

A study of the NF- κ B
signalling pathway in
human acute myeloid
leukaemia.

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Bibliography

Declaration

I declare all work presented in this thesis was undertaken and completed by myself, except where indicated and acknowledged, and that this has not been previously submitted for a degree. All sources of information have been fully acknowledged.

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Abbreviations:

Ab – antibody

ALL – acute lymphocytic leukaemia

AML – acute myeloid leukaemia

AML1/ETO – an oncoprotein

AMMoL – acute myelomonocytic leukaemia

ATG – antithymocyte globulin

ATL – adult T cell leukaemia

ATL – adult T-cell leukaemia

BAFF – B cell activating factor

BCL-2 – B cell lymphoma protein

BCLL – B cell chronic lymphocytic leukaemia

BCR – B cell receptor

Bcr-Abl – Philadelphia chromosome: translocation between breakpoint cluster region gene and V-abl Abelson murine leukaemia viral oncogene homolog 1

Bim – a member of the BCL-2 family of pro-apoptotic proteins

C/EBPs – CCAAT – enhancer binding proteins

CBF β /MYH11 – core binding factor beta/myosin 11 gene fusion

CBP – CREB binding protein

CC – coiled coil

CD – cluster of differentiation

CD45RA – isoform of CD45 antigen

CD45RO – isoform of CD45 antigen

cFLIP – FLICE (caspase-8) inhibitory protein

CLL – chronic lymphocytic leukaemia

CML – chronic myelocytic leukaemia

COX-2 – cyclooxygenase-2

DD – death domain

DIC – disseminated intravascular coagulation

DIC – disseminated intravascular coagulation

DNA – deoxyribonucleic acid

EBV – Epstein-Barr virus

EGFR – epidermal growth factor receptor

ERK – extracellular signal related kinase

FAB – French-American-British

FDP – fibrinogen-fibrin degradation products

GC – glucocorticoids

G-CSF – granulocyte colony stimulating factor

GM-CSF - granulocyte macrophage colony stimulating factor

HAT – histone acetylase

HCL – hairy cell leukaemia

HDAC – histone de-acetylase

HLA-DR – an MHC class II cell surface receptor encoded by the human leukocyte antigen complex

HO-1 – heme-oxygenase 1

Hsp27 – heat shock protein 27

HTLV-1 – human T cell leukaemia virus type 1

IFN α – interferon alpha

Ig – Immunoglobulins

IL – interleukin

iNOS – inducible nitric oxide synthase

I κ B α – inhibitor of kappa B alpha

KSHV – Kaposi's sarcoma – associated herpes virus

LDL – low density lipoprotein

LGLL – large granular lymphocytic leukaemia

LGLs – large granular lymphocytes

LMP-1 – latent membrane protein

LSC – leukaemia stem cell

LZ – leucine zipper

M0, M1, M2, M3, M4, M5, M6, M7, M8 – morphology codes used in the French-American-British system of classification for acute myeloid leukaemia

MAL – megakaryocytic acute leukaemia

MCL-1 – induced myeloid leukaemia cell differentiation protein

MDR-1 – multi drug resistance 1

MDS/MPD – myelodysplastic/myeloproliferative

MEK – mitogen activated protein kinase kinase

MHC – major histocompatibility complex

MoAbs – monoclonal antibodies

NEC – neonatal necrotizing enterocolitis

NF- κ B – nuclear factor kappa B

NIK – NF- κ B inducing kinase

NK cells – natural killer cells

NLS – nuclear localisation signal

NON/SCID – non-obese diabetic severe combined immunodeficient

p16 – protein 16

p53 – protein 53

PEST – proline – glutamate – serine – threonine sequence

PKA – protein kinase A

PKA – protein kinase A

PKC – protein kinase C

PML – pro-myelocytic leukaemia

PML/RAR α – promyelocytic leukaemia/retinoic acid alpha gene translocation

qRT PCR – quantitative real time polymerase chain reaction

RANK-L – receptor activator of NF- κ B ligand

RHD – rel homology domain

RIP – receptor-interacting protein

S – serine

TAD – transcriptional activation domain

TAK – thylakoid membrane threonine kinase

TdT – terminal deoxynucleotidyl transferase

TNF – tumour necrosis factor

TNFR – tumour necrosis factor receptor

T-PLL – T cell prolymphocytic leukaemia

TRAF – tumour necrosis factor receptor associated factor

TWEAK – TNF-related weak inducer of apoptosis

uPA – urokinase plasminogen activator

WHO – World Health Organisation

XIAP – X-linked inhibitor of apoptosis protein

Abstract

The transcription factor NF- κ B can play both a protective and destructive role in cells. NF- κ B protects by signalling for the immune system when cells fall under attack from pathogens. NF- κ B signalling becomes a danger to cells when signalling becomes constitutive and can promote excessive inflammation and tumourigenesis. Constitutive NF- κ B signalling occurs within acute myeloid leukaemia cells, promoting cell survival. This provides reason to investigate the NF- κ B signalling pathway in greater detail. Prevention of NF- κ B signalling should lead to cell apoptosis of AML cells but this outcome may be affected by any one of the many signalling components that make up the extremely complex NF- κ B signalling pathway. A range of the NF- κ B signalling components within AML cells are investigated in detail this study. It was uncovered that although p50 NF- κ B levels were consistent in AML and control cell samples, I κ B α levels were markedly reduced, leading to greater basal NF- κ B activity in AML cancer cells compared to control non-cancer cells. Furthermore, this inhibited I κ B α level observed in AML cells, appeared to be maintained by autocrine TNF production, as anti-TNF treatment impaired the observed response. These findings indicate that I κ B α levels underlie the high basal NF- κ B activity that is observed in AML cancer cells, and possibly other types of NF- κ B-dependent cancers. Targetting I κ B α may help improve cancer chemotherapeutic effectiveness in AML.

Chapter 1: Introduction

This study investigates one particular type of cancerous disease: acute myeloid leukaemia. It is a potentially lethal cancer affecting the white blood cells, whose cellular signalling becomes disrupted, leading to their aberrant proliferation and growth. The NF- κ B signalling pathway plays a major role in regulating such activities within a cell therefore components of the pathway are studied in a degree of detail to provide an insight into their roles in AML.

1.1 Cancer

Many human tissues undergo rapid and continuous cell turnover to maintain their normal and healthy physiology. For example in colonic mucosa or in the peripheral blood the life span of a mature, differentiated cell can be measured in days or even hours (Dalerba et al 2006). Human tissues maintain this continuous cell turnover through a tightly regulated process of renovation sustained by a small amount of long-lived stem cells with extraordinary expansion potential.

Cancer occurs when this process of cell turnover becomes aberrant. This can occur as a result of both internal factors (such as inherited mutations, hormones, and immune conditions) and environmental/acquired factors (such as tobacco, diet, radiation, and infectious organisms (Anand et al 2008). These environmental factors can cause or enhance abnormalities in the genetic material of cells (Kinzler et al 2002). The mutations found in the cancer cell genome accumulate over the lifetime of the cancer patient (Aggarwal et al 2011). If mutations occur in oncogenes or tumour suppressor genes, the tight regulation of cell turnover is lost, therefore causing cells to proliferate abarrently and form a mass which is known as a tumour. Nuclear factor-kappaB (NF- κ B) is a transcription factor which has an established critical role in cancer (Chaturvedi et al 2011). It has both tumour suppressor and oncogenic properties, either of which can lead to tumourigenesis.

Tumours where the cancerous cells remain clustered together in a single mass are known as benign (Alberts et al 2002). Cancerous cells may gain the ability to break loose from a tumour and invade surrounding tissue. This is a property of malignant tumours. Malignant tumours may enter the bloodstream or lymphatic vessels and form secondary tumours called metastases at other sites within the body (Alberts et al 2002).

Cancers are classified according to the tissue and cell type from which they arise. Cancers that arise from epithelial cells are called carcinomas; those arising from connective tissue or muscle cells are called sarcomas; and cancers that do not fit into either of these two broad categories include cancers derived from cells of the nervous system, and leukaemias (Alberts et al 2002).

1.2 Leukaemia

1.2.1 Introduction

This section will give an introduction into Leukaemia. It will initially introduce leukaemia as a disease in more general terms, then progress to provide more detail about the specifics of various types. The French-American-British and World Health Organisation systems of leukaemia classification are described and for each class there is a brief summary of their epidemiology, symptoms, immunology, causes, diagnosis and treatment.

Leukaemia is a cancer of the blood and bone marrow that involves an increase in white blood cell count to a level that is above normal. Control mechanisms in the blood are disrupted causing the bone marrow to produce excess numbers of abnormal white blood cells that do not become mature, thus affecting their ability to function properly. Overproduction of white blood cells displaces the production of other blood cells and components which leads to the demise of normal properties and functions of the blood system.

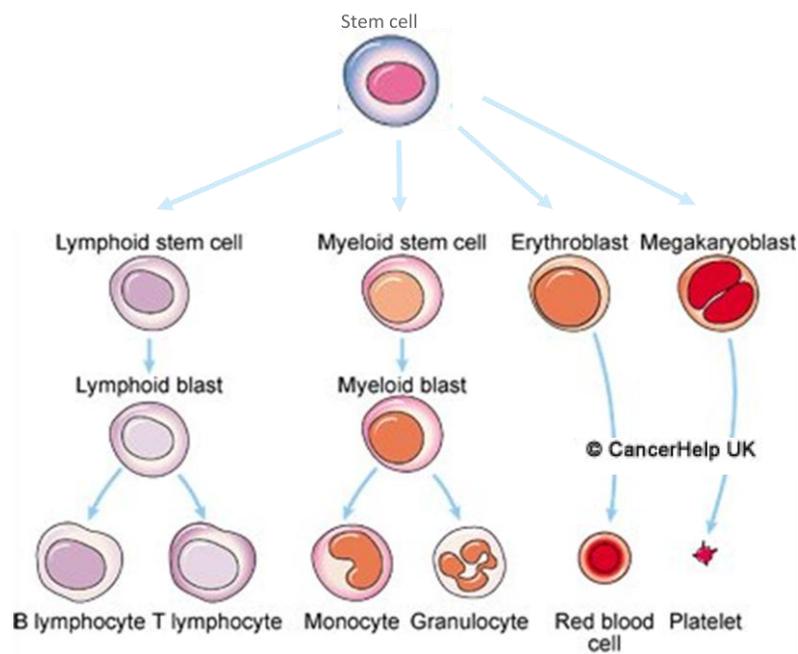
Leukaemia is a disease that varies greatly among patients. Therefore to aid communication between patients, medical professionals, researchers and the general public, leukaemia is divided clinically and pathologically into separate classes. Such classification is explored in the following section.

1.2.2 Classification

Leukaemia can be classified into being either lymphoid or myeloid, depending on which lineage of white blood cell is affected. Lymphoblastic or lymphocytic leukaemias can develop due to defects in lymphoid progenitor cells which would normally mature to form infection-fighting lymphocytes of the immune system. B cells, T cells or natural killer (NK) cells can become affected. Myeloblastic or myelocytic leukaemias arise due to disorders in myeloid

progenitor cells which prevent proper maturation and differentiation into basophils, neutrophils, eosinophils and monocytes which are immature macrophages.

Further classification reflects the speed of progression of the disease, either chronic or acute.



Intro Figure 1: The formation of blood cells of different lineages from a stem cell. [www.cancerhelp.org.uk].

Acute leukaemias progress rapidly due to the rapid accumulation of immature blood cells. These are the most common forms of leukaemia in children and require immediate treatment. Chronic leukaemias have an excessive build up of relatively mature, abnormal white blood cells but take months or years to progress. Eventually though, because the abnormal cells are produced at a much higher rate than normal cells, a high proportion of abnormal cells will accumulate in the blood. Chronic leukaemia treatment does not necessarily need to happen immediately as some chronic leukaemia treatment can be most effective if treatment follows a monitoring period first. Chronic leukaemia is more prevalent in the older population. Chronic leukaemias are a rare disease in childhood, around 98% of childhood leukaemias are acute (Cwynarski et al 2003).

Such classification gives four main groups of disease: Acute Lymphoblastic Leukaemia (ALL), Acute Myeloid Leukaemia (AML), Chronic Lymphoblastic Leukaemia (CLL) and

Chronic Myeloid Leukaemia (CML). As previously defined, the different categories of leukaemia each have their own variations of cause, symptoms and groups of people who are most affected.

1.2.2.1 Chronic leukaemias

Chronic myelogenous leukaemias (CML)

CMLs are defined by the WHO as myeloproliferative diseases that are characterised by the presence of the Philadelphia chromosome (Vardiman et al 2002). The Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22 which leads to the fusion of the BCR and ABL genes in haematopoietic progenitor cells. This in turn leads to production of chimeric proteins with increased tyrosine kinase activity. CML in children is characterised by the same molecular, cytogenetic and morphologic features that are observed in adults, with classical Philadelphia-positive (Ph+) CML (Grier et al 1992). CML is characterised by a biphasic or triphasic clinical course in which a benign chronic phase is followed by transformation into an accelerated and blastic phase, which is often fatal (Faderl et al 1999).

CML is treated with chemotherapy reagents such as hydroxyurea or busulfan to maintain haematologic control; however these chemotherapeutic drugs cannot change the course of the disease (Faderl et al 1999). More recently, CML have received a standard treatment of imatinib which is an inhibitor of BCR-ABL tyrosine kinase. Also, interferon alpha (IFN α) is an alternative treatment in the early chronic phase for patients who do not tolerate imatinib (Hehlmann et al 2007). Depending on their age and donor availability, CML patients can be treated with allogenic stem cell transplantation if other treatment fails. Complete cytogenetic remission is achieved by 87% of patients (Hehlmann et al 2007).

Chronic lymphocytic leukaemia (CLL)

CLL is the most common human leukaemia. It is a malignancy of mature B cells with rearranged immunoglobulin (Ig) genes (Rosenwald et al 2001) and characterised by the accumulation of monoclonal B cells with the appearance of small mature lymphocytes with a characteristic immunophenotype (Hamblin et al 1999). Such cells are typically positive for CD5, CD23 and CD19 however surface immunoglobulins (IgM and IgD) are sparse (Hamblin et al 1999).

Neoplastic cells in patients with CLL over express the proto-oncogene protein Bcl-2, providing them with resistance to apoptosis (Hamblin et al 1999). Proto-oncogene Bcl-2 can occur as a result of a chromosomal translocation between chromosomes 14 and 18 however T- and B-lymphoid cells have found to be present with the bcl-2 protein but without the 14;18 chromosomal translocation (Pezzella et al 1990).

It has been suggested by Rosenwald et al (2001) that CLL may be separated into two different diseases: one derived from an Ig-un-mutated pre-germinal centre B cell and the other derived from an Ig-mutated B cell that has passed through the germinal centre. This model of two CLL diseases has been further supported by findings that Ig-un-mutated and Ig mutated CLL patients have different clinical courses (Hamblin et al 1999). Rosenwald et al investigated this two disease model and found that B cell activation genes were differentially expressed between the two Ig-mutational subgroups in CLL. This gave the possibility that more aggressive clinical behaviour of the Ig-un-mutated subtype is caused by signalling pathways downstream of the B cell receptor (BCR).

1.2.2.2 Acute Leukaemias

Acute Lymphoblastic Leukaemia (ALL)

Acute leukaemias are classified into acute myeloid leukaemia subtypes (see below) and acute lymphocytic leukaemia (ALL). ALL is a malignant disorder of lymphoid progenitor cells which affects both children and adults; however the peak prevalence is in children between the ages of two and five years. The cure rate for this particular disease is above 80% for children. Less than five percent of cases are associated with inherited or predisposing genetic syndromes, with ionising radiation or with exposure to specific chemotherapeutic drugs (Pui et al 2008). The exact pathogenetic events that occur in the development of ALL are unknown however it is thought to originate from genetic lesions in blood-progenitor cells that are committed to differentiate in the T-cell or B-cell pathway, which include mutations that provide cells with the capacity for unlimited self-renewal and mutations that cause stage-specific developmental arrest (Pui et al 2004). Cells become affected by ALL disease mutations in several ways: immunoglobulin or T-cell receptor genes have clonal rearrangements, antigen receptor molecules are expressed, and cell surface glycoproteins tend to resemble those of early developmental stage normal T and B lymphocytes.

Initial treatment of ALL consists of a combination of antibiotic drugs such as anthracyclines which provides a remission rate of up to 80% (National Cancer Institute 2010). If a patient experiences relapse, bone marrow transplantation may be considered (Bortin et al 1980). Treatment is generally more successful in younger patients which could be related to increased incidence of the Philadelphia chromosome (Ph1) in older ALL patients (Hoelzer et al 1988).

1.2.2.3 Rare Leukaemia Subtypes

Hairy Cell Leukaemia (HCL)

Some rarer types of leukaemia are not included in the acute/chronic and myeloid/lymphocytic classification system, one of which is hairy cell leukaemia (HCL). HCL is a lymphoid neoplasm that is one of the most treatable haematologic malignancies thanks to the development of effective agents such as nucleoside analogues and monoclonal antibodies (MoAbs) (Ravandi 2009). The median age for HCL presentation is about 55 years (the range is 24-81 years). The leukaemic cells affected are neoplastic B cells with cytoplasmic hair-like projections that infiltrate the bone marrow, liver, spleen and lymph nodes (Ravandi 2009). The nucleolus is not apparent or non-existent. Symptoms of HCL include anaemia, thrombocytopenia, leukopenia, pancytopenia and splenomegaly (Ravandi 2009).

T cell prolymphocytic leukaemia (T-PLL)

TPLL is the most common type of mature T-cell leukaemia affecting adults, particularly men (Matutes and Estella 1998). It is characterised by a rapidly increasing peripheral blood lymphocyte count, bone marrow involvement and splenomegaly (Catoysky et al 2001). T-PLL cells appear slightly larger than normal lymphocytes in peripheral blood or bone marrow aspirate smears. Also, each cell has a prominent nucleolus, irregular nuclear contours, and abundant non-granular and basophilic cytoplasm with protrusions or blebs (Garand et al 1998). T-PLL cells are of mature T-cell lineage: positive for T-cell markers and negative for terminal deoxynucleotidyl transferase (TdT) (Valbuena et al 2005). The neoplastic cells are usually positive for CD4 and possibly CD8 also (Catoysky et al 2001).

Large granular lymphocytic leukaemia (LGLL)

Large Granular Lymphocytes (LGLs) in normal adults make up 10-15% of the total peripheral blood mononuclear cells (Lamy et al 1998). LGL leukaemia (LGLL) is described as a clonal disorder involving blood, marrow and the spleen (Loughran et al 1985) and can be split into two LGLL disorders based on either the T-cell or NK-cell lineage. T-cell LGLL is a rare disease characterised by persistently increased circulating T-cell LGLs with a typical immunophenotype of CD3+, CD4 and CD8+ and cytogenetic or molecular evidence of clonal T-cell lymphoproliferation (Loughran 1993). T-cell LGLL patients will show neutropenia and have recurrent infections (Semenzato et al 1997). Patients with systemic symptoms or infiltration of the spleen, liver, or bone marrow could be classified in the category of NK-cell leukaemia (Lubomir et al 2006).

