytometer 48 hours post-transfection showed high levels of EGFP expression in HEK 293 cells following transfection with TurboFect (95% of cells were EGFP-positive) (Figure 5.3-d); however, less than 2 % of NB4 cells were EGFP-positive 48 hours after transfection with TurboFect (Figure 5.3-e and -f).

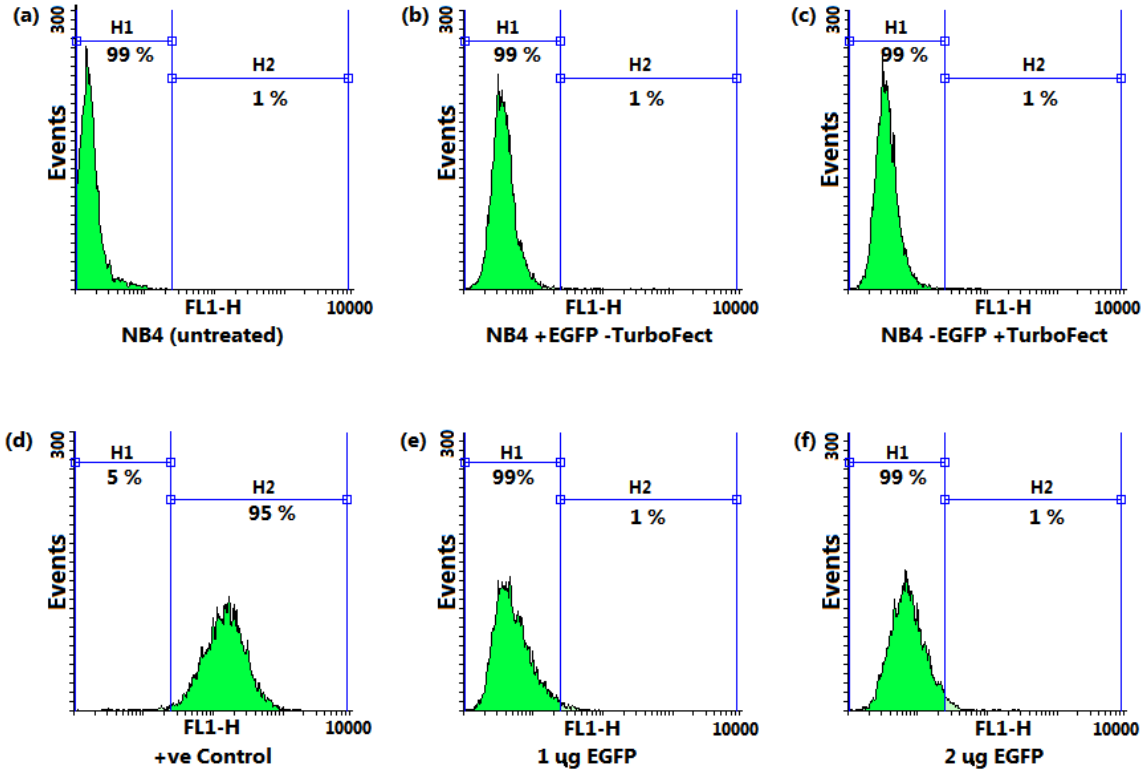

Figure 5.3: Flow cytometric evaluation of pEGFP transfection efficiency 48 hours post-transfection in NB4 cells using the transfection reagent TurboFect

NB4 cells were transfected with 1µg of EGFP plasmid and the green signal for EGFP expression was detected via the FL1-H channel. Region H1 reflects the percentage of events (cells) negative for the EGFP signal, while region H2 reflects the percentage of events with a positive signal for EGFP. NB4 cells were isolated by setting the gate region R1 on the scatter plot of non-transfected NB4 cells. Controls for the acquisition of flow cytometer data included: **(a)** non-transfected NB4 cells (NB4 untreated), **(b)** NB4 cells incubated with only pEGFP and without reagent (NB4+GFP-TurboFect), **(c)** NB4 cells incubated with TurboFect reagent only without pEGFP (NB4-EGFP+TurboFect), and **(d)** HEK 293T cells as a positive control for EGFP expression.

Non-chemical methods trialled in this study for transfecting NB4 cells included electroporation and Nucleofection. For the electroporation trial, 1.2×10^7 NB4 cells were electroporated with 1 μg or 2 μg of pEGFP as described by Rizzo et al. (1998). The evaluation of transfection efficiency was performed after 24 hours and 48 hours using a flow cytometer (Figure 5.4). Controls for data acquisition included untreated NB4 cells (Figure 5.4a and b), NB4 cells incubated with pEGFP only (Figure 5.4c), and NB4 cells pulsed with the electroporator without the addition of pEGFP (Figure 5.4d). A low level of EGFP expression was detected in NB4 cells electroporated with 1 μg of pEGFP 24 and 48 hours post-transfection (Figure 5.4e and f). NB4 cells showed better levels of EGFP expression when transfected with 2 μg of pEGFP, with a maximum of 41 % of electroporated NB4 cells demonstrating positive EGFP expression.

Introducing plasmid DNA into NB4 cells using Nucleofection was the final trial performed in this study. Optimisation included three different Nucleofection solutions; SE, SG and SF, which were tested with 16 different pre-set Nucleofection programs on the Amaxa Nucleofector machine. The CTCFL-EGFP construct was utilised in the optimisation of NB4 cells Nucleofection, with 0.5 μg of CTCFL-EGFP used with 20 μl of the test solution for each Nucleofection. Each reagent had to be tested in the 16 Nucleofection programs, including one program for the control, which was basically the addition of the same plasmid and solution volume but without Nucleofection (no pulse). Nucleofected cells were examined for a CTCFL-EGFP signal 24 hours post-Nucleofection using a fluorescent microscope. Assessment revealed the highest efficiency to be in NB4 cells Nucleofected using the SF solution, where 40 % to 70 % of the examined NB4 cells demonstrated a positive signal for CTCFL-EGFP expression. The Nucleofection program which produced the highest efficiency was EN-138, where 70 % of cells expressed CTCFL-EGFP in combination with the SF solution.

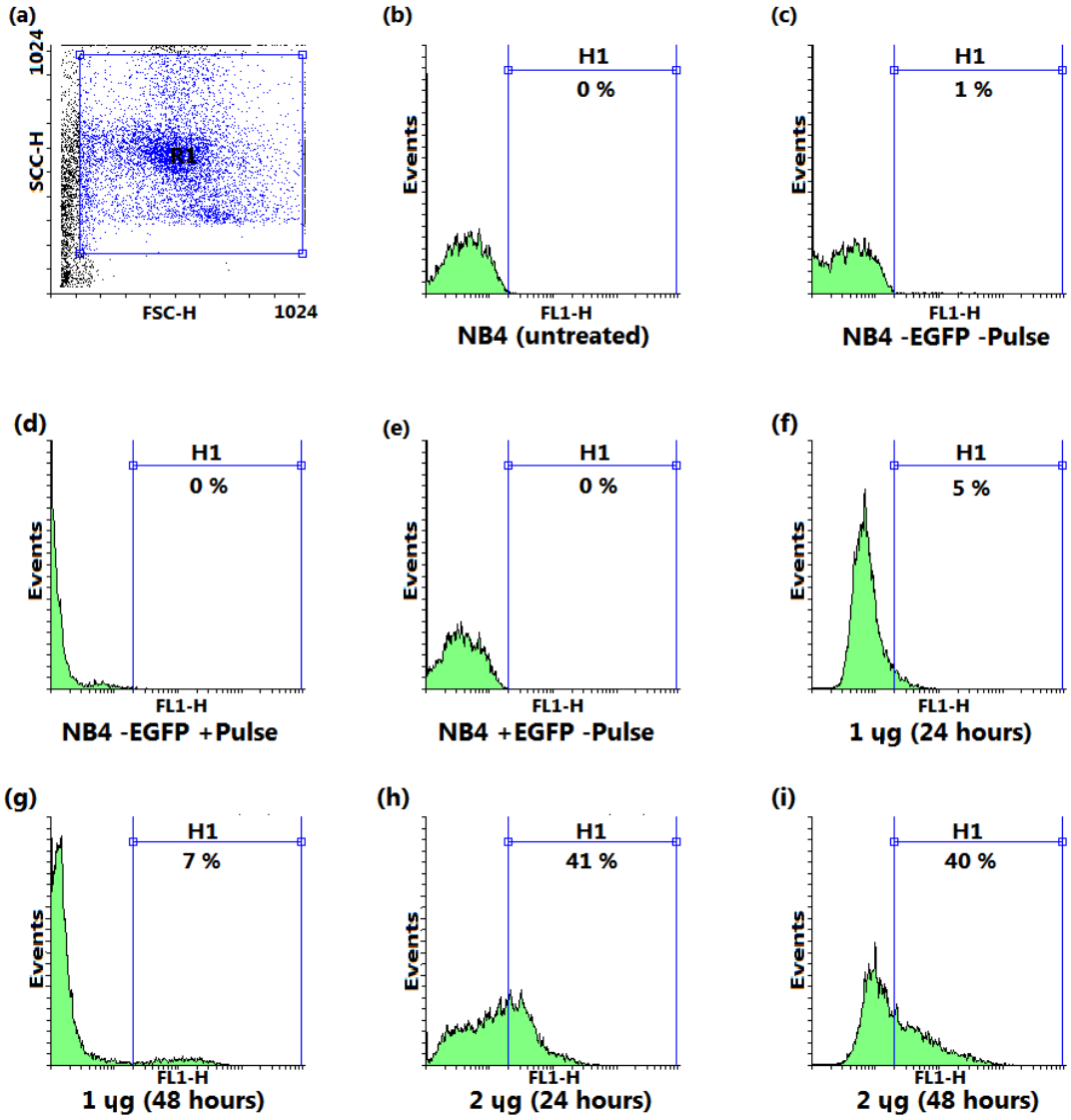

Figure 5.4: Flow cytometric evaluation of pEGFP transfection efficiency 48 hours post-transfection in NB4 cells using electroporation

NB4 cells were transfected using electroporation according to the protocol of Rizzo et al. (1998) and the green signal for EGFP expression was detected via the FL1-H channel. Region H1 reflects the percentage of events (cells) negative for the EGFP signal, while region H2 reflects the percentage of events with a positive signal for EGFP. **(a)** Cells were isolated by setting the gate region R1 on the scatter plot of non-transfected NB4 cells. Controls for the acquisition of flow cytometer data included: **(b)** non-transfected NB4 cells (NB4 untreated), **(c)** NB4 cells incubated only with EP buffer but without pEGFP and no pulse in the electroporator (NB4–GFP–Pulse), **(d)** NB4 cells incubated in EP buffer with pEGFP and without a pulse (NB4+EGFP–Pulse), and **(e)** NB4 cells without pEGFP but pulsed in the electroporator (NB4–GFP+Pulse). **(f)** Transfected NB4 cells (NB4 +EGFP +Pulse) were examined for the EGFP signal after 24 hours and **(g)** 48 hours. Only 7 % of the examined events gave a positive signal for EGFP. After 48 hours of incubation the percentage of positive events for the EGFP signal increased to 39 % of electroporated cells.

Western blot analysis was used to verify the overexpression of CTCFL in NB4 cells when Nucleofected with CTCFL-pCMV6 plasmid, CTCFL-EGFP and the empty vector, pCI. Therefore, 2×10^6 NB4 cells were Nucleofected with 4 μg of CTCFL-pCMV6 in 100 μl of SF solution then incubated with complete RPMI for 24 hours. Whole cell lysates were prepared from the Nucleofected cells after 24 hours of incubation and the samples subjected to Western blot analysis as described in Section 2.9. Levels of CTCFL expression were compared in the Nucleofected cells. NB4 cells incubated with CTCFL-pCMV6 plasmid and SF solution without Nucleofection (mock) were considered to be a negative control (Figure 5.5). Western blot analysis showed that CTCFL was overexpressed in NB4 cells Nucleofected with CTCFL-pCMV6, and the overexpression of CTCFL was also detected in NB4 cells Nucleofected with CTCFL-EGFP, whilst NB4 cells Nucleofected with pCI empty vector or non-Nucleofected cells appeared to express CTCFL at lower levels (Figure 5.5).