LGLL comprises 2-5% of all T-cell and NK-cell malignancies. T-cell LGLL cells often have a normal karyotype however when chromosomal aberrations occur, there is inversion of 12p and 14q, deletion of 5q and trisomy of chromosomes 3, 8 and 14 (Loughran et al 1985). In patients with NK-cell LGLL the most frequent clonal chromosomal abnormality is the deletion of the 6q chromosome. Patients are often treated with methotrexate, cyclophosphamide and cyclosporine in the first instance (Battiwalla 2003). Other therapies include treatment with prednisone, nucleoside analogues, haematopoietic growth factors (G-CSF, GM-CSF), antithymocyte globulin (ATG) and splenectomy, however this is only rare (Mercieca et al 1994, Lamy et al 1995, Bargetzi et al 1996, Gentile et al 1996).

Adult T-cell leukaemia (ATL)

Human T-cell leukaemia/lymphoma virus type 1 (HTLV-1) causes neoplastic transformation of human T-cells in infected individuals, leading to development of adult T-cell leukaemia (ATL). Viral proteins act to increase the responsiveness of T-cells to extracellular stimulation, modulate proapoptotic and antiapoptotic gene signals, enhance T-cell survival, and avoid immune recognition of the infected T-cells (Franchini et al 2003). The virus disrupts several signalling pathways involved in immune function of T-cells thus promoting T-cell proliferation (Franchini et al 2003). Genetic changes such as the mutation of p53 and deletion of p16 occur in some cases of ATL which give a poor prognosis (Yamada et al 1997). The immunological phenotype of neoplastic cells is helper T-cell, CD3+, CD4+, L-selectin+, CD25+, CD45RA+, HLA-DR+, CD29- and CD45RO- in peripheral blood (Tobinai et al 1992). Acute ATL is characterised by a massive infiltration of the peripheral blood by ATL cells,

while the ATL lymphoma is characterised by the presence of less than 1% leukaemic cells on a blood smear and major involvement of lymphoid organs (Nicot 2005).

There are four types of ATLL (acute, chronic, smouldering and lymphoma) with varying symptoms and responses to treatment (Shimoyama 1991). Acute ATLL is for example characterised by symptoms such as fever, cough, lymphadenopathy, skin lesions, hepatosplenomegaly, marked leukocytosis and hypocalcaemia which is frequently associated with lytic bone lesions and generalised bone resorption. The mean survival time for this specific disease is 6 months with a poor response to chemo- or radiotherapy (Nicot 2005).

1.3 Acute Myeloid Leukaemia

1.3.1 Introduction

Acute Myeloid Leukaemia (AML) is a heterogeneous clonal disorder of haemopoietic progenitor cells which lose the ability to differentiate normally and to respond to normal regulators of proliferation. With this and a decreased rate of self-destruction, cells accumulate in the bone marrow and other organs. Within a year of developing the disease, if treatment is not given, fatal infection, bleeding or organ infiltration will occur (Estey and Dohner 2006). Elderly patients do not survive treatment well and only reach a 10% cure rate due to cytogenetic abnormalities. Patients younger than 60 respond better to cytotoxic chemotherapies and have a cure rate ranging from 20-75%, again depending on leukaemia cell cytogenetics.

It was proposed by McCulloch et al (1983) that there is an existence of leukaemia stem cells (LSCs) in AML. Studies have shown that only a small defined subset of leukaemic cells was consistently clonogenic and capable of generating leukaemia when transplanted in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice (Jordan 2007). Leukaemia is a disease determined by a few LSCs that undergo an aberrant and poorly regulated process of organogenesis analogous to that of normal haematopoietic stem cells (HSCs).

AML is the most common myeloid leukaemia with a median age of presentation at 70 years and it is more prevalent in men than women. Risk factors for acquiring AML include benzene

exposure, most of which is received through cigarette smoking. Ionising radiation is another large risk factor, for example people who have survived atomic bombs have a greater risk due to abnormalities of chromosomes 5 and 7 (Nakanishi et al 1999). Also, AML is common in nuclear industry workers (Cardis et al 1995) and those who fly extensively in commercial jet planes (Gundestrup and Storm 1999). The third main risk factor for AML is cytotoxic chemotherapy. AML can develop after 5-10 years following exposure to alkylating agents which cause monosomies or deletions in chromosomes 5 or 7. AML can also develop 1-5 years post-treatment with doxorubicin and etoposide, drugs which interact with DNA topoisomerase II, causing abnormalities in chromosome 11 and translocations between chromosomes 15 and 17 (Estey and Dohner 2006).

Treatment for AML should be aggressive to achieve complete remission. Sixty to 70% of adults with AML may achieve complete remission following induction therapy and more than 25% can expect to survive three or more years and may be cured (National Cancer Institute 2010). Treatment consists of a combination of anthracycline drugs such as daunorubicin, idarubicin or cytarabine (Tallman et al 2005). There are two phases of therapy. The first phase involves drug treatment to attempt to produce complete remission. Complete remission is reached when marrow has less than 5% blasts, neutrophil count is greater than 1000 and platelet count is greater than 100000 (Cheson et al 2003). The second phase aims to prolong the complete remission. Patients are treated with cytarabine or anthracycline. A remission length of three years means the likelihood of relapse falls to less than 10% (Estey and Dohner 2006). All treatment success depends on various factors relating specifically to the patient. Increased morbidity during induction appears to be inversely correlated with age (National Cancer Institute 2010). Other factors include central nervous system involvement with leukaemia, systemic infection at diagnosis, elevated white blood cell count ($>100000/\text{mm}^3$), treatment-induced AML, history of myelodysplastic syndromes and other antecedent haematological disorders (National Cancer Institute 2010).

1.3.2 French-American British classification system for AML

The French-American-British (FAB) nomenclature system for AML helps to define which type of cell has become the leukaemic cell and also its degree of maturity. Morphology of cancerous cells are viewed by light microscopy or their cytogenetics are studied to define which category they come under. Different categories respond differently to treatment however other factors previously described such as age and previous treatment still

contribute to treatment outcome. This means each patient should be treated individually according to their health history, age and genetics, not just by AML subtype alone.

Types of AML according to the FAB classification system range from M0 to M8. M0 is a minimally differentiated acute myeloblastic leukaemia which represents 2-3% of all cases of AML (Kumar et al 2007). The blasts in type M0 cannot be recognised as myeloid based on morphology and cytochemistry alone, however they are agranular and show no signs of having Auer rods. Immunophenotyping of M0 cells shows they have myeloid antigens such as CD13, CD33, CD34 and Human leukocyte antigen (HLA-DR) (Testa et al 2002). There is often loss of the long arm of chromosome 5 and 7 and higher expression of the multidrug resistance glycoprotein p170 (Lichtman et al. 2005).

M1

M1 is an acute myeloblastic leukaemia with too many immature white blood cells accumulating in the blood and bone marrow (Bennett et al 2008).

M2

M2 is acute myeloblastic leukaemia with granulocyte maturation. Patients generally display dysplastic features in myeloid and erythroid lineages with reduction in megakaryocytes (Taj et al 1995). Such characteristics are present in patients with a reciprocal translocation between chromosomes 8 and 21, t(8;21). The DNA before translocation would normally code for two separate proteins RUNX1 and ETO, however translocation causes the two proteins to be transcribed and translated into a single large protein known as M2 AML. This allows the cell to divide unchecked which leads to the development of leukaemia (Taj et al 1995).

M3

M3 is an acute promyelocytic leukaemia (APL) where an abnormal number of immature promyelocytes accumulate in the blood. M3 AML is characterised often by a chromosomal translocation between the retinoic acid receptor alpha gene on chromosome 17 (RAR α) with the promyelocytic leukaemia gene (PML) on chromosome 15, t(15;17)(q22;q12) (Pecorino 2008). The fusion protein binds with enhanced affinity to sites on the cell's DNA, this

enhances interaction of nuclear co-repressor molecule with histone deacetylase, which in turn blocks transcription and differentiation of granulocytes (Rambaldi 2002).

A particular symptom of APL is coagulopathy which is the susceptibility to excessive bleeding and insufficient blood clotting. The majority of APL patients are affected by disseminated intravascular coagulation (DIC) (Tallman et al 1993). This is due to hypofibrinogenemia, increased fibrinogen-fibrin degradation products (FDP), and prolonged prothrombin and thrombin times (Barbui et al 1998). APL is also characterised by the presence of faggot cells. The cytoplasm of faggot cells contains multiple Auer rods (Lazarchick 2009). Auer rods are cytoplasmic inclusions found only in the leukocytes of AML (Pearson 1986). Auer rods appear as clumps of asurophilic granular material that aggregates in elongated needles in the cytoplasm of leukaemic blasts. They are composed of fused lysosomes and contain peroxidase, lysosomal enzymes and large crystalline inclusions (Hütter et al 2007).

M4

AML type M4 is known as acute myelomonocytic leukaemia (AMMoL) or AML-M4 with bone marrow eosinophilia (AML M4Eo). This FAB category commonly has abnormalities of chromosome 16q22, for example: del(16)(q22), inv(16)(p13q22) and t(16;16)(p13;q22) (Arthur et al 1983, Le Beau et al 1983 and Testa et al 1984). Patients with AML M4Eo have a young median age (Bernard et al 1989) and often have a high peripheral white blood cell count and organomegaly and generally have a high response rate to induction chemotherapy (Adriaansen et al 1993). Relapses occur in the central nervous system relatively frequently as leptomeningeal disease and intracerebral myeloblastomas (Holmes et al 1985). Blast cells, monocytic cells and eosinophil cells can be recognised within each AML M4Eo. Eosinophils are often dysplastic with abnormal eosinophilic granules and varying numbers of basophilic-staining granules (Holmes et al 1985).

M5

AML type M5 is further divided into two subtypes: AML M5a (acute monoblastic leukaemia) and 5b (acute monocytic leukaemia). Both are defined when 80% or more of the leukaemic cells are monocytic in their lineage, including monoblasts, promonocytes and monocytes. To distinguish between the two, the relative proportions of monoblasts and promonocytes are noted. In type 5a, 80% or more of the monocytic cells are monoblasts whereas in type 5b,

the majority of monocytic cells are promonocytes (Brunning et al 2001). Type 5a AML has more frequent aberrations of 11q23/MLL than type 5b, and both have higher frequencies than all other AML subtypes (Haferlach et al 2002). Chromosome abnormalities of 11q23 have been related to poor prognosis and short, event-free survival (Thirman et al 1993). There is also a higher incidence of FLT-3 length mutations in AML 5b compared with AML 5a (Haferlach et al 2002).

M6

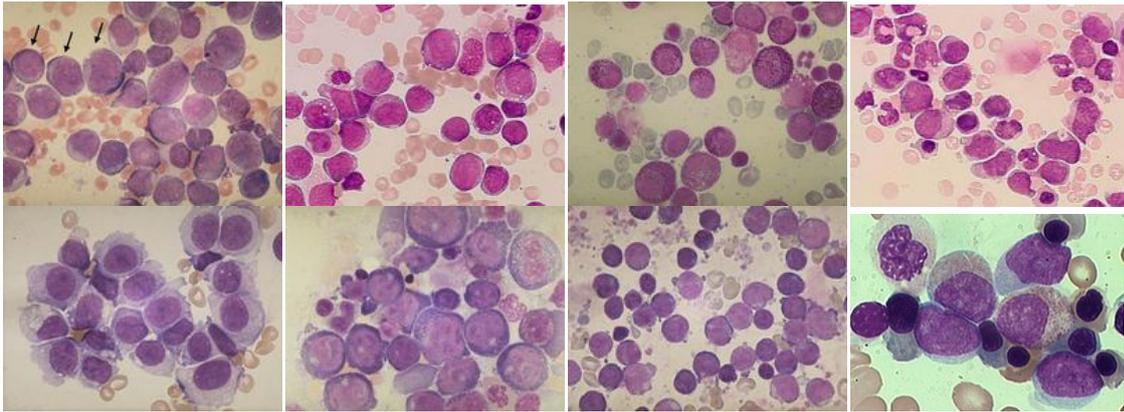
AML type M6 is known as acute erythroleukaemia, a rare subtype of AML with poor prognosis and commonly associated with previous diagnosis of myelodysplastic syndrome (MDS) (Santos et al 2009). Jogai et al (2001) have found blasts in peripheral blood, dyserythropoiesis, dysmegakaryopoiesis and dysplasia in granulocytic cells in many M6 AML patients. Many M6 AML cases have an anomaly named chromosome torsion by Glaser et al. Such an anomaly consisted of the chromatids of the p- and q-arm being located side by side rather than in the usual tandem alignment.

M7

Type M7 AML is known as acute megakaryoblastic leukaemia. Children with constitutional trisomy 21 (Down's syndrome) have a 500-fold increased risk of developing acute megakaryoblastic leukaemia (Hitzler et al 2003). Hitzler et al have found somatic mutations of the gene encoding the haematopoietic transcription factor *GATA1* in acute megakaryoblastic leukaemia blasts of patients with Down's syndrome which has suggested a significant role for these mutations in the development of leukaemia. Type M7 AML typically has a translocation of chromosome 22 involving the megakaryocytic acute leukaemia (*MAL*) gene (Mercher et al 2001).

M8

Finally, type M8 AML is known as acute basophilic leukaemia (ABL) and is another rare form of acute leukaemia. Blast cells are morphologically undifferentiated and coarse basophilic granules can be recognised. Myeloid antigens such as CD9 or CD25 are strongly expressed however lymphoid, erythroid or megakaryocytic markers are not expressed significantly. 1-A pure ABL is a type of monophenotypic ABL where only the basophilic lineage is affected. This is classified as AML M8, which has suspected c-MYB (myeloblastosis) oncogene involvement (Duchayne et al 1999).



Intro Figure 2: The subtypes of AML according to the FAB classification system. Left to right, top to bottom: M1, M2, M3, M4, M5, M6, M7, M8.

1.3.3 World Health Organisation classification system for AML

The FAB classification system is not always used to define AML type because it is based on bone marrow and peripheral blood blast counts without considering clinical and biological variables such as cytogenetics and the degree and number of cytopenias (Lee et al 2003). Also, in many cases there may be no correlation between cell morphology and genetic defects or underlying genetic and molecular defects cannot be identified (Vardiman et al 2002). There also exists the World Health Organisation (WHO) classification system of AML which aims to give more helpful clinical and prognostic information. WHO classification for AML is shown in Intro Table 1 (Vardiman et al 2002).

1. Acute myeloid leukemia with recurrent genetic abnormalities

- Acute myeloid leukemia with t(8;21)(q22;q22), (AML1/ETO)
- Acute myeloid leukemia with abnormal bone marrow eosinophils and inv(16)(p13q22) or t(16;16)(p13;q22), (CBFβ/MYH11)
- Acute promyelocytic leukemia with t(15;17)(q22;q12), (PML/RARα) and variants
- Acute myeloid leukemia with 11q23 (MLL) abnormalities

2. Acute myeloid leukemia with multilineage dysplasia

- Following MDS or MDS/MPD
- Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineages

3. Acute myeloid leukemia and myelodysplastic syndromes, therapy-related

- Alkylating agent/radiation–related type
- Topoisomerase II inhibitor–related type (some may be lymphoid)
- Others

4. Acute myeloid leukemia, not otherwise categorized

- Acute myeloid leukemia, minimally differentiated
- Acute myeloid leukemia without maturation
- Acute myeloid leukemia with maturation
- Acute myelomonocytic leukemia
- Acute monoblastic/acute monocytic leukemia
- Acute erythroid leukemia (erythroid/myeloid and pure erythroleukemia)
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis
- Myeloid sarcoma

Intro Table 1: WHO classification of AML

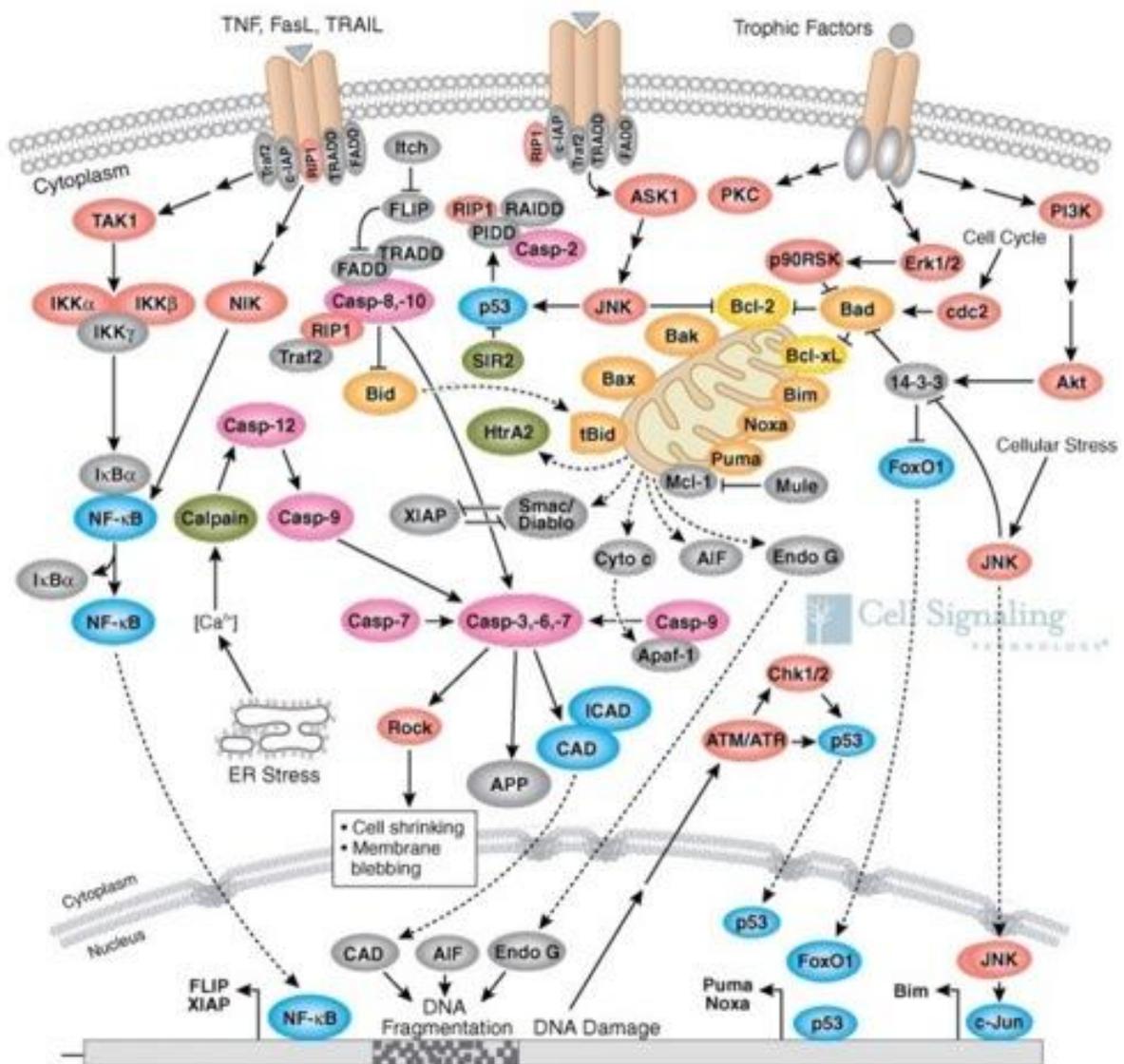
1.4 Apoptosis

1.4.1 Introduction

AML in particular is a disease that is greatly affected by the amount of apoptosis occurring in the cancerous cells. Generally, there is a decreased rate of self-destruction of white blood cells which leads to an accumulation of these cells in the bone marrow and in other organs. This is the cause behind many of the symptoms of AML including increased frequency of

infection and increased bruising. This chapter will explain the link between apoptosis and leukaemia on a molecular level.