The viability of the transfected NB4 cells in all the experiments were always assessed 24 hours post-transfection prior to assessing the transfection efficiency. This was accomplished by gently re-suspending the incubated cells in each well of a plate, and taking a 10 μl sample of the suspended cells and mixing it with 10 μl of trypan blue dye before examining under a light microscope for viability. Over 95 % of the cells were observed to be viable, except after electroporation, which seemed to slightly affect cell viability, as only 79 % of cells were noted as viable and 18 % as necrotic.

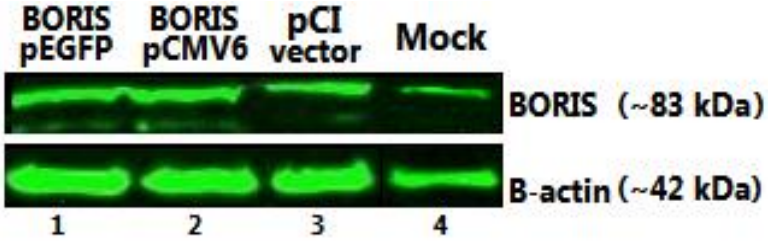

Figure 5.5: Expression of CTCFL in transfected NB4 cells using the Amaxa Nucleofector system

After selecting the optimum Nucleofection solution and pre-set Nucleofector program, NB4 cells were Nucleofected with (**lane 1**) CTCFL-pEGFP, (**lane 2**) CTCFL-pCMV6, and (**lane 3**) pCI empty vector. Whole cell lysates of the Nucleofected NB4 cells were assessed via Western blot analysis. (**lane 4**) NB4 cells incubated with Nucleofection solution and CTCFL-pCMV6 but without a Nucleofector pulse were designated as the negative control in this analysis (mock). NB4 cells Nucleofected with CTCFL-pEGFP and CTCFL-pCMV6 demonstrated over-expression of CTCFL protein in comparison to CTCFL levels in NB4 cells Nucleofected with an empty vector or mock NB4 cells. β-actin was used as a loading control.

5.3.3 Optimisation Studies for siRNA Transfection in NB4 cells

For the knock-down of CTCFL, several chemical and non-chemical methods were trialled. Chemical reagents included the Dharmafect optimisation system for siRNA transfection and the siGLo Red transfection indicator was used in these studies. ON-TARGET plus SMARTpool Human CTCFL siRNA was used for the actual knock down of CTCFL.

The Dharmafect optimisation system is comprised of four different reagents; DharmaFECT 1, DharmaFECT 2, DharmaFECT 3 and DharmaFECT 4. Three different concentrations of each DharmaFECT reagent, 2 µl, 4 µl and 8 µl, were tested in combination with three siGLO concentrations, 25 nM, 50 nM, and 100 nM. Only untreated NB4 cells were utilised at this stage as a negative control for flow cytometer data acquisition and analysis. The cells were examined for the siGLO red indicator 24 hours post-transfection using a fluorescent microscope and a positive signal (fluorescence) was not detected in the inspected cells. The transfection efficiency was assessed again 48 hours post-transfection using a flow cytometer, where the inspected population was gated from the scatter plot of untreated NB4 cells. The transfection efficiency was determined as the percentage of events within the gated population that exhibited a strong level of fluorescence, and cells with signals adjacent to untreated NB4 cells and negative controls were excluded. Comparing the transfection efficiency data for the four DharmaFECT reagents, the highest efficiency was noted in NB4 cells transfected using 0.4 µl of DharmaFECT 2 reagent with 50 nM of the transfection indicator siGLO, where 23 % of cells demonstrated a siGLO-positive signal.

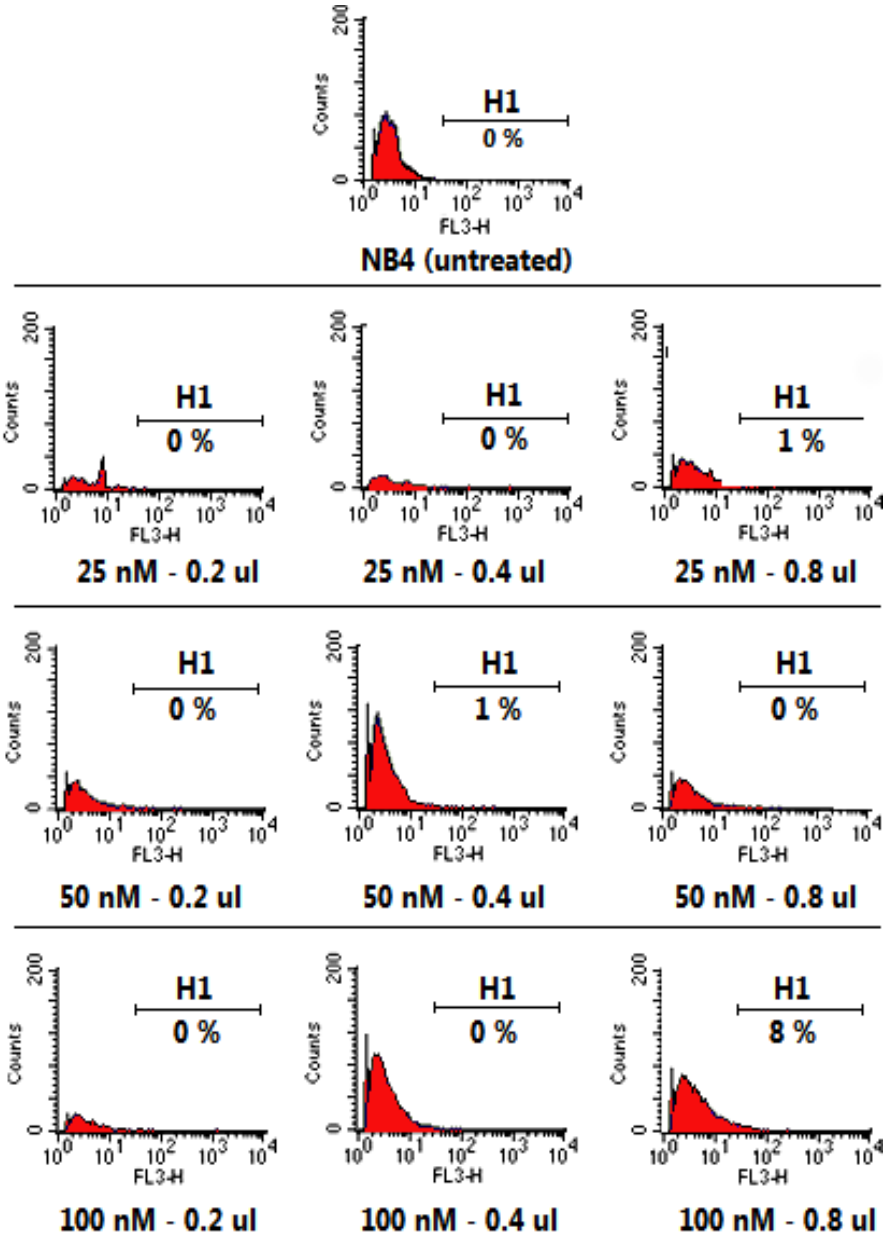

Figure 5.6: Flow cytometric evaluation of siRNA transfection efficiency 48 hours post-transfection in NB4 cells using DharmaFECT 1 transfection reagent

NB4 cells were transfected with 25, 50 or 100 nM of siGLO red transfection indicator in combination with 0.2, 0.4 or 0.8 µl of DharmaFECT 1 reagent. NB4 cells not treated with siGLO or reagent were used as a negative control (top histogram). The fluorescent signal from siGLO was detected by the FL3 red channel and the examined population was gated on the scatter plot of untreated NB4 cells. Region H1 reflects the percentage of cells (events) with a positive siGLO signal. The transfection efficiency achieved using DharmaFECT 1 reagent with NB4 cells did not exceed 8 % of the detected events using 100nM of siGLO and 0.8 µl of DharmaFECT1.

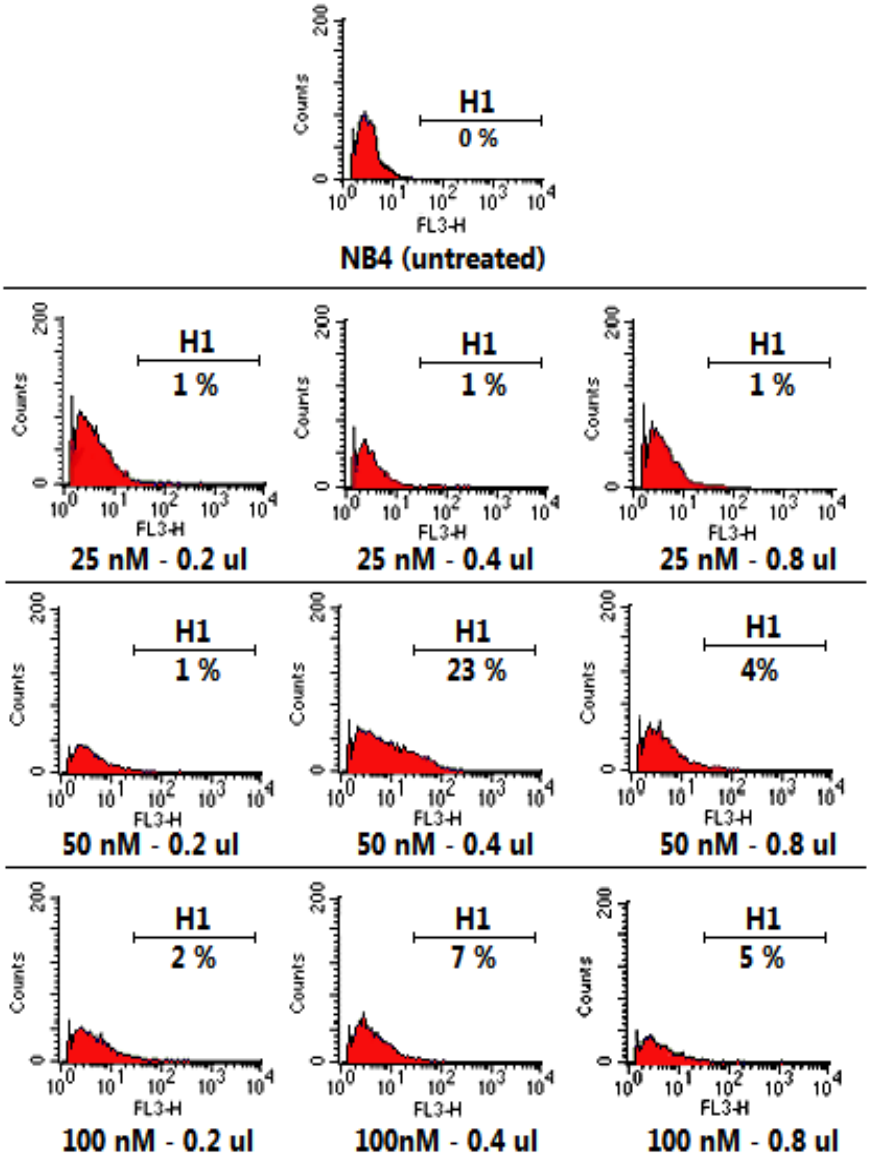

Figure 5.7: Flow cytometric evaluation of siRNA transfection efficiency 48 hours post-transfection in NB4 cells using DharmaFECT 2 reagent

NB4 cells were transfected with 25, 50 or 100 nM of siGLO red transfection indicator in combination with 0.2, 0.4 or 0.8 µl of DharmaFECT 2 reagent. NB4 cells not treated with siGLO or reagent were used as a negative control (top histogram). The fluorescent signal from siGLO was detected by the FL3 red channel and the examined population was gated on the scatter plot of untreated NB4 cells. Region H1 reflects the percentage of cells (events) with a positive siGLO signal. The transfection efficiency achieved using DharmaFECT 2 reagent with NB4 cells did not exceed 23 % of the detected events using 50nM of siGLO and 0.4 µl of DharmaFECT2.