Apoptosis is a homeostatic mechanism of programmed cell death that occurs throughout normal development and ageing to maintain steady and healthy cell populations in tissues. Cells are threatened by bacterial and viral diseases, and also by cytotoxic and mutagenic effects of DNA damaging agents such as: UV light, ionising radiation, chemicals in foodstuffs, air- and water-borne agents, methylating species and reactive oxygen species (Norbury et al 2001). Damaged DNA within cells must be repaired, or the cell must undergo apoptosis to ensure the damage is not replicated and passed on to daughter cells.



Intro Figure 3: Cellular signalling leading to apoptosis [www.cellsignal.com].

1.4.2 Apoptosis and leukaemia

Many forms of leukaemia involve a continuous increase in white blood cells counts due to defects in apoptotic machinery rather than increased cell proliferation alone (Kitada et al 1998). For example, B-cell chronic lymphocytic leukaemia (B-CLL) is characterised by the accumulation of resting, long-lived, CD5+ B cells in blood which express high levels of the antiapoptotic proteins such MCL-1 and BCL-2 (Robertson 1996). BCL-2 is a proto-oncogene which delays the onset of apoptosis in cells. BCL-2 over expression is associated with resistance to chemotherapy and inhibition of apoptosis (Panayiotidis 1993). When CLL cells are cultured in vitro, cells with high levels of BCL-2 survive longer than those with low levels which suggests an inherent survival advantage by BCL-2 (Kumudha et al 2010). MCL-1 is an oncogene that promotes tumorigenesis and protection against apoptosis and drug resistance of malignant cells. MCL-1 expression is required in tumours to maintain cell viability and promote cell differentiation, and its down-regulation induces apoptosis (Adida et al 2000).

CML has a translocated Philadelphia chromosome, forming the hybrid Bcr-ABL protein, an oncoprotein which is believed to inhibit the apoptotic response, providing a survival advantage for the leukaemic cell clone (Sears et al 2010).

In AML and other lymphoid malignancies, glucocorticoids (GCs) are common components of many chemotherapeutic treatments. Upon relapse however, patients can become resistant to GC treatment due to defects in apoptotic machinery (Tissing et al 2003). Heidari et al (2010) have recently shown that down regulation of the antiapoptotic BCL-2 family proteins, particularly MCL-1, enhances GC-induced cell death. MCL-1 is targeted by GX15-070 (obatoclax) which induces caspase-3 cleavage and increases a population of Annexin V-positive cells (Heidari et al 2010).

Lu et al (2006) and Heidari et al (2010) have shown that BIM, a pro-apoptotic BH3-only protein, is up regulated by treatment with dexamethasone (Dex) in ALL cells. BIM plays an important role in Dex-induced apoptosis (Lu et al 2006). Co-treatment with Dex and MEK/ERK inhibitors both up regulates BIM and aids BIM dephosphorylation/activation, promoting apoptosis in a variety of ALL cells (Rambal et al 2009).

Gonzalez et al (2010) have investigated the effect that Heat-shock protein-27 (Hsp27) on leukaemic cells. Hsp27 associates with components of the extrinsic and intrinsic apoptotic

pathways and inhibits the execution of apoptosis. Elevated expression of Hsp27 accentuates tumorigenicity and the resistance to chemotherapy of malignant cells (Hansen et al 1999) which is why high expression of Hsp27 in leukaemia is associated with a bad prognosis (Thomas et al 2005). Gonzalez et al (2010) have recently shown that apigenin, a common plant dietary flavonoid found in parsley and celery, can be used as an anti-cancer compound against Hsp27. Apigenin induces phosphorylation of Hsp27 which modulates its oligomerisation properties thus modulating the cytoprotective activity of Hsp27 (Kato et al 1994). Apigenin also has cytotoxic flavonoid activity against Hsp-27 which is more effective following the phosphorylation step (Gonzalez et al 2010)

Survivin, an inhibitor of apoptosis protein, is over expressed in the blasts of AML patient samples (Carter et al 2001). Survivin suppresses cell death induced by a variety of stimuli including tumour necrosis factor (TNF), Fas, menadione, staurosporine, etoposide, paclitaxel and growth factor withdrawal (Ambrosini et al 1997). Disruption of survivin expression causes cell death and cell division defects, resulting in polyploidy and multinucleated cells (Fraser et al 1999). XIAP is another inhibitor of apoptosis protein whose expression levels in primary AML samples is variable and is found to be inversely correlated with AML patient survival (Tamm et al 2000).

Finally, NF- κ B signalling largely affects the outcome of a cells response to apoptotic signals. Activation of NF- κ B is required for a successful immune response and to amplify the survival and proliferation of cells (Demchenko et al 2009). NF- κ B has been found to be constitutively active in most tumour cell lines (Aggarwal 2004) which allows NF- κ B to mediate inflammation and tumorigenesis and prevent a sufficient level of apoptosis required to maintain a healthy number of blood cells present in the population.

1.5 Nuclear Factor-kappaB

1.5.1 Introduction

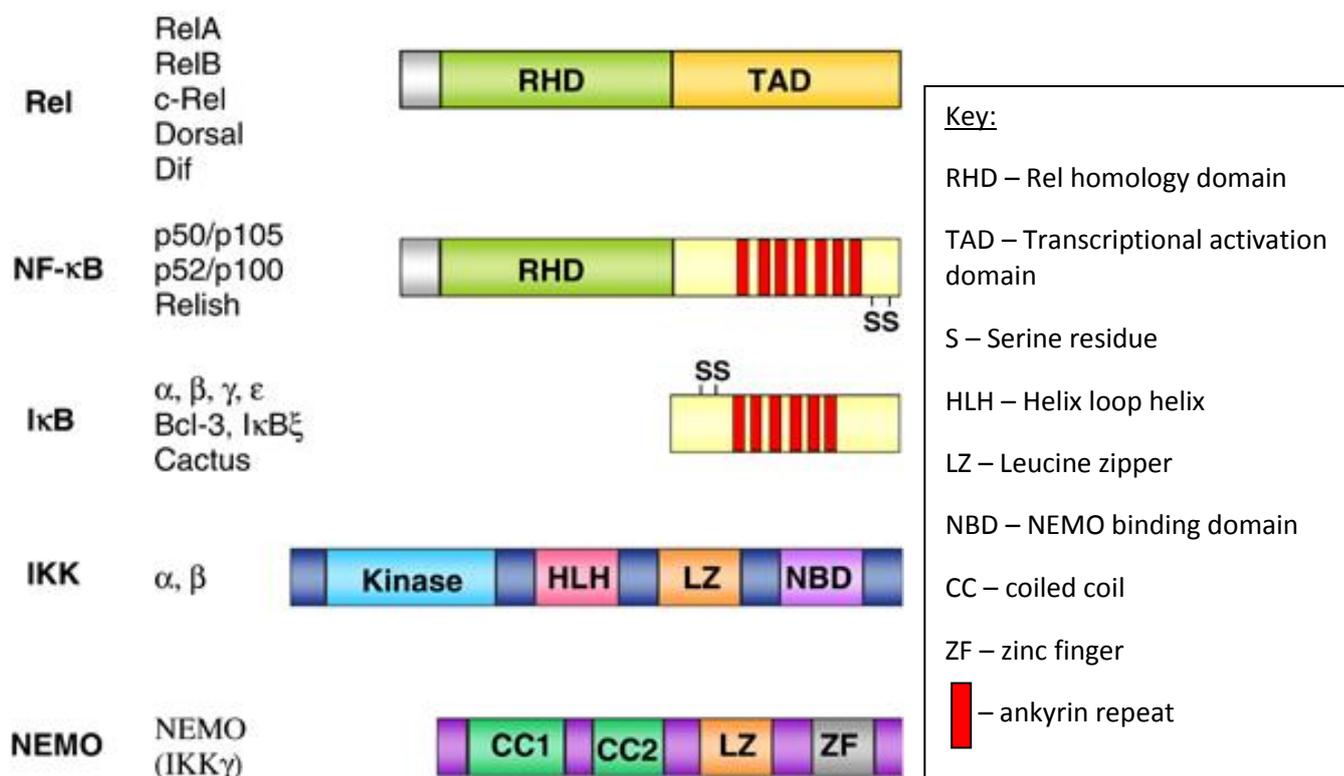
NF- κ B (or nuclear factor for the kappa-light-chain-enhancer of activated B cells) was discovered by David Baltimore in 1986 via its binding to a sequence in the kappa light chain enhancer in B cells. Proteins in the NF- κ B family are dimeric transcription factors composed of five different subunits: p65 (RelA), RelB (p65), cRel, p50 and p52 (Harald et al 2010). They control cellular responses such as inflammation, immune response, differentiation,

proliferation and apoptosis (Kempe et al 2005). NF- κ B proteins do this in response to stimuli such as stress, cytokines, free radicals, ultraviolet radiation, oxidised low density lipoproteins (LDLs), and bacterial or viral antigens (Brasier et al 2006). Normally, NF- κ B has a role in regulating the immune response to infection, however NF- κ B can become dysregulated leading to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development.

1.5.2 NF- κ B proteins and their structure

NF- κ B 1 and NF- κ B 2 proteins are different in that they are synthesised as large precursors p105 and p100 which undergo processing to become the mature p50 and p52 subunits. This processing is mediated by the ubiquitin/proteasome pathway and involves selective degradation of their C-terminal region which contains inhibitory ankyrin repeats (Gilmore et al 2006). While the generation of p52 from p100 is a tightly regulated process, p50 is produced from constitutive processing of p105 (Karin et al 2000).

All proteins in the NF- κ B family have a conserved DNA-binding/dimerisation domain called the Rel homology domain (RHD) in their N-termini (Intro Figure 3). The RHD contains a nuclear localisation sequence (NLS) which is involved in dimerisation, sequence specific DNA binding and interaction with the inhibitor I κ B proteins (Ghosh et al 1998). NF- κ B dimers bind to κ B sites within the promoters/enhancers of target genes and regulate transcription through the recruitment of co activators and co repressors (Hayden and Ghosh 2008). A subfamily of NF- κ B proteins including RelA, RelB and c-Rel have a transactivation domain (TAD) in their C-termini, which also has sequences important for nuclear localisation and I κ B inhibitor binding (Gilmore et al 2006). p50 and p52 may repress transcription unless associated with a TAD-containing NF- κ B family member, as they do not have a TAD of their own (Hayden and Ghosh 2008).



Intro Figure 3: Structure of core NF-κB signalling proteins. [Gilmore 2006].

1.5.3 NF-κB signalling

In un stimulated cells, NF-κB proteins normally reside in the cytoplasm in an inactive state where their nuclear localisation sequence (NLS) is masked by either one of two ways (Hayden et al 2008). The different ways in which the NLS of NF-κB proteins is masked distinguishes between the canonical and non-canonical NF-κB signalling pathways. In the canonical or classical pathway, a dimer of NF-κB interacts intermolecularly with an inhibitor of κB (IκB) protein forming an inactive ternary complex (Scheidereit et al 2006). In the non-canonical or alternative NF-κB signalling pathway, blockade of the NLS occurs by intramolecular binding of an inhibitory domain. The inhibition comes from the IκB protein-like inhibitory domains on the C-terminal parts of the large 105 and 100 kDa precursor proteins of NF-κB subunits p50 and p52 (Ghosh et al 1998 and Scheidereit et al 2006). Receptor-mediated activation of the canonical NF-κB pathway occurs within a matter of minutes and does not require new protein synthesis, however activation of the non-canonical NF-κB pathway takes several hours and does require new protein synthesis of NIK (Matsuhima et al 2001 and Zarnegar et al 2008).

1.5.3.1 The non-canonical or alternative NF- κ B signalling pathway

The non canonical pathway is activated by lymphorganogenic cytokines, such as lymphotoxin- α - β -heterotrimers, and B cell survival and maturation factors, such as CD40 ligand (CD40L) and BAFF (B cell activating factor)(Derudder et al 2003 and Claudio et al 2002). Lymphotoxin- α - β , CD40L and BAFF belong to the TNF super family whose members signal through trimeric receptors (TNF receptors or TNFRs) and signalling proteins called TNFR-associated factors (TRAFs) (Bishop et al 2004). Other members of the TNF super family which signal in this way are RANKL (receptor activator of NF- κ B ligand) and TWEAK (TNF-related weak inducer of apoptosis) (Claudio et al 2002). TRAFs are the key intermediates in both the classical and alternative NF- κ B signalling pathways (Hacker et al 2006).

Activation of the non-canonical NF- κ B signalling pathway requires degradation of TRAF proteins. TRAF2 and TRAF3 are inhibitors of non-canonical NF- κ B signalling (Gardam et al 2008). Activation of the non-canonical pathway depends on receptor-induced TRAF3 degradation and stabilisation of NIK (Liao et al 2004) as TRAF3 is a negative regulator of the activation of NIK. In the resting state, TRAF3 induces NIK ubiquitination and degradation (Liao et al 2004). Upon receptor activation, TRAF2 ubiquitinates and activates cIAP1 and cIAP2 to induce degradative Lys48-linked polyubiquitination of TRAF3. cIAP1 and cIAP2 are RING-containing components of the non-canonical pathway which belong to the IAP (inhibitors of apoptosis) family (Vallabhparpu et al 2008). Degradation of TRAF3 prevents targeting of newly synthesised NIK by a TRAF2-cIAP1-cIAP2 ubiquitin ligase complex, allowing NIK accumulation and activation through autophosphorylation (Vallabhparpu et al 2008).

NIK (NF- κ B inducing kinase) is a positive regulator of the processing of the *NF- κ B 2* gene product p100 into the mature NF- κ B subunit p52 (Xiao et al 2001). P100 has several properties that are likely to be important for NF- κ B regulation: selective regulation of specific NF- κ B complexes such that in its absence, a subset of inducible NF- κ B responses (e.g. RelB-dependent gene expression) is lost; selective function of I κ B subunits downstream of specific signalling pathways; and regulation of basal activity of specific NF- κ B dimers (Hayden and Ghosh 2008). NIK is a potent and specific inducer of p100 processing which acts via I κ K α subunits only, I κ B β and NEMO are not involved (Hacker and Karin 2006). NIK directly phosphorylates and activates I κ K α (Hacker and Karin 2006) which then induces site-

specific phosphorylation of p100 by I κ K α and ubiquitination of p100. This leads to p100 processing (as opposed to degradation) in the 26s proteasome, producing the mature p52 NF- κ B subunit (Xiao et al 2001).

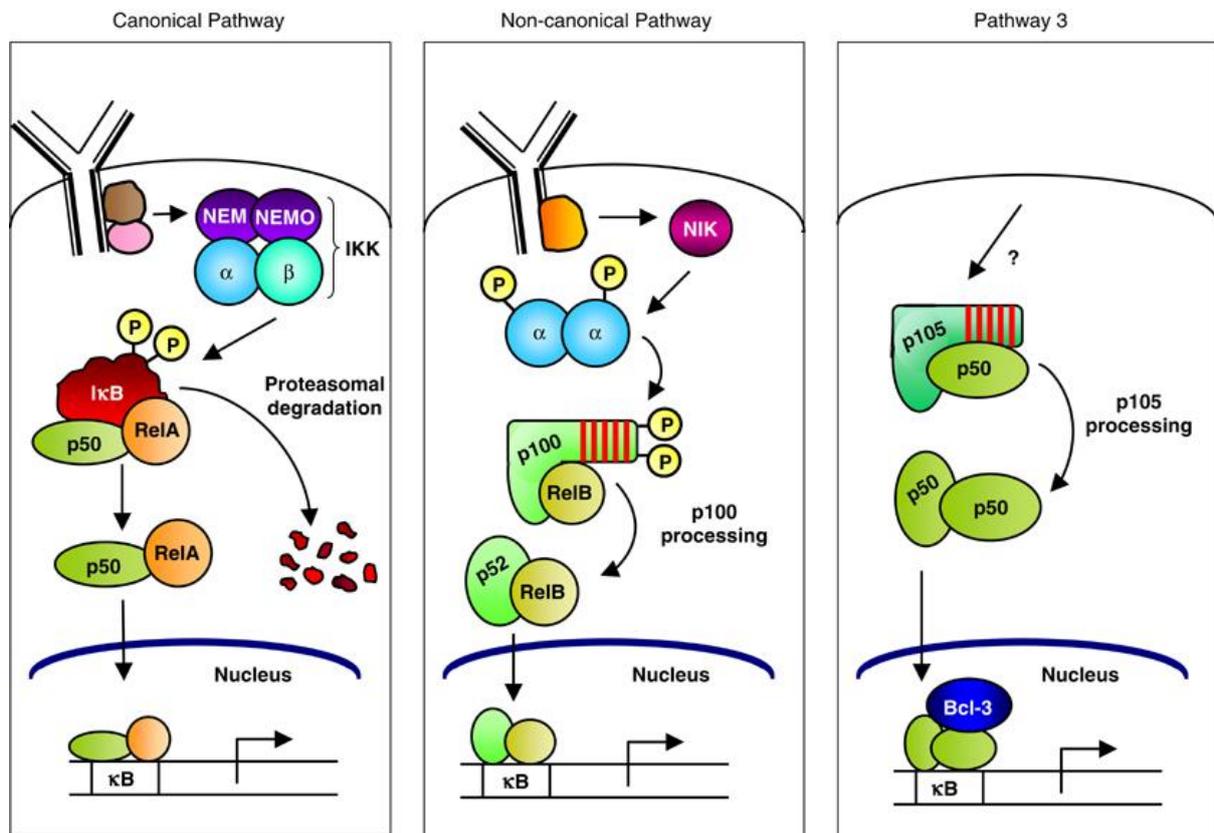
Constitutive processing of p100 is suppressed by a processing-inhibitory domain (PID) which can be found at the C-terminal region of p100 (Xiao et al 2001). A low level of constitutive processing of p100 to p52 does however occur in a cell type-specific manner. This is also dependent on I κ K α and NIK (Qing and Xiao 2005 and Xiao et al 2004). Once the mature p52 subunit has been generated, it will associate with RelB to gain the use of RelB's TAD domain, then translocate into the nucleus as a heterodimer. p52 containing NF- κ B dimers drive a transcriptional response that is distinct from that induced by the canonical, I κ B α -regulated pathway (Hayden and Ghosh 2008). Because p100 undergoes constitutive processing in certain tissues, RelB/p52 heterodimers may exhibit constitutive activation.

1.5.3.2 The classical or canonical NF- κ B signalling pathway

The canonical NF- κ B pathway is initiated by activation of the TRAF adaptor proteins and the subsequent stimulation of the I κ B kinase (IKK) complex which contains the kinases I κ K α , IKK β and the structural/regulatory component NEMO or I κ K γ (Hayden et al 2008). The NF- κ B precursor protein p105 undergoes processing via the proteasome to give the mature subunit p50 (Moorthy et al 2006). When p105 is bound to NF- κ B complexes, it appears that processing is inhibited and instead induced degradation is favoured (Cohen et al 2001). This means that unprocessed p105 acts as an I κ B protein that binds NF- κ B dimers and can be inducibly degraded upon I κ K activation (Hayden and Ghosh 2008). The activated I κ K complex phosphorylates I κ B proteins which triggers their rapid proteasomal degradation. I κ B proteins in unstimulated cells provide inhibition by masking the NLS of p65 which sequesters the NF- κ B dimers away from κ B elements and inhibits transcription (Hayden and Ghosh 2008). I κ B protein degradation leaves the NF- κ B p65:p50 dimers free from inhibition in the cytoplasm as the NLS of both p50 and p65 subunits become exposed, allowing them to translocate into the nucleus. Cells that lack all three I κ B subunits show relatively normal nuclear and cytoplasmic p65 distribution but significantly increased basal NF- κ B dependent gene expression. This suggests that regulation of the transcriptional activity of NF- κ B by I κ Bs is partly independent of cytoplasmic sequestration (Tergaonkar et al 2005).