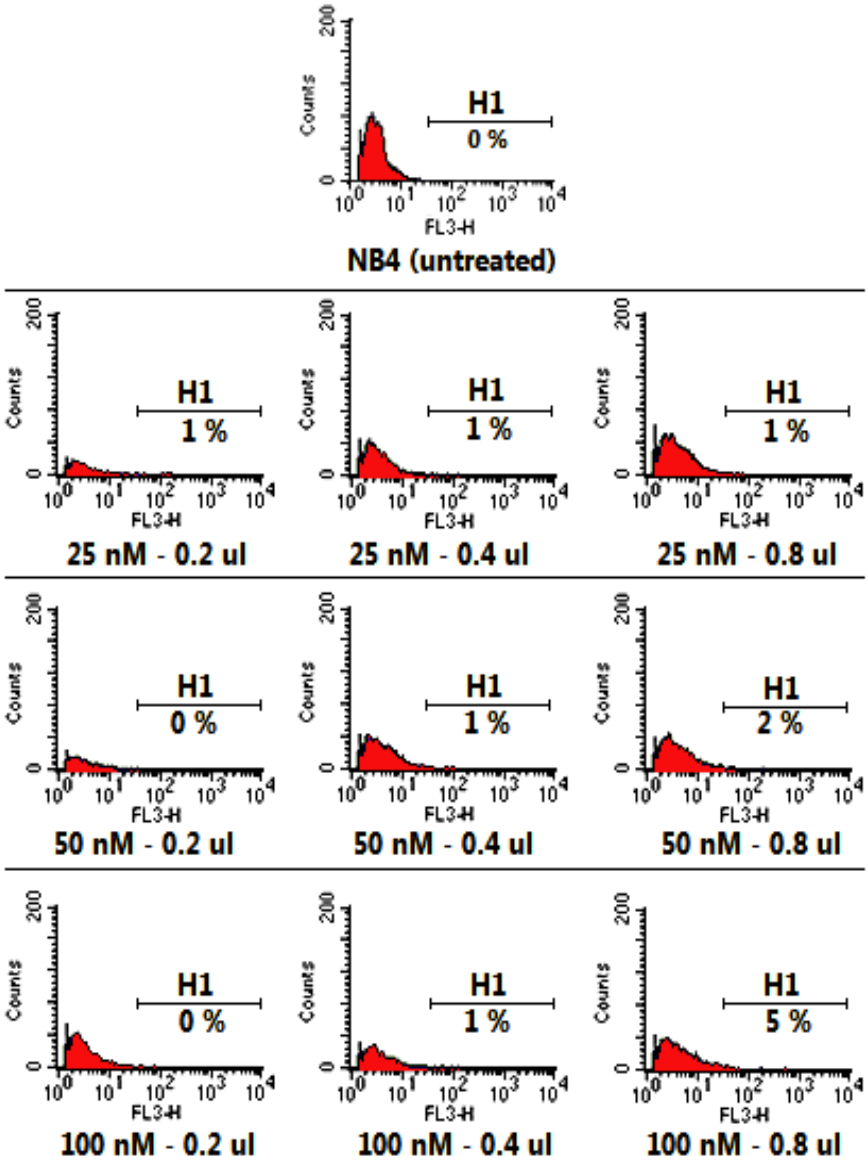

Figure 5.8: Flow cytometric evaluation of siRNA transfection efficiency 48 hours post-transfection in NB4 cells using DharmaFECT 3 transfection reagent

NB4 cells were transfected with 25, 50 or 100 nM of siGLO red transfection indicator in combination with 0.2, 0.4 or 0.8 µl of DharmaFECT 3 reagent. NB4 cells not treated with siGLO or reagent were used as a negative control (top histogram). The fluorescent signal from siGLO was detected by the FL3 red channel and the examined population was gated on the scatter plot of untreated NB4 cells. Region H1 reflects the percentage of cells (events) with a positive siGLO signal. The transfection efficiency achieved using DharmaFECT 3 reagent with NB4 cells did not exceed 5 % of the detected events using 100nM of siGLO and 0.8 µl of DharmaFECT3.

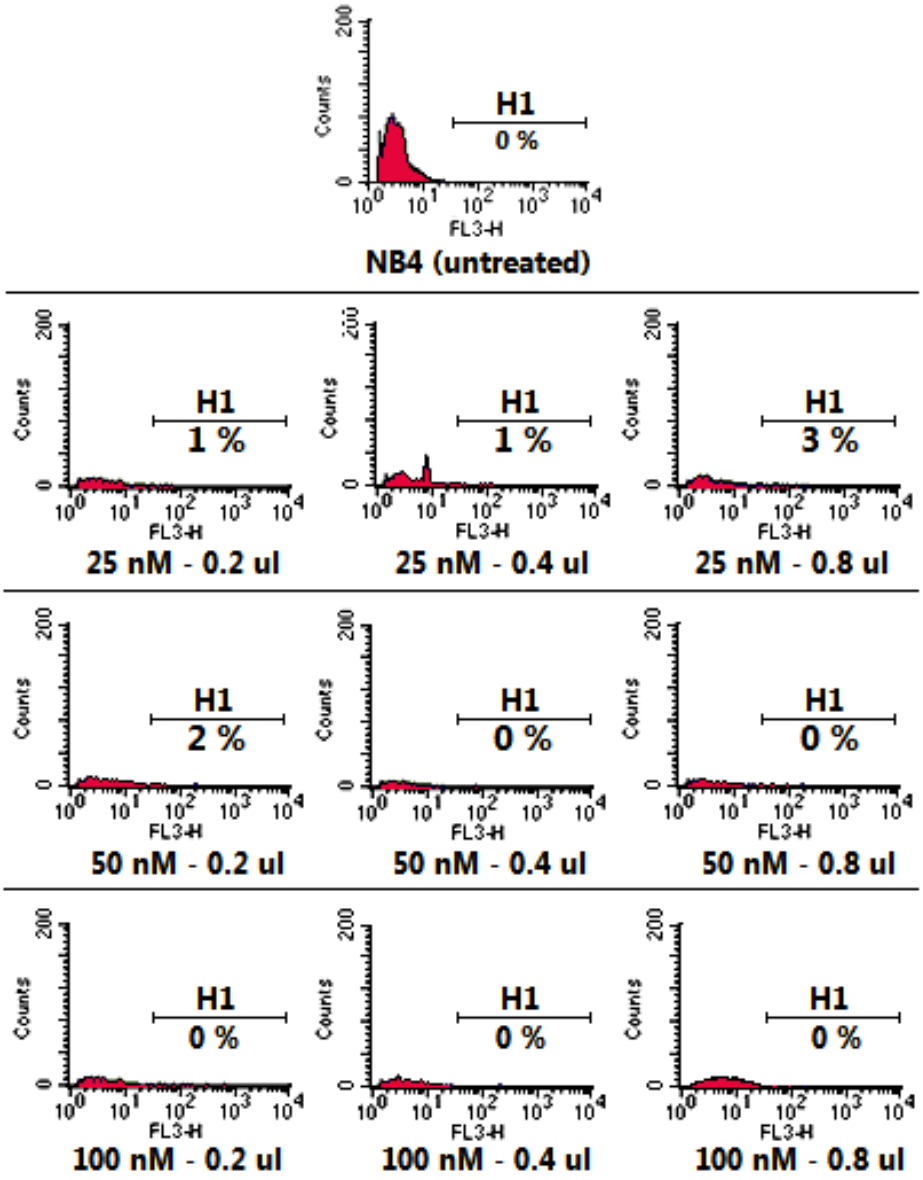

Figure 5.9: Flow cytometric evaluation of siRNA transfection efficiency 48 hours post-transfection in NB4 cells transfected using DharmaFECT4 transfection reagent

NB4 cells were transfected with 25, 50 or 100 nM of siGLO red transfection indicator in combination with 0.2, 0.4 or 0.8 µl of DharmaFECT 4 reagent. NB4 cells not treated with siGLO or reagent were used as a negative control (top histogram). The fluorescent signal from siGLO was detected by the FL3 red channel and the examined population was gated on the scatter plot of untreated NB4 cells. Region H1 reflects the percentage of cells (events) with a positive siGLO signal. The transfection efficiency for all combinations was ≤ 1%.

Non-chemical transfection methods trialled for the knock-down of CTCFL protein by siRNA transfection included electroporation and Nucleofection. NB4 cells were transfected with different concentrations of siGLO red transfection indicator (25 nM, 50 nM or 100 nM) as described previously (Tartey and Takeuchi, 2014). The transfection efficiency of the NB4 cells was then assessed using a fluorescent microscope 24 hours post-transfection and using a flow cytometer 48 hours post-transfection. The electroporation efficiency for delivering siGLO into the cells was significantly low, regardless of the concentration of siGLO (Figure 5.10).

Optimisation of the Nucleofection of siGLO into NB4 cells was carried out as described for plasmid DNA, using Nucleofection solutions SE, SF and SG in combination with the 15 Nucleofection programs and 50 pmol of siGLO. Again, NB4 cells demonstrated a high transfection efficiency when Nucleofected with SE solution in combination with the EN-138 pre-set Nucleofector program, with 70 % of Nucleofected cells showing a positive siGLO signal. Knock-down of the CTCFL protein level was verified using Western blot analysis, which revealed that NB4 cells Nucleofected with CTCFL siRNA exhibited a much lower level of CTCFL compared to NB4 cells Nucleofected with siGENOME non-targeting siRNA or NB4 cells incubated with Nucleofection solution and CTCFL siRNA without a Nucleofector pulse (Figure 5.11). As in the optimisation studies for plasmid DNA transfection, the viability of the transfected cells with siRNA were assessed 24 hours post-transfection and less than 5 % of the incubated cells were found to be non-viable.

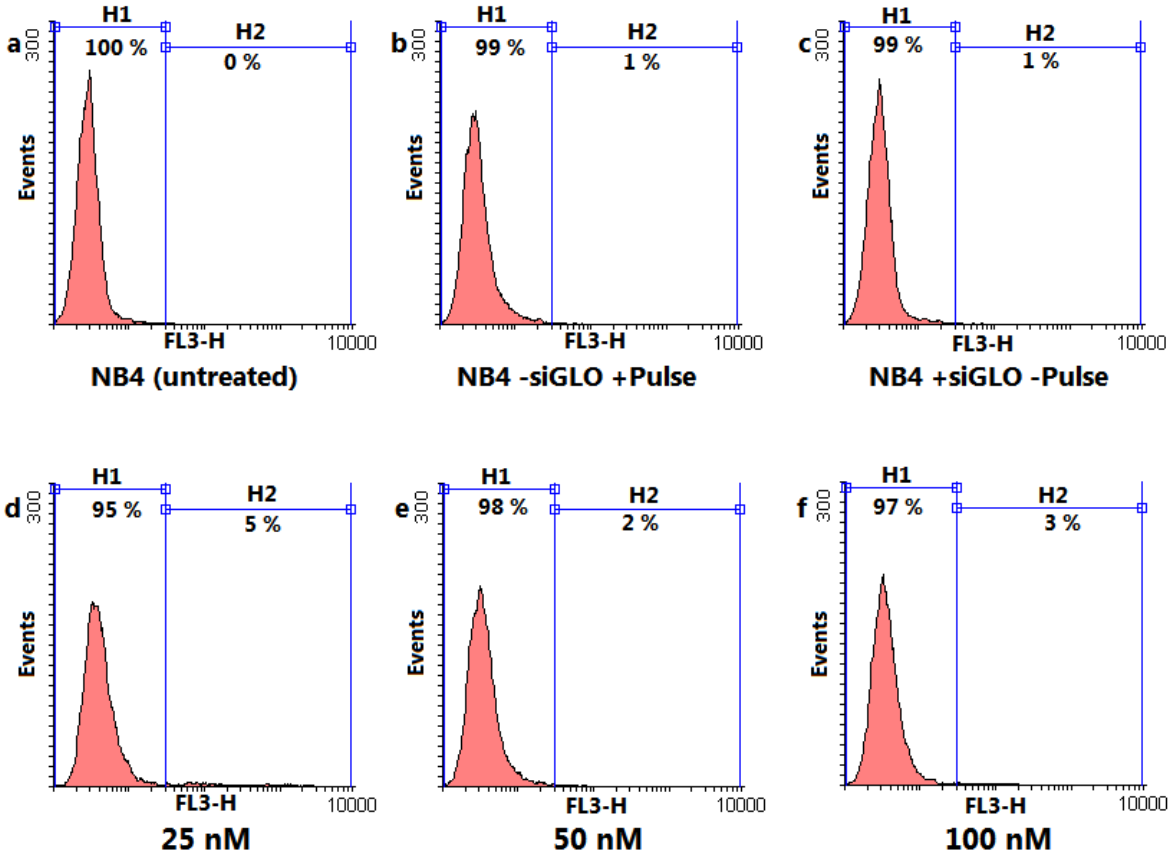

Figure 5.10: Flow cytometric evaluation of siRNA transfection efficiency 48 hours post-transfection in NB4 cells treated using electroporation