Downstream of I κ B degradation, it has been shown that phosphorylation of p65 by protein kinase A (PKA) is crucial for NF- κ B transcriptional activity (Chen and Greene 2004). PKA can be found in a complex with cytosolic NF- κ B :I κ B complexes and phosphorylates p65 at a serine residue after degradation of I κ B α . This promotes the interaction of p65 with the transcriptional co activators CREB-binding protein (CBP) and p300 (Zhong et al 1998). There are also other kinases such as MSK1 and MSK2 (mitogen- and stress-activated protein kinase) which phosphorylate serine residues of p65 (Hayden and Ghosh 2008). Finally, I κ K α and I κ K β are involved in the direct phosphorylation of p65 (Chen and Greene 2004) and CK2 inducibly phosphorylates p65 following IL-1 or TNF α stimulation (Bird et al 1997). p65 can increase transcription when it is acetylated by CBP/p300 and associated histone acetylases (HATs) (Chen and Greene 2004). The acetylation of a particular lysine (Lys310) has most clearly been shown to enhance transcriptional activity without altering DNA or I κ B binding (Chen et al 2002)

To summarise, in the non-canonical NF- κ B pathway TRAF and NIK are sufficient to activate I κ K α in a NEMO-dependent manner. In the canonical NF- κ B signalling pathway, signalling relies on both TRAF and RIP proteins as well as the kinase TAK1. NEMO is required for signalling in all canonical NF- κ B pathways. Pathways may be categorised as non-canonical and canonical based on the requirement for NEMO or on the specific I κ B protein that is phosphorylated and degraded/processed. I κ B α , I κ B β and I κ B ϵ for canonical and p100 for non-canonical. All aspects of the NF- κ B signalling pathway are shown in the figure below.



Intro Figure 4: Components of the canonical, non-canonical and alternate NF-κB signalling pathways. [Gilmore 2006].

1.5.4 Termination of the NF-κB response

Transcription of target genes is regulated through posttranslational modifications of NF-κB that affect the ability of NF-κB dimers to interact with transcriptional co-activators. NF-κB - dependent transcription of IκB proteins and additional mechanisms targeting DNA-bound NF-κB dimers terminate the response (Hayden and Ghosh 2008). Active, DNA-bound NF-κB is suppressed once signalling is terminated. This is possibly done when promoter-bound p65 may be subject to proteasomal degradation (Sacconi et al 2004). IκKα is also involved in termination of inflammatory transcriptional responses in macrophages, possibly by promoting the nuclear degradation of both p65 and c-Rel (Lawrence et al 2005, Li et al 2005).

1.5.5 TNF-induced NF-κB signalling

TNFα binds to TNFR1 which recruits TRAF2 to the receptor through its interaction with TRADD (Hsu et al 1996). TRAF2 has E3 ubiquitin ligase activity and TRAF5 is also involved

with TNFR1 signalling (Nakano et al 1999). I κ K may be recruited to the TNFR1 receptor through RIP1 kinase (Hayden and Ghosh 2008). RIP family kinases are characterised by their conserved serine/threonine kinase domains (Meylan and Tschopp 2005). There are seven RIPs in the family and RIP1 has a death domain (DD) which mediates interaction with other death domain-containing adaptors and receptors (Hayden and Ghosh 2008). Receptor-interacting proteins (RIPs) act both upstream of and with TRAF proteins to activate I κ K. RIP proteins act as true adaptors in NF- κ B signalling pathways by interacting with upstream signalling cassettes through well characterized protein-binding domains. RIP1 binds to NEMO and is essential for TNF α -induced I κ K and NF- κ B activation (Hsu et al 1996 and Kelliher et al 1998).

1.5.6 NF- κ B and cancer

The activation of NF- κ B can produce both beneficial and damaging effects on human health. While NF- κ B is needed for proper immune system function, inappropriate NF- κ B activation can mediate inflammation, tumour cell survival, proliferation, invasion, angiogenesis and metastases (Kunnumakkara 2009). Inflammatory agents, carcinogens, tumour promoters and the tumour microenvironment can activate NF- κ B (Bharti and Aggarwal 2002). Cancer is a pro-inflammatory disease (Balkwill and Mantovani, 2001) and most inflammatory agents mediate their effects through the activation of NF- κ B.

NF- κ B is rarely found to be constitutively active in normal cells except for proliferating T cells, B cells, thymocytes, monocytes and astrocytes. NF- κ B is however constitutively active in tumour tissues derived from patients with multiple myeloma (Feinman et al 1999), AML (Griffin 2001), ALL (Kordes et al 2000), CML, (Baron et al 2002) and prostate (Palayoor et al 1999) and breast cancers (Nakshatri et al 1997). The constitutive activation of NF- κ B may be caused by mutation of I κ B α (Wood et al 1998), enhanced proteasomal activity (Miyamoto et al 1994), or enhanced inflammatory cytokine expression (O'Connell et al 1995). This may occur as a result of the effects of the carcinogens such as DMBA and cigarette smoke, and of tumour promoters such as phorbol esters (Banerjee et al 2002 and Anto et al 2002). TNF may be the most potent activator of NF- κ B, it mediates carcinogenesis through induction of proliferation, invasion and metastasis of tumour cells (Shishodia et al 2003). c-Rel itself is an oncogenic protein of the NF- κ B family which is found to be amplified in Hodgkin's lymphomas, diffuse large B cell lymphomas, and some follicular and mediastinal B cell lymphomas (Gilmore et al 2004). Other oncogenes involved in cellular transformation via the effects of NF- κ B include Ras, *c-myc*, Pim-2, Kaposi's sarcoma-associated herpes virus

(KSHV) proteins, EBV latent membrane protein (LMP)-1 and human T lymphocytic leukaemia virus (HTLV)-1 (Aggarwal 2004).

Both cytokines and cytokine receptors either are regulated by NF- κ B or mediate proliferation through activation of NF- κ B (Aggarwal 2004). For example IL-1 β is a growth factor for AML and it activates NF- κ B to mediate its proliferative effects (Osborn et al 1989). NF- κ B activation also has a role in mediating angiogenesis and cellular invasion in tumours. Gene products involved in tumour invasion such as matrix metalloproteinases (MMP), urokinase type of plasminogen activator (uPA) and interleukin-8 (IL-8) are regulated by NF- κ B signalling, particularly the degradation of I κ B α (Novak et al 1991). Growth factors controlled by NF- κ B activation that regulate angiogenesis are VEGF, TNF, IL-1 and IL-8 which are produced by macrophages, neutrophils and other inflammatory cells (Loch et al 2001). NF- κ B regulates the expression of various adhesion molecules including ICAM-1, VCAM-1 and ELAM-1 (van de Stolpe et al 1994) which mediate metastasis of cancerous cells, along with inducible nitric oxide synthase (iNOS) (Thomsen and Miles 1998).

Inflammation mediates tumorigenesis. NF- κ B links this relationship in various ways. Greten et al (2004) demonstrated expression of I κ K β in enterocytes is important during early stages of tumour initiation and promotion by expression of antiapoptotic proteins whereas in myeloid cells, I κ K β contributed to initiation and promotion through expression of tumour growth factors. Activation of NF- κ B by endotoxin in cancer cells produces inflammation-induced tumour growth through expression of TNF and inhibition of NF- κ B mediates tumour regression through TRAIL (Luo et al 2004). NF- κ B activation is required for LPS-induced tumour growth in the CT26 colon cancer cell line and TNF mediates LPS-induced tumour growth and NF- κ B activation (Luo et al 2004).

The p53 protein has long been implicated at multiple levels with the NF- κ B pathway. The importance of both NF- κ B and p53 in cancer has promoted significant research into the relationship between these two transcription factors (Hayden and Ghosh 2008). P53 and p65 have been reported to directly compete for CBP/p300 (Webster and Perkins, 1999) which has been suggested to promote their cross-repression.

Deregulation of NF- κ B activity has been increasingly implicated in neoplastic transformation and cancer progression (Sun and Xiao 1993). Adult T cell leukaemia can be caused by

infection by human T cell leukaemia virus type 1 (HTLV-1). HTLV-1 has a viral protein Tax which persistently activates NF- κ B. Tax protein works by activating the I κ K complex in a NEMO-dependent manner (Yamaoka et al 1998). Tax physically interacts with p100, this recruitment leads to the phosphorylation and processing of p100 (Xiao et al 2001). Tax however is not detectable in fresh ATL cell samples and ATL-derived cell lines despite there being reported constitutive I κ K activity and aberrant expression of p52 in such ATL cells (Hironaka et al 2004). Such findings have suggested that Tax-independent constitutive NF- κ B activation in ATL cells involves the non-canonical signalling pathway (Miura et al 2005). This theory has been supported by findings from that NIK is over-expressed at the pre-translational level in ATL (Saito et al 2008).

1.5.7 NF- κ B and chemotherapy resistance

It has been shown by many investigations that NF- κ B is constitutively activated in several human tumours (Camp et al 2003). NF- κ B activation may play a role in oncogenesis by stimulating cell growth and inhibiting apoptosis and providing a survival advantage for transformed cells (Camp et al 2003). NF- κ B liberation allows nuclear translocation and subsequent induction of target genes that code for cell cycle regulatory genes such as cyclin D1 that stimulate cell proliferation and antiapoptotic proteins such as survivin (Hinz et al 1999).

Chemotherapy and radiation therapies are generally targeted to components of the apoptotic pathway however it has become obvious that NF- κ B has a role in inducible chemoresistance (Camp et al 2003). Unfortunately, agents such as cytokines, chemotherapeutic agents and radiation that induce apoptosis also activate NF- κ B (Beg and Baltimore 1996). This indicates that NF- κ B is part of the auto-defence mechanism of the cell thus may mediate desensitisation, chemoresistance and radioresistance (Wang et al 1999).

Most chemotherapeutic drugs used today activate NF- κ B and induce resistance to chemotherapeutic agents. NF- κ B mediates chemoresistance by its close relationships with MDR1, COX-2, survivin, Bcl-2/bcl-xL, IAP-1, IAP-2, XIAP, cFLIP, adhesion molecules, AKT/PI3K, mTOR signaling, PTK, 5-LOX, EGFR, IL-6, HER-2, PKC/PKA, cyclin D1, C-myc, integrins, and STAT3. Aspirin is an established NF- κ B blocker and an anti-inflammatory agent shows promise in the treatment of certain cancers. Blockers of NF- κ B such as

polyphenols are nontoxic and should be beneficial both in the prevention and treatment of cancer (Aggarwal et al 2004).

1.5.8 NF- κ B activation prevents tumorigenesis

It appears thus far that NF- κ B would be a perfect target for anti-cancer therapies however NF- κ B has a dual nature in which either its activation or inactivation may lead to tumorigenesis (Aggarwal 2004). For example, NF- κ B mediates a switch from cell proliferation in basal cells of the epidermis to growth arrest and differentiation in suprabasal cells (Seitz et al 1998). If the pharmacologic inhibitor of NF- κ B is applied to intact skin, epidermal hyperplasia occurs (Seitz et al 1998) showing that in this tissue NF- κ B is important for cellular growth inhibition. Another study by Dajee et al (2003) has shown that blocking NF- κ B triggers invasive human epidermal neoplasia because in normal human epidermal cells NF- κ B triggers cell-cycle arrest.

1.5.9 NF- κ B and AML

When healthy monocytes and macrophages receive signalling from TNF, NF- κ B becomes activated which protects these cells from apoptosis via transcription of antiapoptotic genes such as Fas-associated protein with death domain-like interleukin-1 (IL-1)-converting enzyme (FLICE/caspase-8)-inhibitory protein (FLIP) and inhibitor of apoptosis-1 (Tucker et al 2004). Such healthy cells can become sensitive to cell death signals after the inhibition of NF- κ B following treatment with anti-TNF (Liu et al 2004).

In AML cells however, there is a limited effect of NF- κ B inhibition on apoptosis following treatment with anti-TNF (Rushworth et al 2008). This inability to induce apoptosis comes from the cytoprotective gene heme oxygenase-1 (HO-1). HO-1 is induced in response to NF- κ B inhibition and superinduced when in combination with TNF activation. When HO-1 expression is inhibited, AML cells become susceptible to TNF-induced apoptosis (Rushworth et al 2008).

TNF is a recognised activator of NF- κ B which in AML cells protects transformed cells from cell death.

AML blasts have constitutive NF- κ B activation (Guzman et al 2001) which protects the leukaemic cells from apoptosis. This protective effect can be removed by inhibiting NF- κ B

signalling which can be done by treating AML cells with proteasome inhibitors, which rapidly induces cell death (Guzman et al 2001). Another method by which NF- κ B has been inhibited is with dominant-negative alleles of the NF- κ B regulatory molecule I κ B α (Epinat et al 1999).

1.6 AIMS

The aim of this work was to understand the significance of NF- κ B in human AML by:

- Comparing the expression levels of NF- κ B signalling components between human AML samples and control cells
- Understanding the mechanism controlling I κ B expression relative to other NF- κ B signalling components.

Chapter 2: Materials and Methods

2.1 Human Research/Patient Rights

Primary AML cells were obtained under local ethical approval (LREC ref 07/H0310/146). Informed consent was obtained from patients and any identifying information was anonymised according to the approved ethics code of practice.

2.2 Materials

The AML-derived cell lines THP-1, HL60, U937, and AML 193 cells were obtained from the European Collection of Cell Cultures. Recombinant human TNF was purchased from R&D Systems (Minneapolis, MN). Antihuman HO-1 antibody was purchased from Assay Designs (Ann Arbor, MI). Antihuman FLIP antibody was purchased from Abcam (Cambridge, MA). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were obtained from Sigma-Aldrich (St Louis, MO), unless indicated.

2.3 Methods

2.3.1 Cell culture

All cells including cell lines, AML primary cells and primary control cells were cultured in RPMI-1640 supplemented with 10% FCS and 1% L-glutamine and incubated in a humidified atmosphere at 37°C with 5% CO₂. AML cells were obtained from AML bone marrow following the protocol described below. AML bone marrow samples were obtained from the Norfolk and Norwich University Hospital.

2.3.2 Isolation of AML cells

Blood from AML patients was treated as described below to isolate AML cells.

2.3.3 Isolation of mononuclear cells

A butterfly needle and 50 ml syringe was used to extract 45 ml blood from a healthy donor. The blood was added to 5 ml Sodium Citrate in a 50 ml Falcon tube (1:10 dilution) and inverted to prevent blood clotting. Blood was mixed with 50 ml of 50% Hank's solution. Histopaque solution (15 ml) was added to each of four 50 ml falcon tubes. Blood mixed with Hank's solution was distributed evenly between the four Falcon tubes (25 ml in each): blood was carefully and slowly added to the tubes which were held at an angle to ensure there was

no mixing of blood into the Histopaque solution. Blood was spun in a centrifuge at 1800 rpm (402 rcf) for 20 min with the brake off. After spinning, the layer of monocytes that formed in the tube were removed by pipetting and transferred to a new 50 ml falcon tube. Cells were spun at 1500 rpm for 10 min then supernatant was removed. Cells were re-suspended in 50 ml Hank's solution. At this point, 20 µl of cells were taken for a cell count. Cells were spun again in the 50 ml falcon tube at 200 g, supernatant was removed, cells were re-suspended in 50 ml Hank's solution and spun again at 200xg. Supernatant was removed and cells were re-suspended in 300 µl of buffer for magnetic labelling. Buffer was made up of 0.5% Bovine-Serum Albumin, 2mM EDTA and PBS pH 7.2.

2.3.4 CD34+ cell magnetic labelling and separation

Throughout the process, cells were kept cool and pre-cooled solutions were used. The volumes for magnetic labelling were for up to 10^8 total cells, when there were fewer or higher cell numbers, volumes were scaled down or up accordingly. To the 300 µl of cells in buffer, 100 µl of FcR blocking reagent was added, followed by 100 µl of CD34 MicroBeads. This was mixed well and refrigerated for 30 min (4-8 °C). Cells were washed by adding 5-10 ml of buffer and centrifuged at 300xg for 10 min. Supernatant was completely removed and cells were re-suspended in 500 µl of buffer.

MACS separation column (size MS) was placed in the magnetic field of a suitable MACS Separator. MS Columns were prepared by rinsing with 500 µl of buffer. Cell suspension was added to the column. Unlabelled cells that passed through the column were collected and the column was washed with 500 µl of buffer three times. New buffer was only added when the column reservoir was empty. The column was removed from the separator and placed on 15 ml falcon tube for collection of cells. One ml of buffer was pipetted onto the column and magnetically labelled cells were flushed out immediately by firmly pushing the plunger into the column. CD34+ cells were collected in an eppendorf tube.

2.3.5 RNA extraction

Cells suspended in media were added to an eppendorf tube and spun at 13000rpm for 2 min to create a cell pellet. Supernatant was removed by pipetting. Cells were re-suspended in nucleic acid purification lysis solution (Applied Biosystems). RNA was extracted using the ABI Prism 6100 Nucleic Acid PrepStation set to RNA cell method. RNA was collected in Bio Rad individual PCR tubes and stored at -20°C unless immediately used.

2.3.6 Reverse Transcription

Master mix containing random hexamers (Quiagen), MgCl₂, 10x reverse transcription buffer, dNTPs, Reverse Transcriptase and RNase (all Applied Biosystems) was made up and 6 µl of this was added to 4 µl of RNA. PCR tubes containing master mix and RNA were put into the C1000 Bio Rad Thermal Cycler machine and set to 42°C for 15 min, 95°C for 3 min then cooled to 4°C. Once the reaction had stopped, tubes were removed from the Thermal Cycler and 90 µl of double distilled water was added to each sample of cDNA.

2.3.7 Real-time PCR

cDNA samples were taken and 5 µl from each was added to an individual well in the 96 well PCR plate. Master mix was made up from SYBR green (Roche), relevant primers (Primers (Invitrogen) diluted at 15 µl forward primer+15 µl reverse primer and 270 µl double distilled water) and double distilled water. Of this master mix, 15 µl was added to each well of the PCR plate already containing cDNA. The 96 well plate was inserted into the Light Cycler 480 qRT PCR machine (Roche) and programmed to run for 55 amplification cycles and to measure the melting curves for each sample.

2.3.8 Immunostaining and image acquisition

Cell samples used for immunostaining included HL60 and THP1 cell lines and various AML patient samples. Cells were suspended in media and 150µl of this was added for each sample in a Shandon Double Cytofunnel (Thermo Scientific). Cell samples were spun onto glass Poly Prep slides (Sigma-Aldrich) in a Cytospin 4 machine (Thermo Shandon) set to 500rpm for 5 min. A ring was drawn around each cell sample using a liquid repellent slide marker pen. Cells were fixed in 4% paraformaldehyde, pH 7.4 for 15 min. Cells were washed in PBS: PBS was added, left to wash for 5 min, removed by pipetting and then repeated once. Cells were blocked for 15 min using 30% goat quench (in 50 ml: 15 ml goat serum 30%, 35 ml gelatine quench buffer). Goat quench was removed by pipetting and cells were permeabilised for 20 min in 0.5% Triton X-100 in 30% goat quench. Permeabilisation solution was removed and cells were incubated for 1 hour at 4°C in primary antibody. Antibodies were diluted to 0.5 µl in 1000 µl 30% goat quench. Cells were washed in PBS for 5 min three times then incubated at 4°C in secondary antibody for 40 min. Cells were washed in PBS for 5 min three times. DAPI was added to the cells at a concentration of 1 in 2000 and left for 20

min. DAPI was removed and cells were mounted in Fluoromount G and cover slips were added. Stained slides were stored at 4°C.