NB4 cells were electroporated as described by Mathieu et al. (2008) except that here siGLO Red was used as a transfection indicator (**a**) Controls for population and parameter set up included NB4 cells grown in standard culture without the addition of siGLO or reagent (NB4 untreated). Other controls included (**b)** cells incubated with siGLO alone and without a pulse (NB4+siGLO–Pulse), and (**c**) cells pulsed but without the addition of siGLO (NB4–siGLO+Pulse). siGLO was added to NB4 cells (**d**) 25 nM, (**e**) 50nM, and (**f**) 100 nM of siGLO and electroporated with 260 v and 1050 µF. The H1 region reflects the percentage of events in the electroporated population which exhibited a MFI adjacent to the main fluorescence intensity of non-electroporated NB4 cells, while the H2 region reflects the percentage of events with a positive siGLO signal.

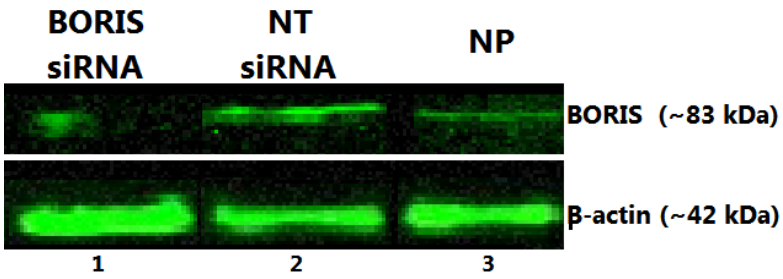

Figure 5.11: Western blot analysis of the knock-down of CTCFL in transfected NB4 cells using Nucleofection

After optimisation of the instrument settings and solution, NB4 cells were transfected using the Amaxa Nucleofection system. (**lane 1**) 2×10^6 NB4 cells were Nucleofected with 100 pmol/µl of ON-TARGET plus SMARTpool Human CTCFL siRNA (CTCFL siRNA). (**lane 2**) For the negative control the same number of NB4 cells was Nucleofected with siGENOME non-targeting siRNA (NT siRNA), or (**lane 3**) NB4 cells were incubated with CTCFL siRNA and solution but not subjected to a Nucleofection pulse (NP). CTCFL levels were significantly lower in NB4 cells transfected with CTCFL siRNA in comparison to NB4 cells Nucleofected with non-target siRNA or NB4 incubated with transfection indicator and solution with no pulse.

5.4. Discussion

Manipulating CTCFL levels is a key step in order to study the effects of altering CTCFL protein levels on the immunological function and physiological characteristics of human PMNs. This enables the function of CTCFL protein in the human PMNs as a transcription factor to be understood, and may also indicate if it is involved, either directly or indirectly, in the regulation of the function of PMNs. The manipulation of CTCFL levels in a human PMN cell model, the NB4 cell line, was achieved using transient transfection, which is the temporary introduction of ectopic nucleic acid material into cells by chemical or physical means. As naturally non-adherent cells, the NB4 cell line is intrinsically resistant to accepting foreign nuclear material using conventional methods of transfection. Viral stable transfection methods have been proven to yield the efficient transfection of nucleic acid material in suspension cells, particularly NB4 cells (Ji et al., 1994; van Horssen et al., 2012). In addition, the stable transfection with the CTCFL gene would be a practical choice, especially if further studies on transfected cells were to be undertaken. However, viral methods for transfection were not favoured in the optimisation studies, as this method introduces several issues and thus requires further processing to overcome these. For example, viral methods can induce an immunological reaction or insertional mutagenesis in the transfected cells (Abdallah et al., 2011; Kim and Eberwine, 2010). In addition, viral methods require additional safety precautions and take a longer time to produce.

For the successful transfection of nucleic acid material in NB4 cells, several options were considered. Commercial reagents designed for the transfection of difficult-to-transfect cells or earlier published transfection protocols with reported success in transfecting NB4 cells were selected for the trials. The selected concentrations of nucleic material and

transfection reagents to be trialled were based on published literature and manufacturer recommendations for the cell type and transfected nucleic material. The transfected NB4 cells were always selected to have a low passage number (less than 30 passages) and to be in logarithmic growth at the time of transfection. In addition, the viability of the cells was always assessed following transfection (usually 24 hours post-transfection) in order to confirm cell viability and to exclude solutions or substrate concentration that had a toxic effect.

The commercial chemical methods that were assessed in this study were X-treme GENE HD (a non-liposomal multicomponent reagent), TurboFect (a liposomal reagent) for plasmid DNA transfection, and DharmaFECT 1, 2, 3, and 4 for siRNA transfection. The optimisation study suggested that chemical transfection methods were the least effective method for transfecting NB4 cells with either plasmid DNA or siRNA.

The non-chemical methods tested were electroporation and the advanced version, Nucleofection. Both of these methods are based on using an electrical pulse to create transient small pores within the cell membrane in order to deliver nucleic acid into the cell. Nucleofection differs in having pre-set electrical pulsation programs which have been developed specifically for difficult-to-transfect cells, such as primary mammalian cells, stem cells, and suspension cell lines. The flow cytometer data suggested that Nucleofection was the best method for transfecting NB4 cells with both pEGFP and siGLO. This finding was confirmed by Western blot analysis which showed that the CTCFL protein was overexpressed in Nucleofected cells transfected with the CTCFL-pCMV6 plasmid construct and knocked-down in NB4 cells Nucleofected with siRNA targeting CTCFL. In total, 70 % of Nucleofected NB4 cells were successfully transfected with either plasmid DNA or siRNA.