Slides were viewed and images were acquired using a Zeiss AxioCam MRm fluorescent microscope.

2.3.9 Anti-TNF treatment

A stock solution of anti-TNF was diluted using double distilled water to the required concentration of 3 µg/ml and added to HL60, U937, and THP1 cells plated at 1×10^5 cells per well in 12 well plates. Each well contained 2 ml of media. Other wells containing the cell lines were also left as controls – no anti-TNF was added. Cells were incubated for the required time periods (0, 4, 8, 24, 48 or 72 hours) following the anti-TNF treatment.

2.3.10 MTS assays

Following the appropriate incubation periods, a sample of cells were taken from their plates by pipetting. Cells were transferred into MTS plates with 100 µl of cells in each well. 15 µL of MTS was added to each well of the MTS plate containing a cell sample. Cells were incubated with MTS for 45 min then cell death was measured using a BMG Labtech MTS plate reader.

2.3.11 Protein extraction

Cells required for protein analysis were spun at 13000 rpm for 10 seconds. Once a pellet had formed, the supernatant was removed and the pellet re-suspended in 40 µl of cell lysate (mixed by pipetting the solution repeatedly up and down).

2.3.12 Western blotting

Resolving gel (12%) was made containing distilled water, 30% acrylamide mix, 1.5M TRIS (pH 8.8), 10% Sodium Dodecyl Sulphate (Melford), 10% ammonium persulphate and TEMED. Resolving gel was pipetted between two glass plates and left to set. A few drops of butanol were added to the top of the gel to level it out and remove bubbles. Stacker gel was made using distilled water, 30% acrylamide mix, 1.0M TRIS (pH 6.8), 10% SDS, 10% ammonium persulphate and TEMED. Stacker gel was added to the top of the glass plates on

top of the resolving gel. A comb was added to create the wells, each tooth of the comb had a width of 4 mm.

Once the gels are set, the lane combs and the glass plates were removed from their holders and transferred into an electrophoresis tank into which enough running buffer was added to cover the glass plates containing the gel. Running buffer was made up of 30g glycine (Fisher Scientific), 6g TRIS Base Ultra Pure (Formedium), 24 ml 10% SDS and 2 l of distilled water. Buffer was pipetted in and out of each well to clean it. From each protein sample, 20-40 μ l was carefully pipetted into each well with a DNA ladder in one of the end wells. The gel was run for 40 min at 200V.

Membranes were activated in methanol for 10 seconds then washed three times with distilled water and placed in transfer buffer until needed. Membranes were also marked on the side to face the gel. Transfer buffer was made up from 30g glycine, 6g TRIS and 2l of water. Once the gel was stopped, buffer was removed and the tank and gel were washed with distilled water. Gel was removed from the glass plates and transferred onto the cassette in the order: cassette black outer casing, fibre pad, filter paper, gel, activated membrane, filter paper, fibre pad, red cassette outer casing. All components were pre-soaked in transfer buffer. Before locking the cassette closed, bubbles were removed from between the gel and the membrane by rolling a tube across the fibre pad. The cassette was put into the transfer tank in the correct orientation along with a cooling unit of ice. The tank was filled with the correct amount of transfer buffer according to the amount of cassettes in the tank then run at 100V for 1hour.

Cassettes and membranes were removed, then membranes were blocked with milk (2.5 g milk powder (Marvel), 50 ml 1xPBS) for 1hour whilst rocking. Membranes were washed in PBS with 0.1% TWEEN three times for 10 min each time. Membranes were incubated in primary antibody (IkBa in 1 in 1000 dilution in milk) overnight at 4°C. Membranes were washed four times for 10min each time with PBS and 0.1% TWEEN then secondary antibody was added (Rabbit pAb to Mouse IgG (HRP) Abcam) for an hour.

Following the washing of the secondary antibody from the membranes, activator reagents were added to the membranes. Reagent A: 68 mM p-coumaric acid – 5 g in DMSO (22.3 mg in 2 ml). Reagent B: 1.25 mM luminol – 1g in 0.1M TRIS pH8.5 (44.3 mg in 200 ml). Five

millilitres of Reagent B and 50 µl of Reagent A were added to the membranes along with 1.5 µl of H₂O₂ (30%).

Blots were visualised using x ray film.

2.3.13 Drug treatment

Cells were plated at 1x10⁵ cells per well in 12 well plates. Cytarabine was added to the cells at the necessary time periods at concentrations of 0.5 µM and 1 µM. A set of cells were left as controls with no cytarabine added. Daunorubicin was added to cells at 0.2 µM and 0.3 µM concentrations. Cells were analysed for cell death using MTS assay as described above.

The table below lists the sequences specific to each primer used for real time PCR.

Primer	Sequence (5' to 3')
NIK Forward	CCC AGG CTG AGT GTG AGA AT
NIK Reverse	CGG ACT GGC TGT ACT GTT TG
p65 Forward	GGC GAG AGG AGC ACA GAT AC
p65 Reverse	CCT GGT CCT GTG TAG CCA TT
p100 Forward	ACA GAT GGC CCA TAC CTT CA
p100 Reverse	GAT AGG TCT TTC GGC CCT TC
p105 Forward	ACA GAT GGC CCA TAC CTT CA
p105 Reverse	AGG TAG TCC ACC ATG GGA TG
cRel Forward	TCC ATT CAA TGT CCC TGA AA
cRel Reverse	CCA TGT TCA TCA GGG AGA AAA
IκBα Forward	GAG CCC TGG AAG CAG CAG
IκBα Reverse	CTC CGC CAC TTA CGA GTC C
RelB Forward	CTT ACG GAG GCC GAG CAG
RelB Reverse	TTC AGT TCT TTG GCC TCT TG

Table 1: Sequences specific to each primer used for real time PCR.

Chapter 3: Expression levels of NF- κ B signalling
molecules in human AML

3.1 Introduction

In the majority of cell types, NF- κ B is found in the cytoplasm in its inactive form. Upon stimulation from factors such as TNF, the inhibition of NF- κ B signalling is lost, and so begins the cascade of signalling events which lead to the nuclear translocation of NF- κ B. Once in the nucleus, NF- κ B forms an active transcriptional complex with DNA encoding inflammatory, pro-apoptotic and anti-apoptotic genes (Zhou et al 2003)

3.1.1 NF- κ B in AML

In unstimulated primary AML cells, studies have detected aberrant NF- κ B activity (Monica et al 2001). NF- κ B signalling provides cells with antiapoptotic activity and is therefore considered to be an important survival factor for AML cells. Because NF- κ B has a major role in mediating cell survival, it has become a target for cancer therapy (Wang et al 1996).

Treating healthy monocytes or macrophages with TNF in physiologic conditions does not induce cell death because the NF- κ B signalling pathway becomes activated and triggers the expression of antiapoptotic genes (Want et al 1998). Cell death will only occur in such cells when NF- κ B signalling is inhibited (Liu et al 2004). NF- κ B inhibited AML cells do not however respond in the same way to TNF treatment and apoptosis does not occur (Rushworth and MacEwan 2008).

NF- κ B signalling components have been investigated and the following results gathered because of the constitutive activation of NF- κ B in monocytes of AML patients (Guzman et al 2001) and its great influence on the survival of such cells.

3.2 mRNA expression of canonical NF- κ B subunits in AML

Components of the canonical and non-canonical NF κ B signalling pathway were investigated in healthy CD34+ cells, AML cell lines (HL60, U937 and THP1), and AML cell patient samples to compare their mRNA levels. The aim of this was to discover whether their levels of mRNA were increased, decreased or remained the same in AML compared to healthy CD34+ cells. The result of this investigation hoped to give a clearer idea of any effect a change of expression could have on the NF κ B signalling pathway and ultimately, the final signalling outcome and fate of the cell.

p105 mRNA expression.

A Student's T-test was performed on the data for p105 (Figure 1A), comparing the control CD34+ cells to all of the AML cell samples. The result was 0.852 (to three significant figures) which shows that there is no significant difference between the CD34+ cells and the AML patient samples collectively.

When the results are viewed more closely however, the majority of the results for AML samples are below 0.5 besides AML samples 103, 212 and 314 in particular. So with these three results removed and the T-test is re-calculated, the result is 0.00632 which shows that there is a significant difference in mRNA levels of p105 between the majority of AML samples and the CD34+ cell samples.

A further T-test was carried out on the data for the cell lines' mRNA levels for p105 compared with those of the AML patient samples. This test was performed to determine whether the mRNA data for the cell lines was representative for that of actual AML patient samples. The result of the T-test was 0.613 which shows that there was no significant difference between the data for the AML cell lines and for that of the AML patient samples. This confirms that the data collected from the AML cell lines was indeed representative of data from AML cell samples.

p65 mRNA expression.

A Student's T-test was performed on the data for p65 (Figure 1B), comparing the control CD34+ cells to all of the AML cell samples. The result was 0.519 which shows there is no significant difference in mRNA levels between CD34+ cells and AML patient samples. However, much like the results for p105, the levels of p65 in AML samples 103, 212 and 314 are particularly elevated. So a separate T-test was performed for the p65 results without the above three AML samples to see if this made a difference. The second T-test result was 0.923 which strengthens the hypothesis that there is no significant difference in p65 mRNA levels whether they are measured in AML or healthy CD34+ cells.

Once again, a further T-test was carried out to compare the mRNA levels for p65 in AML cell lines and AML cell samples. The result of the T-test was 0.660 which meant there was no significant difference between the two sets of results. This means that using HL60, U937 and

THP1 cell lines to measure cellular p65 mRNA levels would generally be indicative of the same mRNA levels in AML patient samples.

cRel mRNA expression.

A Student's T-test was performed on the data for cRel (Figure 1C), comparing the control CD34+ cells to all of the AML cell samples. The result was 0.365 which shows that there is no significant difference in cRel levels between the CD34+ control cells and the AML cell samples. Therefore these results show that cRel mRNA levels are neither reduced nor increased generally in AML. On the other hand, these results do show that there is a great deal of variation in cRel mRNA levels between patients with AML.

A T-test was performed on the cRel mRNA results from the AML cell lines and AML cell patient samples. The result was 0.169, meaning there was no significant difference between the two sets of results, which leads to the conclusion that the AML cell lines may be used to determine cRel mRNA levels in AML.

IκBα mRNA expression.

A Student's T-test was performed on the data for IκBα (Figure 1D), comparing the control CD34+ cells to all of the AML cell samples. The result was 0.00000840 concluding that there is a significant amount of difference in IκBα levels between CD34+ cells and AML samples. IκBα levels are significantly reduced in AML cells when compared with healthy CD34+ cells. A T-test was also performed to compare the AML cell line data with that of the AML cell patient samples. The result of this test was 0.696 which gives the conclusion that there is no significant difference between the two sets of data, again leading to the notion that AML cell lines are behaving in the same way as AML cell patient samples.

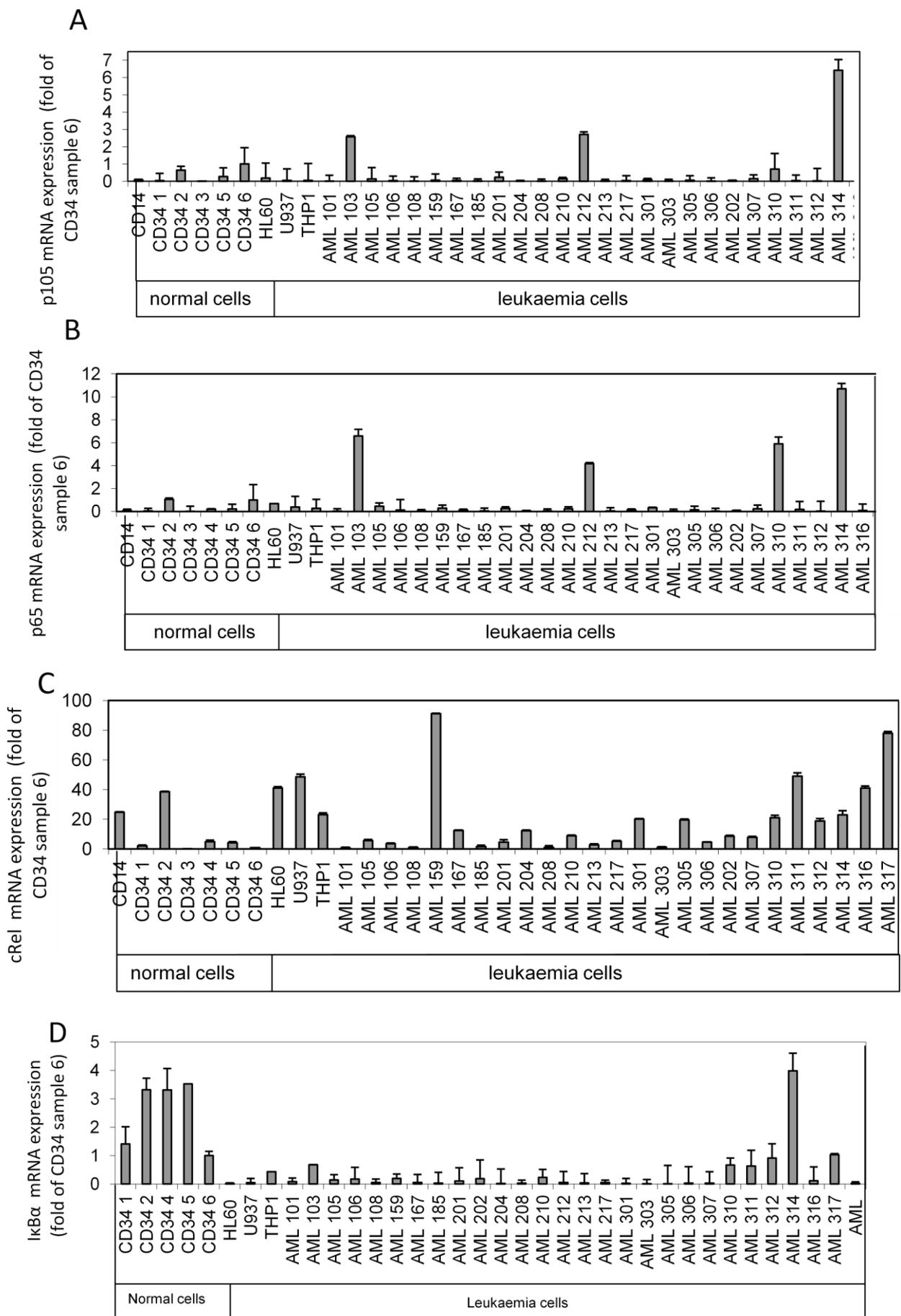


Figure 1 mRNA expression of canonical NF-κB subunits in AML

Total mRNA were extracted from CD34+ cells, AML cell lines and AML patient samples and subjected to real time PCR to measure the mRNA levels of p105 (A), p65 (B), cRel (C) and IκBα (D). The data are expressed as mean values with standard deviation represented by the error bars. n=3

3.3 mRNA expression of non-canonical NF- κ B subunits in AML

RelB mRNA expression.

A Student's T-test was performed on the data for RelB (Figure 2A), comparing the control CD34+ cells to all of the AML cell samples. The result was 0.0000264 which shows that there is a significant difference between the sets of data for RelB mRNA levels in CD34+ cells and AML cell patient samples. mRNA levels for RelB are significantly reduced in AML cell samples when compared to those of healthy CD34+ cell samples.

A separate T-test was performed on the data for RelB to compare mRNA levels between the AML cell lines and AML cell samples. The result of this test was 0.984 which shows there is no significant difference between the two sets of data. This lack of difference shows that in the case of RelB mRNA levels, AML cell lines and AML cell samples are very similar.

p100 mRNA expression.

A Student's T-test was performed on the data for p100 (Figure 2B) to compare mRNA levels of the control CD34+ cells to those of all the AML cell samples. The result was 0.478 which shows there was no significant difference between the two sets of data. Even with the high levels of p100 mRNA recorded for AML samples 310, 311, 312, 314, 316 and 317 removed from the T-test, the result is still 0.184 which brings about the same conclusion. Therefore from these results, it appears that p100 mRNA levels remain unchanged when healthy cells transform into leukaemic AML cells.

Another T-test was performed to compare the p100 mRNA levels for the AML cell lines and the AML cell patient samples. The result was 0.703 which means there is no significant difference between the two sets of data. This means that mRNA levels of p100 in cell lines closely resembles those of AML cell patient samples.

NIK mRNA expression.

A Student's T-test was performed on the data for NIK (Figure 2C) to compare mRNA levels of the control CD34+ cells to those of all the AML cell samples. The result was 0.0653 which shows there is no significant difference between the CD34+ cells and the AML cell patient

samples. This suggests that there is no difference in mRNA levels of NIK between healthy CD34+ cells and leukaemic AML cells.

As the results are viewed in the form of a graph, it does however appear that NIK mRNA levels are generally a little lowered in AML cell patient samples. If, like for the results of p100 mRNA AML samples; 310, 311, 312, 314, 316 and 317 are removed and the T-test is repeated, the result becomes 0.0102 which does show a significant difference between the two sets of data. This would suggest that there may be a significant decrease in NIK mRNA levels in AML cell patient samples compared to healthy CD34+ cells, however the results for those six AML cell samples shall not be disregarded.

Another T-test was carried out to compare the data for mRNA levels of NIK in AML cell lines and the AML cell samples. The result was 0.0675 which shows there is no significant difference between the two sets of data. This suggests that NIK mRNA levels in AML cell lines HL60, U937 and THP1 are indicative of those in leukaemic cells in AML patients.