Many future investigations depend on the successful manipulation of CTCFL levels in NB4 cells, which has been achieved here. These investigations may be able to answer questions regarding the involvement of CTCFL in the regulation of the physiology and function of PMNs. For example, data from Chapter 3 suggested a decline in CTCFL levels as cells became more differentiated, and the potential relationship between the expression of CTCFL and the neutrophilic differentiation of the PML cell line, NB4. However, this raises the question of whether CTCFL is actively involved in the regulation of the differentiation PMNs or whether it is regulated by other transcription factors. If CTCFL is actively involved in the regulation of PMN differentiation, then the overexpression of CTCFL in PMNs would affect or impair their differentiation, while CTCFL knock-down would promote the differentiation of NB4 cells. In fact that transcription factors are indispensable regulators for the production and development of PMNs and that their knock-down could lead to substantial impairment of PMN survival or could even lead to cell death (van Horssen et al., 2012). Cell death was not observed in these experiments, as cells maintained viability when CTCFL levels were knocked-down in NB4 cells, which may suggest an indirect role for CTCFL in regulating the survival of PMNs. Other aspects of PMN function, for example the expression levels of immunity receptors, such as CD14, HLA-DR or TLR, may be altered as NB4 cells commence differentiation. In addition, the phagocytic activity and levels of NO production when CTCFL levels are manipulated in PMNs need to be considered and it would be interesting to examine the levels of immunity receptors and phagocytic activity of PMNs after manipulating CTCFL expression. Not all NB4 cells subjected to Nucleofection would exhibit the overexpression or knock-down of CTCFL protein at the same level, as 30 % of cells did not take up nucleic material, therefore, cells would need to be labelled and collected using fluorescent activated cell sorting (FACS) to differentiate between

successfully manipulated cells and cells which did not incorporate any nucleic material or had a low percentage of transfection efficiency.

Chapter 6 - **General Discussion and Conclusion**

The main aim of this investigation is to study the expression of CTCFL in the human polymorphonulcear neutrophils (PMNs) and its relation to the physiology and function of these cells. CTCFL is a transcription factor and a cancer testis (CT) antigen that was first detected exclusively in the male germline and not in soma (Klenova et al., 2002). The investigation is based on the findings of earlier report by D'Arcy et al in 2006. The report suggested CTCFL is expressed in the PMNs of breast cancer patients at levels which correlate to the size of the tumor and that CTCFL is not detected in healthy individuals who have no familial history of cancer. Based on these findings the report suggested CTCFL to be a potential marker for breast cancer diagnosis/prognosis. Furthermore, the report hypothesised that the source of CTCFL detected in the PMNs of breast cancer patients could be the tumor cells. This could be either by activating CTCFL expression in PMNs or through passive accumulation of CTCFL molecules through phagocytosis. These two hypotheses were not yet investigated. In another report by the same author, CTCFL was found to be aberrantly expressed in breast tumors (D'Arcy et al., 2008). However, a later report suggested differently that CTCFL may not to be expressed in all breast tumor cell lines (Hines et al., 2010). More recently CTCFL was reported not to be as exclusive as once thought (Jones et al., 2011), and was also detected in other cell types as ovaries and embryonic stem cells (Monk et al., 2008). As CTCFL was not investigated as vigorously as its paralogue CTCF, many aspects need to be addressed in research regarding CTCFL expression in different cell types and at different stages of the cell's life cycle.

The objective in the first part of this investigation was to study the immune- and phenotypical profile of CTCFL-expressing PMNs cell line model (NB4 cell line) when

induced into differentiation. Taking into account that NB4 cells are promyelocytic leukemia, we observed the changes in the phenotype profile that co-occurred when the levels of CTCFL were directed to decline due to the induction of neutrophilic differentiation. This co-occurrence of phenotypical alterations does not necessarily indicate a direct involvement of CTCFL in the regulation of these characteristics, but an overview of phenotypical changes occurring when CTCFL levels are changed in these cells. For example, the onset of apoptosis and the concurring of CTCFL levels decline in differentiating pormyelocytes. The profiling of these cells included assessing their survival, onset of apoptosis, phagocytic activity and generation of nitric oxide, and immunophenotyping of surface receptors involved in the main immunological function of PMNs, phagocytosis. Antigen presenting molecules were also investigated. PMNs are not classically known as antigen presenting cells, however, under certain conditions, PMNs could act similarly (Beauvillain et al., 2007). CTCFL was suggested to be a transcription factor that is capable of genome-wide alterations in gene expression through altering DNA methylation patterns. Accordingly, it is possible that CTCFL may be involved in the differentiation, survival and apoptosis of cells. In this part we also attempted investigate the correlation between CTCFL expression and cellular differentiation by studying the co-localization of CTCFL protein with a differentiation marker in promyelocytic leukemia, PML protein during neutrophilic differentiation. Our findings suggested a possible association between the two proteins but were not conclusive, and further protein-protein interaction study could be carried out.

In the second part of this work the effect of breast malignancy on CTCFL expression in the PMNs was investigated peripheral human. Earlier report suggested CTCFL to be expressed at high levels in the PMNs of breast cancer patients and was not detected in the peripheral PMNs of healthy individuals. In contrast to D'Arcy's report, verification

experiments done in this study detected CTCFL at low levels in the PMNs of healthy donor. As proper controls were employed in these experiments, we could deduce that the used antibody in this work is has a higher sensitivity than previously used CTCFL antibodies and that CTCFL is expressed in the PMNs of healthy individuals at low levels. The findings in this part of the investigation therefore suggested that CTCFL could be upregulated rather than activated in the PMNs of breast cancer patients. In an attempt to investigate the effect of breast tumor on the human PMNs, a panel of experiments included culturing PMNs collected from healthy donors with the serum of breast cancer patients, as well as culturing donor PMNs with the breast cancer cell line, CAMA-1. These experiment seemed to show that the serum of healthy donors does not contain any elements that are capable of influencing CTCFL levels or actively down-regulate CTCFL expression. Also, the serum of breast cancer patients may contain elements that sustain existing CTCFL levels, but does not lead to the upregulation of these levels. The findings of this part should be investigated further by assessing CTCFL mRNA levels using reverse transcriptase – polymerase chain reaction (RT-PCR).

In the third part of this investigation, optimisation studies were carried out for the upregulation and knock down of CTCFL protein using transient transfection. The objective of the optimisation studies was to manipulate CTCFL levels in order to investigate the correlation between CTCFL and the different aspects of PMNs immunological functions, e.g. the expression of immunity surface receptors. PMN cell line models are grown in suspension and thus inherently hard to transfect cell, as they are in many cases does not uptake nuclear material when using conventional transfection techniques. After many optimisations, it was possible to transfect NB4 cells using the Amaxa nucleofector system, with a 70% success rate for transfecting CTCFL plasmid DNA as well as small interfering RNA targeting CTCFL protein.

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