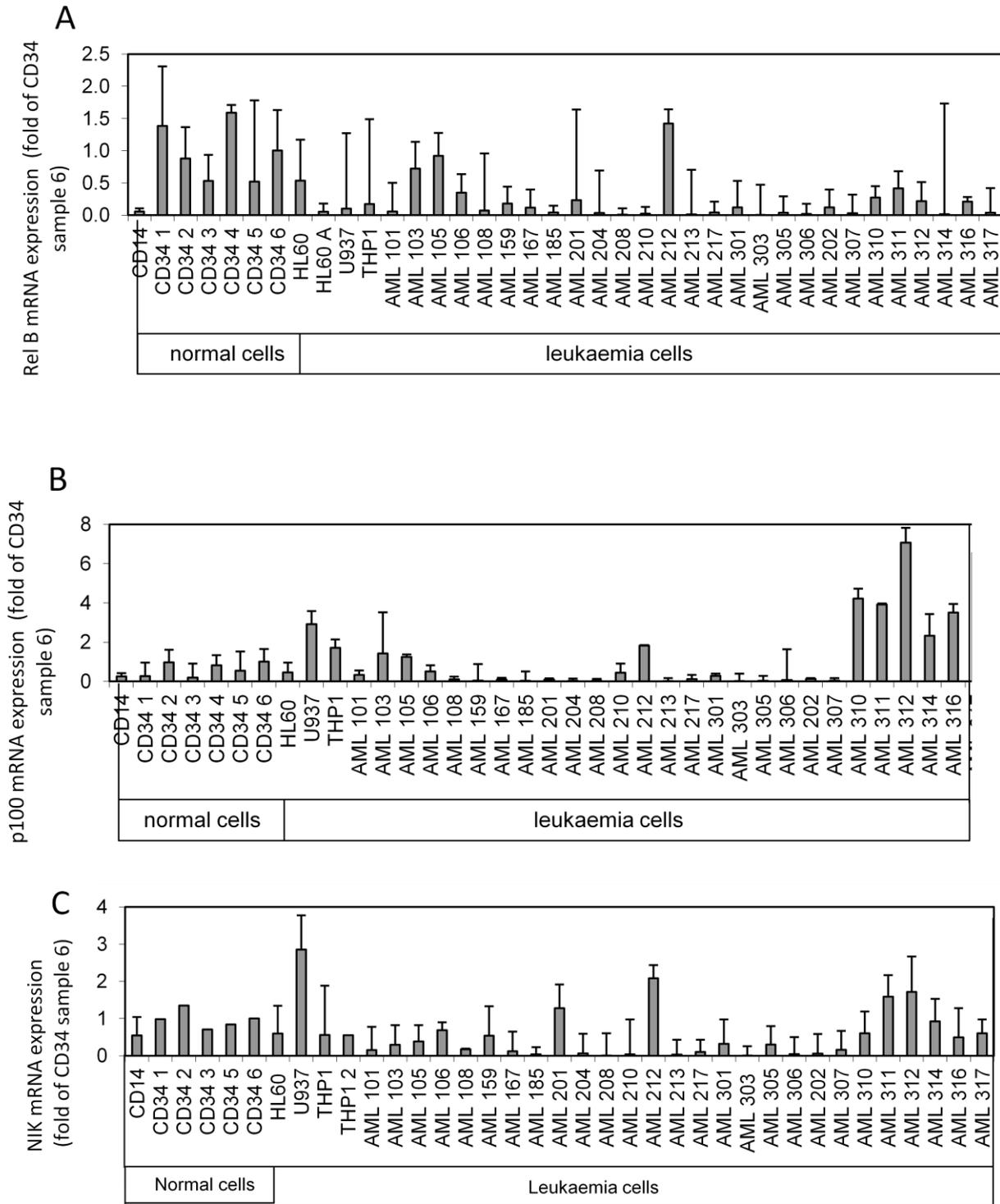


Figure 2 mRNA expression of non-canonical NF- κ B subunits in AML

Total mRNA were extracted from CD34⁺ cells, AML cell lines and AML patient samples and subjected to real time PCR to measure the mRNA levels of RelB (A), p100 (B), NIK (C). The data are expressed as mean values with standard deviation represented by the error bars. n=3

3.4 Protein expression of canonical NF- κ B subunits in AML

Much like the investigation for mRNA expression levels of both canonical and non-canonical NF κ B signalling pathways in AML, protein expression was also analysed.

It is generally thought that mRNA expression levels closely reflect expression levels of the proteins for which they code for. Some studies however have shown moderate and varied correlations between the two (Guo et al 2008), which suggests that mRNA expression may sometimes be a useful tool in predicting protein expression levels but it should not be regarded as an exact representation.

Expression levels of the proteins p50, p65, I κ B α , RelB, p100 and NIK were investigated in a representative number of AML cell patient samples. The aim of this was to discover where in the cell the proteins were being expressed in AML cells.

p50 protein expression.

It appears from the set of images for p50 in Figure 3 that there is more p50 protein expression in the cytoplasm of the AML patient's cell than there is in the nucleus. The fluorescent staining showing the presence of the p50 protein within the cell is a lot more intense in the periphery of the cell as opposed to the area within the nucleus (defined by the DAPI staining). There does appear to be some p50 present in the nucleus which suggests that the p50 subunit is also in the nucleus of AML samples.

p65 protein expression.

The image in Figure 3 representing p65 protein expression shows low levels of p65 within the nucleus of the AML patient cell and higher levels within the cytoplasm at the periphery of the cell. As with p50, there does appear to be some p65 protein to be found within the nucleus but as the intensity of the staining is much lower than in the cytoplasm. However it does imply p65 activity in these AML cells.

IκBα protein expression

Protein expression of IκBα within the two AML patient cell samples shown in Figure 3 appears to occur mainly within the cytoplasm of the cells. There does appear to be some IκBα expression in the nucleus of the cell but at a lower level than in the cytoplasm.

3.5 Protein expression of non-canonical NF-κB subunits in AML

RelB protein expression

Expression of RelB protein in the AML patient sample shown in Figure 4 clearly appears much greater in the cell's cytoplasm than in the nucleus. There is a definite area in the top image (RelB antibody alone) with very low RelB expression which exactly matches the area marked by DAPI staining as the nucleus. RelB staining is distinct from the DAPI staining which indicates that RelB is not found present in the nucleus.

p100 protein expression

The image in Figure 4 showing p100 protein expression within a cell taken from an AML patient's blood sample shows more p100 protein expression in the cell cytoplasm than in the nucleus. The intensity of the p100 protein expression is a lot greater in the cytoplasm of the cell compared to the nucleus.

NIK protein expression

Expression of NIK protein is shown in Figure 4 in four cells from an AML patient sample. Each cell has an area, represented as the nucleus by DAPI staining, with reduced NIK protein expression. There does appear to be some NIK staining present within the nuclear area which is unexpected as NIK is only required in the cytoplasm.

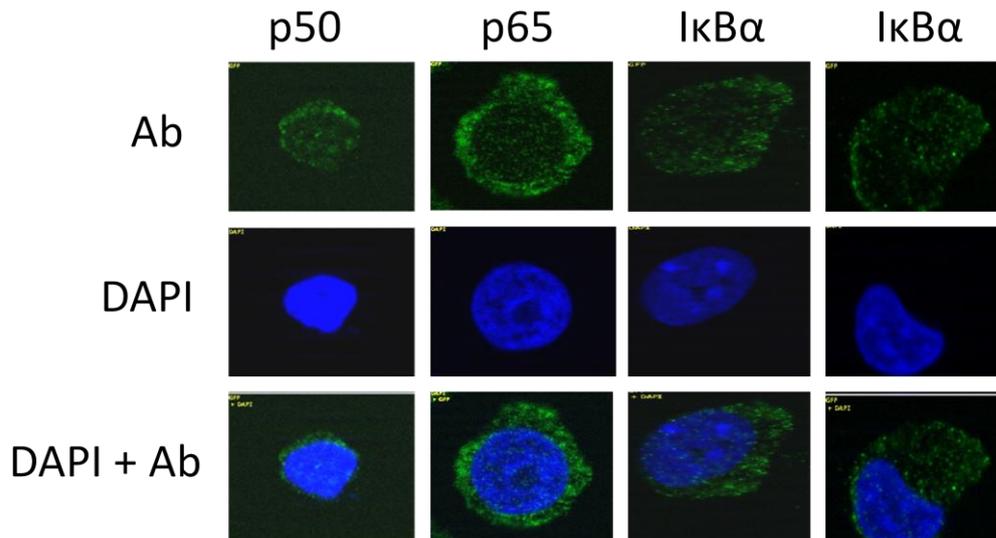


Figure 3 protein expression of canonical NF-κB subunits in AML
 AML patient samples (316, 316, 306 and 310 respectively) were analysed for canonical NF-κB proteins p50, p65 and IκBα distribution within the cell by immunocytochemistry, with DAPI used to stain nuclei. Images show typical results displayed by 10 different patient samples investigated.

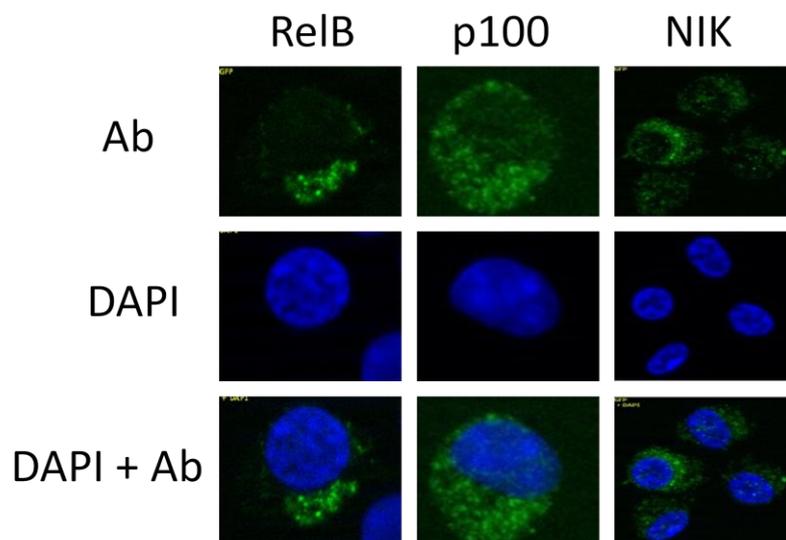


Figure 4 protein expression of non-canonical NF-κB subunits in AML
 AML patient samples (314, 316 and 316 respectively) were analysed for canonical NF-κB proteins RelB, p100 and NIK distribution within the cell by immunocytochemistry, with DAPI used to stain nuclei. Images show typical results displayed by 10 different patient samples investigated.

3.6 Discussion

T-tests carried out to compare mRNA levels for AML cell lines and actual AML cell patient samples showed that there was no significant difference between the two sets of data for any of the NF κ B signalling pathway components. This gives the implication that cell lines may be used to decipher certain aspects of cellular activity within cells of patients with AML. From the results gathered here, this generally appears to be a safe assumption. What these results also show however is the large amount of variability between individuals with AML. Therefore cell lines may be used to give an insight to suggest what may be occurring within AML cells, but may not provide hard evidence from which to draw final conclusions.

Protein expression of NF- κ B subunits from the canonical and non-canonical NF- κ B signalling pathways were investigated by immunofluorescence using cell samples from AML patients to discover where the proteins were being expressed. There was also a question as to whether or not the mRNA results for the signalling components correlated with the protein expression results.

p105

p105 is the precursor to the mature NF- κ B subunit p50. The p50 subunit is generated by proteolytic processing of the 105 kDA precursor protein – the protein is ubiquitinated then processed in the 20 S proteasome (Sears et al 1998). Results for mRNA levels of the p105 subunit showed that it was slightly decreased in AML when compared to normal CD34+ cells. If less p105 mRNA is being transcribed then there will be less translation of the mRNA into p105 protein. Less p105 protein available for processing will mean that there will be less p50 protein present in AML cells. If there is less p50 in AML cells then less p50:p65 dimers will form, for translocation into the nucleus. This ties in well with the immunofluorescence protein staining for p50 showing reduced amounts of it in the nucleus compared with in the cytoplasm. There is less p50 protein in the nucleus of AML cells because there is less of it available to produce dimers for nuclear translocation.

p65

Protein expression of p65 was also low in the nucleus of AML cells. mRNA levels of p65 did not however decrease when measured in AML samples. Low nuclear p65 protein expression could therefore be due to the reduced amounts of p50 protein. It is necessary that p65 forms

dimers with p50 to be able to translocate to the nucleus so if there is less p50 available then less p65 will reach the nucleus.

cRel

The above reasoning for low p65 nuclear protein expression could also be applied to cRel as the results for cRel were very similar to those of p65. mRNA levels for cRel were unchanged in AML when compared with healthy CD34+ cells and cRel protein expression in the nucleus of AML cells was low. The low protein expression of cRel in the nucleus of AML cells may be due to reduced amounts of p50 available to dimerise with it in the cytoplasm.

IκBα

Protein expression of IκBα was present both in the nucleus and in the cytoplasm of the AML patient sample cells. The amount of IκBα in the cytoplasm was higher than in the nucleus and there is a definite difference in the levels of expression in the area marked as the nucleus by the DAPI staining. IκBα is required in the nucleus upon cell activation to dissociate NF-κB from the DNA and transport it back into the cytoplasm to prevent further DNA transcription (Prigent et al 2000). Therefore IκBα protein expression is expected in the nucleus when cells have been stimulated for NF-κB signalling. In unstimulated cells, IκBα protein expression is maintained in the nucleus but at a low level. This is to ensure that once cells become activated, NF-κB will have the ability to become transcriptionally active as the activity of NF-κB will not be inhibited by too much IκBα present (Huang et al 2000).

The main result gained from these investigations is the significant amount of difference in IκBα mRNA levels between CD34+ cells and AML cell samples. IκBα mRNA levels were significantly reduced in AML cells compared to healthy CD34+ cells. This low level of IκBα mRNA present in AML cells is likely to translate into low levels of IκBα protein being present in AML cells. As IκBα plays an important regulatory role in the NF-κB signalling pathway, cells with less available IκBα protein are likely to experience downstream effects.

What events could be occurring upstream of IκBα to cause reduced levels of mRNA for this NF-κB inhibitor? A quicker turnover of IκBα mRNA could be reducing the length of time any one IκBα protein is present within a cell therefore causing an overall reduction of IκBα present in the cell. Epigenetic changes in regulation could be occurring such as the effects of

miRNA or RNA methylation. Finally, inhibition of transcription could be the reason for decreased levels of mRNA present in AML cells in this study.

RelB

AML patient samples showed a significant decrease in mRNA levels of RelB which implies that there would also be low RelB protein expression. When the protein expression of RelB was detected, it was mainly found present in the cytoplasm of the cell. Lower levels of RelB protein in the cell could mean less RelB translocation from the cytoplasm to the nucleus, which would explain the lack of RelB in the nucleus of AML cells.

p100

The mRNA levels for p100 showed no change in AML samples when compared to healthy CD34+ cells however the protein expression for p100 was low in the nucleus of AML cells. p100 is the precursor protein to the mature p52 NF- κ B subunit which is the molecule which translocates into the nucleus therefore p100's presence within the nucleus would not be expected.

NIK

The role of NIK in the non-canonical NF- κ B signalling pathway is to induce p100 processing via I κ K molecules. This process occurs in the cytoplasm of cells therefore NIK protein expression should also be found in the cell cytoplasm. The images showing NIK protein expression do indeed show higher amounts of NIK present in the AML cell's cytoplasm than in the nucleus.

Chapter 4: Understanding the mechanisms
controlling the low expression of I κ B α in AML

4.1 introduction

The main finding from the results in chapter 3 was the low expression of I κ B α mRNA in AML patient samples compared to control cells. Because the NF- κ B signalling pathway has such a central role in controlling cell death, it is important to investigate what may be causing the low expression of I κ B α and ultimately aberrant NF- κ B signalling in AML.

4.1.1 TNF and NF- κ B in AML

Levels of TNF are known to be elevated in AML. TNF is a cytokine that can trigger NF- κ B signalling through the TNF receptors present on the cell membrane. AML cells also have higher basal levels of NF- κ B signalling than healthy cells. I κ B α is the inhibitor of NF- κ B signalling therefore I κ B α must be degraded in order to enable NF- κ B signalling to occur. The elevated levels of TNF present in AML cells could be causing the low expression of I κ B α because TNF signalling via NF- κ B requires low levels of I κ B α present in the cell. Therefore, the effect of TNF on I κ B α will be investigated by removing TNF from AML cell lines to discover what happens in relation to I κ B α when TNF is no longer present.

4.2 Treating AML cells with anti-TNF: the effect on I κ B α mRNA and protein

4.2.1 I κ B α mRNA levels following anti-TNF treatment

To investigate the effect of TNF on the expression of I κ B α mRNA, AML cell lines were treated with anti-TNF then their I κ B α mRNA levels were measured by qRT PCR. The same cell lines were also left untreated and used as controls to compare their I κ B α mRNA levels with the ones that had been treated.

HL60 cells

HL60 cells were treated with anti-TNF then left to incubate for 24 and 48 hours. Following these time periods, cells were taken and analysed by qRT PCR to measure their I κ B α mRNA levels. Figure 5A shows the results from this investigation. In HL60 cells, the levels of I κ B α in the cells incubated for 24 hours have fallen. They decrease further when cells are analysed at 48 hours. The I κ B α mRNA levels in the HL60 cells treated with anti-TNF have fallen to levels lower than in the untreated HL60 cells.

THP-1 cells

THP-1 cells were treated in the same way as the HL60s. Figure 5B shows after 24 hours following anti-TNF treatment, I κ B α mRNA levels have increased slightly above the levels of I κ B α mRNA in the control THP-1 cells after 24 hours. By 48 hours, the I κ B α mRNA levels have almost doubled in comparison to the control cells.

U937 cells

U937 cells that were treated with anti-TNF have a much higher amount of I κ B α mRNA present in their cells 24 hours following treatment than the control cells (Figure 5C). The amount of mRNA present in the treated U937s has more than doubled by 24 hours after treatment. U937 cells left to incubate for 48 hours following anti-TNF treatment have increased levels of I κ B α mRNA compared to the control, but the levels are lower than those measured at 24 hours.

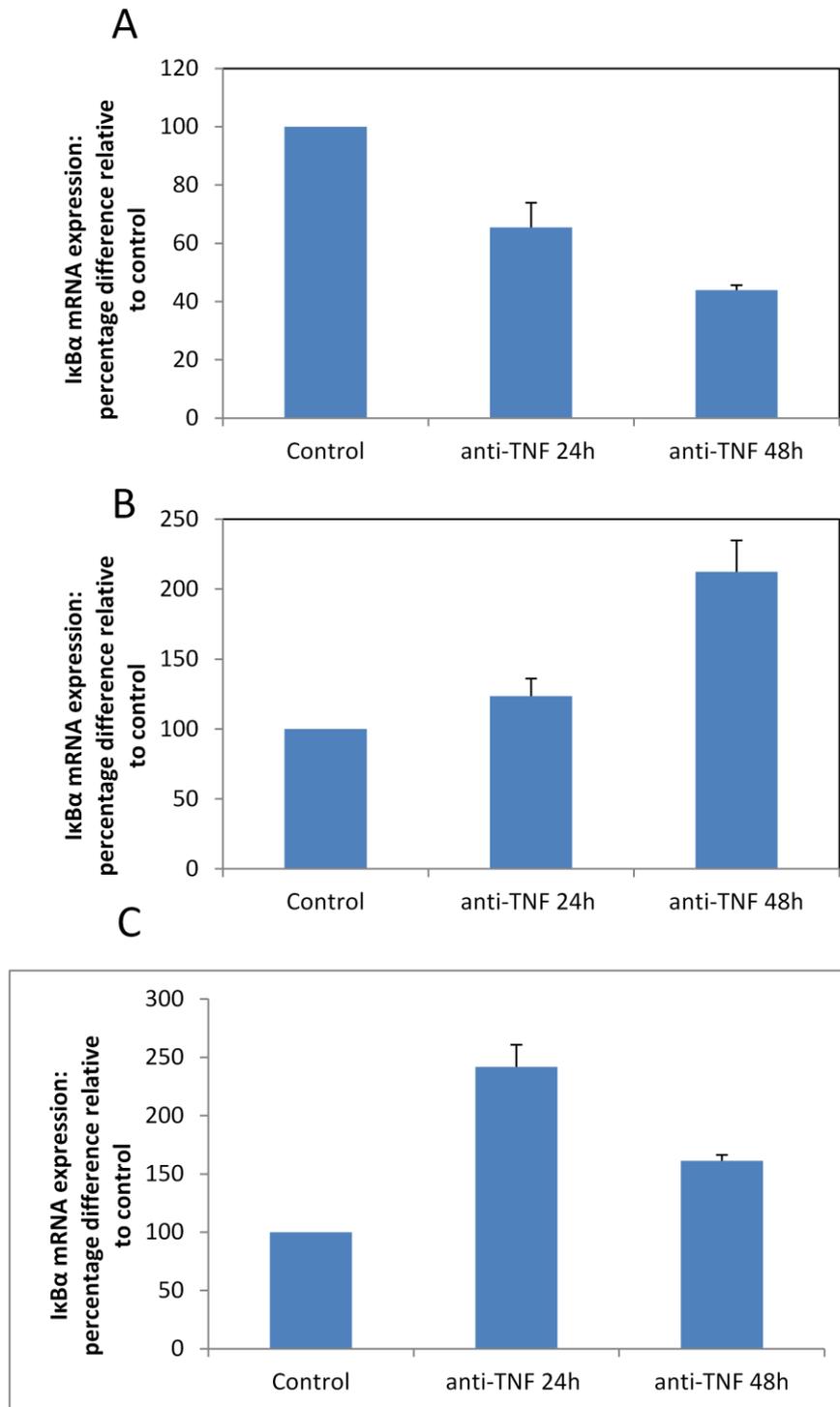


Figure 5 Effect of anti-TNF treatment on mRNA expression of IkBα in AML cell lines.

Cell lines were treated with 5ng/ml anti-TNF then left to incubate for 24 and 48 hours. Following the appropriate time periods, total mRNA were extracted from HL60s (A), THP-1s (B) and U937s (C) and subjected to real time PCR to measure the mRNA levels of IkBα. The data are expressed as mean values n=3

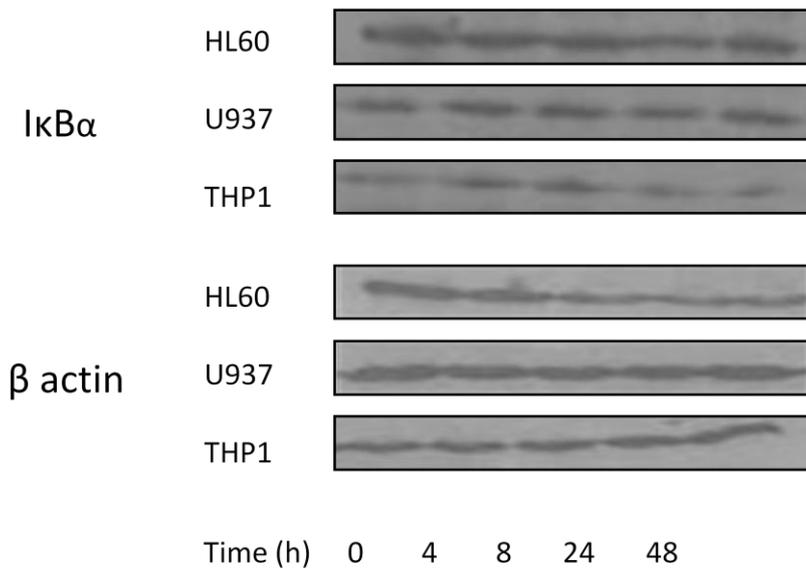


Figure 6: protein expression of IκBα in AML cell lines.

Cell lines were treated with anti-TNF then left to incubate for 0, 4, 8, 24 and 48 hours. Following the appropriate time periods, cells were subjected to western blotting to measure the protein levels of IκBα in HL60s (A), THP-1s (B) and U937s (C). n=3

4.2.2 IκBα protein levels following anti-TNF treatment

A similar investigation into the effect of treating AML cell lines with anti-TNF was carried out again, but this time measurements of IκBα protein were taken at 0, 4, 8, 24 and 48 hour time points following the anti-TNF treatment, and cells taken from the same samples were also used for IκBα protein analysis.

Figure 6 shows the western blots from the measurements of IκBα protein levels in AML cell lines after treatment with anti-TNF. Figure 7 shows these same results following quantification by measuring the intensity of individual bands. All the levels of IκBα protein in figure 7 are calculated as a fold of what they were at time 0, when no anti-TNF has been added. Four hours following anti-TNF addition shows an increase in IκBα protein in HL60, THP-1 and U937 cell lines. By the 8 hour time point, protein levels for IκBα have continued to rise in HL60 and U937 cells. In THP-1 cells however, the protein levels have decreased again, but are still higher than before anti-TNF treatment. By 24 hours, all cell lines have lower IκBα protein levels than when they were at their peak; either at 8 hours for HL60s and U937s, or at 4 hours for the THP-1 cells. Despite the decrease in IκBα protein levels, they are still all higher than they were previous to anti-TNF treatment.

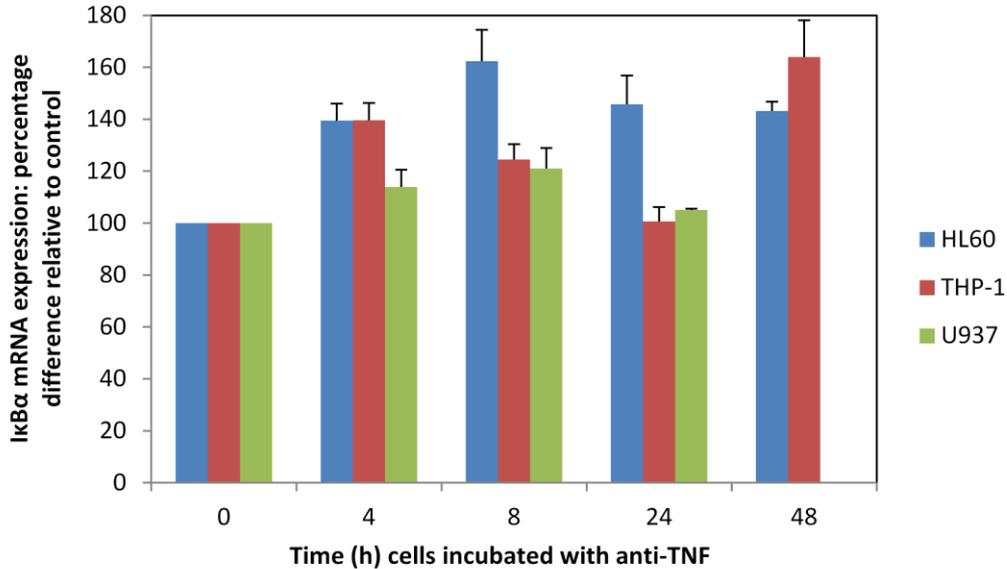


Figure 7 protein expression of IkB α in AML cell lines.

Cell lines were treated with 3 ng/ml anti-TNF then left to incubate for 0, 4, 8, 24 and 48 hours. Following the appropriate time periods, cells were subjected to western blotting to measure the protein levels of IkB α in HL60s (A), THP-1s (B) and U937s (C). The data are expressed as mean values. n=3

4.3 Effect of anti-TNF treatment on AML cell survival

AML cell lines HL60, THP1 and U937 were treated with varying amounts of anti-TNF to discover the effect this had on cell death. The treated cells were compared with cells that had received no anti-TNF treatment. Cell death was measured with an MTS assay.

HL60 cells.

Treating HL60 cells with 1 ng/ml of anti-TNF induced cell death (Figure 8A). It can be concluded that the cell death occurred as a result of the anti-TNF treatment because the results were calculated as a fold of the amount of cell death for HL60 cells to which no anti-TNF was added. This factors out cell death that may have occurred as a result of non-optimal cell conditions. Cell death was measured in the same cell samples over periods of 24 and 48 hours. More cell death occurred in HL60 cells that were treated with 1 ng of anti-TNF for 48 hours.

THP-1 cells

THP-1 cells were treated with 1 ng of anti-TNF for time periods of 24, 48 and 72 hours and the levels of cell death were measured. Figure 8B clearly shows a neat trend in the increase of cell death over the increasing time periods following anti-TNF treatment. By 24 hours following anti-TNF treatment, cell death has increased to levels above the amount of cell death of the untreated cells. At 48 hours, cell death has increased further and MTS readings of cell death showed that the highest amount was occurring once cells had been treated with anti-TNF then incubated for 72 hours.

U937 cells

U937 cells were treated with 1 ng of anti-TNF then left to incubate for 24, 48 and 72 hours (Figure 8C). Cell death is increased following anti-TNF treatment in U937 cells after 24 hours. Cell death appears to decrease after 48 hours as the results show an increase in the number of cells present in the sample on the MTS plate, however this is most likely to be an anomalous result possibly caused by bubbles in the cell solution causing an obstruction to the MTS plate reading. U937 cells were treated with a higher amount of 3 ng of anti-TNF which caused more cell death to occur after 24 hours than when cells were treated with 1 ng of anti-TNF. Cell death increased even further once cells had been incubated for 48 hours with 3 ng of anti-TNF.

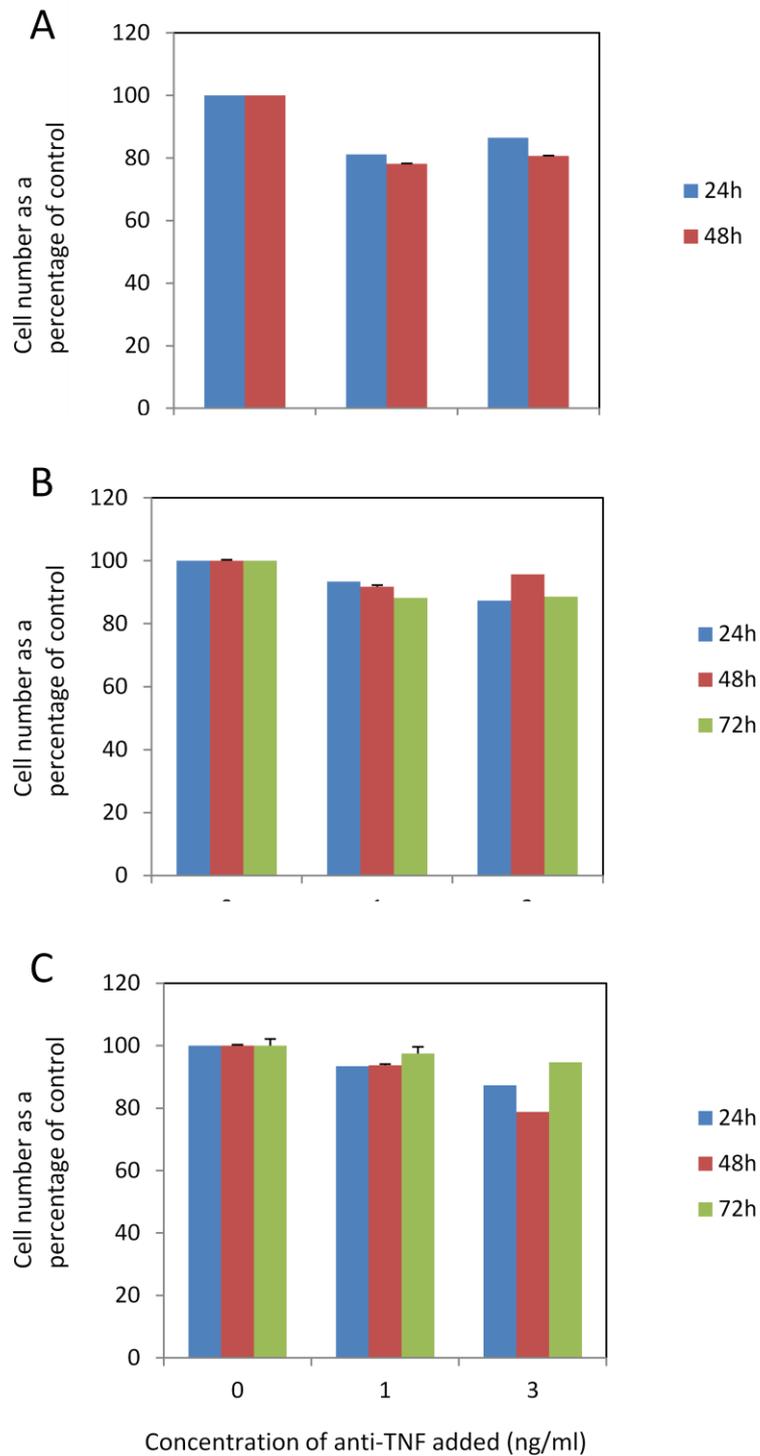


Figure 8 The effect of anti-TNF treatment on cell death of AML cell lines
 AML cell lines (HL60 (A), THP-1 (B) and U937 (C)) were treated with anti-TNF at concentrations of 0, 1 and 3 ng/ml. Cells were incubated for 24, 48, 72 h. Cells were plated onto MTS plates with MTS added, then plates were read to quantify cell death. n=3

4.4 Effect of anti-TNF and drug treatment on AML cell survival

Chemotherapeutic drugs such as Daunorubicin and Cytarabine are established drug treatments for AML (Feldman et al 2008). They both affect cell's DNA which disrupts the cell cycle which leads to cell death. These chemotherapeutic drugs were used in combination with TNF to investigate their combined effect on cell death of AML cell lines HL60, U937 and THP-1. Each cell line was treated with anti-TNF, Daunorubicin, Cytarabine, Daunorubicin with anti-TNF, Cytarabine with anti-TNF and two samples left with no treatment as controls. Cells were left to incubate for 24 and 48 hours to see the effect of the treatments on cell death over time.

HL60 cells.

Treatment with anti-TNF alone causes a small amount of cell death as demonstrated previously by the results in figure 8A. Once again, the levels of cell death in HL60 cells treated with anti-TNF are higher than those of the control cells (Fig 9A), meaning that the cell death that has occurred is as a result of the anti-TNF treatment.

Treating HL60 cells with both types of drug produces a more dramatic effect on the increase of cell death than treating with anti-TNF alone. Daunorubicin appears to have a slightly greater effect on cell death than Cytarabine does. It is clear that increasing the dosage of Daunorubicin also increases the amount of cell death after 24 hours however this effect becomes less obvious after 48 hours. The same effect is not obvious with increasing the dosage of Cytarabine.

A T-test was performed to compare the data collected after 24 and 48 hours. The result was 0.000765 which shows that there is a significant difference between the two sets of data. In this case, that means there was a significant increase in cell death when cells were left to incubate for 48 hours following treatment rather than just 24 hours.

THP-1 cells.

Treatment of THP-1 cells with anti-TNF alone in the previous experiment (figure 8B) clearly showed cell death as a result of this treatment. Such an effect in figure 9B is also obvious

after 48 hours but does not appear to be the case for THP-1 cells after 24 hours. Cells appear to have proliferated according to figure 9B however this is likely to be an anomalous result perhaps caused by an air bubble in the well of the MTS plate.

Treating the THP-1 cells with Daunorubicin and Cytarabine has a greater effect on increasing cell death than when treating the cells with anti-TNF alone. An increase in dosage of both chemotherapeutic drugs has a positive effect on the increase of cell death, particularly with Daunorubicin. Treating cells with a combination of the chemotherapeutic drugs and anti-TNF does not appear to have any beneficial effect on causing cell death in THP-1 cells.

A T-test was performed to compare the results after 24 and 48 hours, the result was 0.697 which shows there was no significant difference between the two sets of results, meaning that there was no significant difference in the amount of cell death after 48 hours compared to that of 24 hours. The difference may not have been significant however studying figure 9B does reveal a slight overall increase in cell death.

U937 cells

U937 cells treated with anti-TNF alone after 24 hours gives an increase in cell death when compared to control cells that were left untreated. The result for 48 hours following anti-TNF treatment would suggest cell proliferation however this result could be anomalous and perhaps due to air bubbles present in the MTS plate well.

Figure 9C shows that treating U937 cells with Daunorubicin increases cell death. Such an effect is increased further by increasing the dosage of Daunorubicin treatment. The same cannot be said however for treatment of U937 cells with Cytarabine which has appeared to actually have no effect on cell death.

Treating U937 cells with Daunorubicin and anti-TNF together has a slight beneficial effect on increasing cell death after 48 hours. After 48 hours, Daunorubicin had a greater effect on increasing cell death in U937 cells. All of the results taken together after 48 hours however were not significantly different from the results taken at 24 hours proven by the result of a T-test being 0.560.

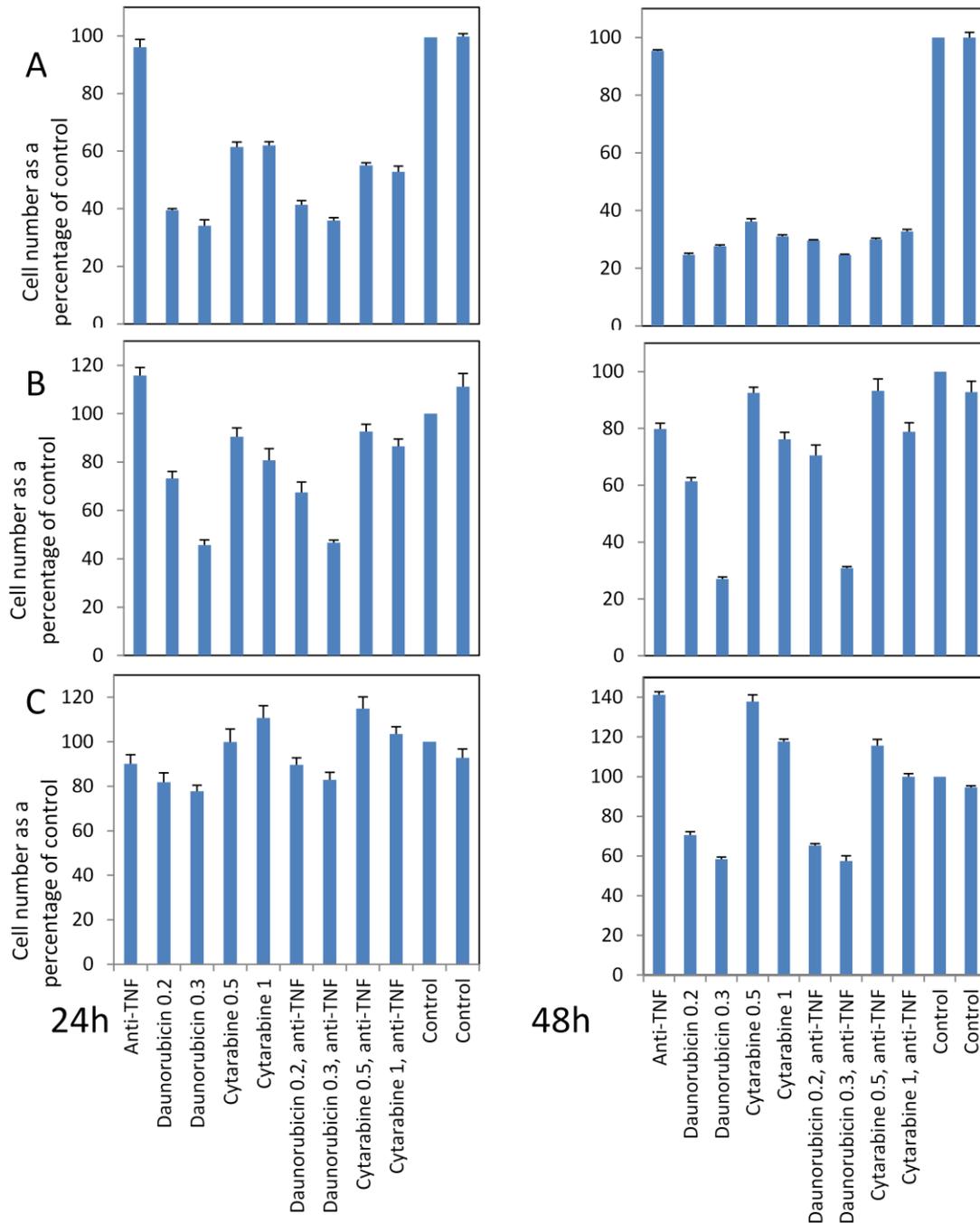


Figure 9 The effect of combinations of anti-TNF, Daunorubicin and Cytarabine treatment on cell death of AML cells after 24 and 48 hours

AML cell lines (HL60 (A), U937 (B) and THP-1 (C)) were treated with anti-TNF, 0.2 μ M Daunorubicin, 0.3 μ M Daunorubicin, 0.5 μ M Cytarabine, 1 μ M Cytarabine, 0.2 μ M Daunorubicin with anti-TNF, 0.3 μ M Daunorubicin with anti-TNF, 0.5 μ M Cytarabine with anti-TNF, 1 μ M Cytarabine and anti-TNF and two cell samples were left as controls. Following either a 24 or 48 hour incubation period, cells were plated onto MTS plates with MTS added, then plates were read to quantify cell death. n=3

4.5 Discussion

4.5.1 Effect of anti-TNF treatment on I κ B α mRNA and protein levels in AML cells.

AML cell lines HL60, U937 and THP-1 were treated with anti-TNF to investigate the effect of reduced TNF signalling on the levels of I κ B α mRNA present within the cells. The data in figure 5 shows that in THP-1 and U937 cells, I κ B α mRNA levels increased following anti-TNF treatment. This result was to be expected.

Previous results from investigations into I κ B α mRNA levels in AML patient samples have shown that I κ B α mRNA is reduced in AML. It is already established that AML has slightly increased TNF levels above those of healthy cells, which therefore lead to the notion that increased TNF is increasing I κ B α levels and not negatively regulating I κ B α . More TNF present in AML cells would lead to more activation of the TNF receptors. This leads to activation of I κ B kinase (IKK) which phosphorylates two serine residues located on the I κ B regulatory domain. I κ B α then becomes degraded in the proteasome and its inhibitory action against NF- κ B subunits is lost. Therefore, treating the AML cells with anti-TNF would prevent TNF signalling and prevent the dysregulation of I κ B α , hence the increase in levels of I κ B α mRNA following anti-TNF treatment.

The results presented in figure 5 for the HL60 cells do not comply with the results for cell lines U937 and THP-1 and are not what was expected. Figure 7 does however show HL60 cells with increased I κ B α protein following anti-TNF treatment. The HL60 cells in this investigation behaved as expected and also in the same way as the other two cell lines, whose I κ B α mRNA also increased following anti-TNF treatment.

What was interesting and perhaps unexpected about the results shown in Figure 7 was that each cell line had a peak in I κ B α protein levels either at 4 hours for THP-1 cells or at 8 hours for HL60s and U937s, then following that peak, I κ B α protein levels decreased again but not to below the original levels before anti-TNF treatment.

4.5.2 Effect of anti-TNF treatment on AML cell survival.

All the AML cell lines used in the investigations into the effect of anti-TNF on cell death showed increased cell death following increased amounts of anti-TNF treatment over longer time periods. Treating cells with 3 ng of anti-TNF had a greater effect on the amount of cell

death that occurred. The effect of the anti-TNF treatment became greater with time, this was apparent as there was more cell death in cell lines that were left to incubate for up to 72 hours.

TNF- α is known to inhibit induce apoptosis in differentiated cells (Chaudhary et al 1997). On the other hand, for early haematopoietic precursors, TNF- α provides survival signals to the cells (Gersuk et al 1998). AML is known to have high levels of TNF which is why investigations were made into the effects of removing TNF from the AML cell lines by addition of anti-TNF. Removal of the TNF signalling in AML cell lines also removes the survival signals from the cells, thus causing more cell death. This is reflected in the results shown in figure 8.

4.5.3 Effect of anti-TNF and drug treatment on AML cell survival.

Figure 9 shows all the results from the investigations into treating AML cell lines (HL60, U937 and THP-1) with combinations of anti-TNF and with the chemotherapeutic drugs Daunorubicin and Cytarabine. The results show that HL60 cells generally show better responses to anti-TNF and drug treatments, with more increases in cell death than with the other two cell lines. There was also a significant increase in cell death of HL60s 48 hours following treatment. The results also collectively show that Daunorubicin had a greater effect on cell death of all cell lines than Cytarabine did.

Chapter 5: Discussion

5.1 Summary

This study investigated the mRNA expression of NF- κ B signalling components in AML. The main results showed that I κ B α and RelB levels were found to be significantly reduced in AML cells compared to healthy CD34+ cells. This led to further investigation into the expression of I κ B α in AML. Basal levels of TNF are higher in AML cells (Bueso-Ramos et al 2004; Rushworth et al 2010), which is likely to have a positive effect on the amount of I κ B α present in AML cells as TNF induces I κ B α expression through feedback by NF- κ B. This effect was investigated by measuring the expression levels of I κ B α following treatment of AML cell lines with anti-TNF. I κ B α mRNA and protein expression showed a significant increase as a result of anti-TNF treatment, suggesting a different mechanism is regulating I κ B α expression in human AML. Cell death was also investigated in AML cell lines treated with anti-TNF. There was more cell death in cells following anti-TNF treatment than in cells left alone. This investigation provides novel information about NF- κ B signalling components in AML and the effect that treating AML cells with anti-TNF has on these NF- κ B proteins and ultimately on cell death.

5.2 reduced I κ B α and RelB expression in AML – variation in AML patients and overall effect

I κ B α and RelB expression levels were significantly reduced in AML cells. This meant that collectively, AML samples had significantly lower expression of I κ B α and RelB compared to the healthy CD34+ cells. When studying the results of AML patient samples individually however, it becomes obvious that generalisations may not be possible due to the large amounts of variation of expression of NF- κ B subunits between AML patients. From these particular results however, it has been shown that I κ B α and RelB in particular have low levels of expression therefore it would be fair to expect the same expression patterns in other AML patients. It would also be reasonable to expect large amounts of variation in the low expression of I κ B α and RelB between AML patients.

Despite different AML patient samples having the same low levels of I κ B α and RelB expression, such patients may still have completely different outcomes to each other in terms of response to treatment, pathology of the disease and survival. The study into mRNA levels of NF- κ B components showed large amounts of variation for each AML patient sample. For example, AML sample 301 had higher levels of p105, p65, cRel, RelB, p100

and NIK than AML sample 185 but had similar low levels of I κ B α . AML sample 307 had higher p105 and cRel than AML sample 301, lower RelB and NIK, and around the same levels of p65, I κ B α and p100. Therefore, I κ B α expression may be reduced in all AML patients however the cell's response to NF- κ B signalling will also depend on the signalling from all the other NF- κ B components put together, both canonical and non-canonical. So predicting a patient's outcome from the disease is an extremely complex matter because all signalling components need to be taken into account and their effects studied as one big picture, rather than individually. This point is demonstrated by large variations in the outcomes of patients even within the same FAB classification group of AML.

5.3 protein expression of NIK and nuclear NIK

Protein expression of the NF- κ B subunits was studied by immunofluorescent cell staining to gain insight into the location of the proteins within the AML cells. NIK protein expression was found in the nucleus of AML cells in this study. NIK is a regulatory kinase responsible for the activation of non-canonical NF- κ B signalling. The main role of NIK requires it to be present within the cytoplasm therefore it is not expected to be present in the nucleus of a cell in large amounts. Previous studies by Birbach et al (2004) have also found NIK in the nucleus of cancerous HeLa cells and have suggested a special role of nuclear NIK for an elevated constitutive NF- κ B activity in those cells.

NIK has been found to undergo nuclear-cytoplasmic shuttling in non activated cells (Birbach et al 2002). NIK has both nuclear localisation signals (NLS) and nuclear export signals (NES) which are both required for the dynamic exchange of NIK between subcellular compartments. The nucleus of a cell is known to be a highly dynamic organelle containing distinct sub-compartments (Carmo-Fonseca 2002). Such compartments are not defined by membranes but are however regarded as distinct compartments. Proteins may accumulate in such compartments by interaction with local high-affinity binding sites (Misteli, 2001). NIK may be held in nuclear compartments such as the nucleolus by interaction with structural motifs in rRNA (Birbach et al 2004).

NIK may be required in the nucleus and nucleolus for many reasons. NIK activates IKK which itself shuttles between the cytosol and nucleus (Birbach et al 2002). IKK can upregulate target genes like NF- κ B by binding to promoter elements, also direct phosphorylation of histones and facilitation of the transcription process (Anest et al 2003 and

Yamamoto et al 2003). NIK may also be required in the nucleus because NIK and IKK directly activate p65 via phosphorylation on the transactivation domain of p65 (Jiang et al 2003). All nuclear functions of NIK and IKK may be regulated by the distribution of NIK between the nucleoli and nucleoplasm (Birbach et al 2004).

5.4 I κ B α protein expression in nucleus and I κ B α protein shuttling

I κ B α protein was mainly found to be expressed in the cytoplasm of the AML patient sample cells. The majority of I κ B α expression is expected in the cytoplasm of unstimulated cells because it is a regulatory I κ B subunit that inhibits the translocation of p50 and cRel/p65 NF- κ B dimers into the nucleus. Upon I κ B α phosphorylation, ubiquitination and degradation, the nuclear localisation signal of the NF- κ B subunits becomes unmasked (Beg et al 1992) leaving them free to translocate to the nucleus. In stimulated cells where the NF- κ B pathway has been activated and transcription of NF- κ B genes is occurring, I κ B α protein synthesis is stimulated so that I κ B α can enter the nucleus of the cell to remove NF- κ B from its gene promoters (Arenzana-Seisdedos et al 1995). The export of such I κ B α /NF- κ B complexes out of the nucleus and into the cytoplasm to restore the pre-stimulation state of the complexes is a process known as postinduction repression (Arenzana-Seisdedos et al 1997).

In HeLa and peripheral blood T-lymphocytes, I κ B α is expressed not only in the cytoplasm of the cells but also in the nucleus in the absence of NF- κ B stimulation (Huang et al 1999). Nuclear I κ B α is maintained at low levels so that when cells are activated, NF- κ B will have the ability to be transcriptionally active without being immediately removed from the nucleus by I κ B α (Huang et al 1999). Huang et al have also found that the dominant nuclear export over nuclear import of I κ B α contributes to the largely cytoplasmic localization of the inactive complexes to achieve efficient NF- κ B activation by extracellular signals.

To summarise, AML cells were found to have higher I κ B α protein expression in the cytoplasm of the cells than in the nucleus. I κ B α is expressed in the cytoplasm of the cell to regulate NF- κ B signalling by inhibition of p50 and RelB/p65 subunits. Some I κ B α expression was present in the nucleus because I κ B α is able to remove the transcriptional activity of NF- κ B subunits by shuttling them out of the nucleus and back into the cytoplasm.

5.5 I κ B α regulation of Rel and NF- κ B family components

Transcription factors in the Rel/NF- κ B family are all related to each other over a region of about 300 amino acids called the Rel Homology Domain (RHD) which governs DNA binding and dimerisation. Each protein has a nuclear localisation signal (NLS) however the various homo- and heterodimers of NF- κ B are maintained in the cytoplasm by I κ B α which masks the NLSs. NF- κ B proteins are free to enter the nucleus following proteasomal degradation of I κ B α . I κ B α degradation occurs following phosphorylation of two serine residues in the N-terminal region of the molecule and consequently ubiquitination of I κ B α (Brown et al 1995).

The interactions between the NLSs of a Rel/NF- κ B dimer and I κ B α have been investigated by Latimer et al (1998). Their study showed that neither the RelA nor c-Rel homodimers require NLSs to bind to I κ B α . On the other hand, their study showed that in the absence of an NLS in RelA or c-Rel, I κ B α has a significantly reduced ability to inhibit DNA binding. Also, complexes of I κ B α with NLS deletion mutants of RelA or c-Rel were significantly less stable than complexes of I κ B α with the wild-type RelA or c-Rel. This therefore suggests that I κ B α contacts one or both of the RelA or c-Rel NLSs to stabilise the interaction but this contact is not required for binding.

The story is different for the interactions between p50 and I κ B α however, where such interactions do require the NLS for binding. There are certain residues present within the p50 NLS that are required for binding with I κ B α , and this depends on their charge. Finally, in a p50-I κ B α complex or a c-Rel-I κ B α complex, the N terminus of I κ B α either directly or indirectly masks one or both of the dimer NLSs (Latimer et al 1998). This is how I κ B α exerts its inhibition on the Rel/NF- κ B dimers within the cytoplasm of cells.

5.6 The I κ B family

There are three members in the I κ B family and they do not all respond in the same way to NF- κ B-inducing signals. The three members are I κ B α , I κ B β and I κ B ϵ (Thompson et al 1995 and Haskill et al 1991). The carboxyl-terminal regions of the precursors for p50 and p52, respectively p105 and p100 can also function as I κ Bs. Each family member contains six copies of an ankyrin repeat which functions as a protein-protein interaction domain (Simeonidis et al 1999). The region carboxyl-terminal to the ankyrin repeats contains a proline (P), glutamate (E), serine (S), and threonine (T) (PEST) sequence regulating basal

level protein turnover and is also required for inhibition of DNA binding whilst the amino-terminal region is the signal responsive domain (Verma et al 1995 and Ghosh et al 1998).

All the IκBs share structural similarities however they exhibit substantial differences *in vivo* which depends on the cell type and on the stimulus (Simeonidis et al 1997, Suyang et al 1996 and Chu et al 1996). Generally, IκBα is rapidly degraded whereas IκBβ and IκBε are degraded more slowly (Thanos et al 1995 and Ghosh et al 1998). Also, IκBα is a stronger inhibitor of NF-κB than IκBβ or IκBε (Simeonidis et al 1997).

As previously mentioned, IκBα does not only inhibit NF-κB signalling by masking the NLSs of Rel/NF-κB dimers, it also enters the nucleus and can inhibit NF-κB-DNA binding. IκBs can promote transport of NF-κB out of the nucleus and into the cytoplasm, thus terminating transcription and resetting the switch (Tran et al 1997). IκBα is a stronger nuclear inhibitor of nuclear NF-κB activity than IκBβ and IκBε does not efficiently enter the nucleus (Arenzana-Seisdedos et al 1995). This further demonstrates the differential inhibitory strengths of IκB proteins.

5.7 Regulation of IκBα

5.7.1 Regulation of IκBα by IKK

Like all inhibitors, there is a mechanism in place to regulate the activity of IκBα to allow NF-κB signalling to occur when required. Potent NF-κB activators can induce almost complete degradation of IκBs, particularly IκBα, within minutes. This process, which is mediated by the 26S proteasome (Alkalay et al 1995) depends on the phosphorylation of two conserved serines in the N-terminal regulatory domain of IκB (Brown et al 1995). Even the substitution of a single serine can considerably inhibit the degradation of IκB, and serines cannot be replaced by threonine showing that they are phosphorylated by a serine-specific kinase (DiDonato et al 1996). Immediately following phosphorylation, IκBs undergo a second post-translational modification of polyubiquitination. Polyubiquitination involves a cascade of enzymatic reactions involving E1, E2 and E3 ubiquitin ligases. The acceptor sites for ubiquitin on IκBα are arginines 21 and 22 which cannot be substituted with lysines because degradation becomes considerably retarded (DiDonato et al 1996 and Scherer et al 1995).

The enzymes that catalyse the ubiquitination of phosphorylated I κ Bs are constitutively active. Therefore the regulation that determines the activity of I κ Bs lies in the phosphorylation step of the two N-terminal serines. The only cases where this does not occur is activation of NF- κ B in response to UV radiation which does not involve I κ B phosphorylation at the N-terminal sites (Bender et al 1998) and also in anoxia which stimulates phosphorylation of I κ B α at tyrosine 42 (Imbert et al 1996). In all other cases however, the control of the phosphorylation of I κ Bs in response to all other NF- κ B activating stimuli lies with the IKK complex.

IKK is a protein complex composed of two catalytic subunits IKK α and IKK β (DiDonato et al 1997) and a third regulatory polypeptide IKK γ (also known as NEMO) (Rothwarf et al 1998). IKK α and IKK β have very similar primary structures with their protein kinase domains found at their N terminus. They also share identical activation kinetics and substrate specificities (Mercurio et al 1997). IKK γ /NEMO does not contain a catalytic domain however IKK activation certainly requires an intact IKK γ subunit for activation as shown by Yamaoka et al (1998) when no IKK or NF- κ B activity was found in IKK γ /NEMO-deficient cells following treatment with cytokines such as TNF and IL-1. The C-terminal region of IKK γ is particularly necessary for the recruitment of upstream activators (Rothwarf et al 1998). The predominant form of the IKK complex comprises of an IKK α -IKK β heterodimer associated with either a dimer or a trimer of IKK γ (Rothwarf et al 1998).

Activation of IKK depends on phosphorylation of its IKK β subunit, and more recently, studies have shown that incubating cells with TNF stimulates the phosphorylation of all three IKK subunits (Delhase et al 1999). Like I κ Bs, IKK α and IKK β are phosphorylated exclusively at serines present on an activation loop (Delhase et al 1999). The phosphorylation moves the activation loop away from the catalytic pocket which allows its interaction with substrates such as I κ Bs (Johnson et al 1996). To summarise, IKK is activated as a result of IKK β phosphorylation and IKK α phosphorylation is not essential for stimulation of IKK activity, therefore the IKK β subunit is the target for upstream activators involved in proinflammatory signalling that are recruited to the complex via IKK γ .

Once activated IKK complexes have phosphorylated the I κ B subunits of NF- κ B-I κ B complexes (triggering their ubiquitin-dependent degradation and therefore activation of NF- κ B), the activated IKK β subunits undergo C-terminal autophosphorylation. When at least nine of the C-terminal serines are phosphorylated, the enzyme reaches a low activity state, facilitating inactivation of IKK by phosphatases once the upstream signal has disappeared.

Without this method of control, prolonged IKK activation would result in prolonged NF- κ B activation followed by increased production of inflammatory mediators. Such mediators can further NF- κ B activation (Barnes et al 1997) which without the efficient process of rapidly terminating IKK and NF- κ B activities, would result in damage to cells and disease. For example, constitutively activated IKK has been detected in Hodgkin's disease cells (Krappmann et al 1999). This results in constitutive NF- κ B activation which protects these cells from induction of apoptosis by radio- and chemotherapy (Bargou et al 1997).

It is likely that elevated NF- κ B and IKK activity is protecting AML cells from apoptosis and that this protection would continue to have an effect despite treatment. Elevated IKK activity may be the cause of the low levels of I κ B α found in AML cells in this study, more I κ B α phosphorylations would occur, leading to increased polyubiquitination and hence a higher rate of I κ B α degradation in the proteasome.

5.7.2 Regulation of I κ B α by RelB

RelB is a transcription activator of chemokines and TNF- α in macrophages (Weih et al 1997) however it has been found by Xia et al (1999) that in fibroblasts RelB plays the role of transcription suppressor of these genes. RelB shares many common features of the NF- κ B family but unlike other NF- κ B family members, RelB cannot form homo-dimers and only associates efficiently with p50 and p52 (Dobrzanski et al 1994). The RelB heterodimers have a much lower affinity for I κ B α than other NF- κ B complexes do and are less susceptible to inhibition by I κ B α (Lernbecher et al 1994). Due to the inefficient binding of RelB to I κ B α , RelB is allowed to enter and remain in the nucleus (Dobrzanski et al 1994). Whilst in the nucleus, RelB can strongly induce the expression of I κ B α and therefore may indirectly inhibit RelA or c-Rel activities by driving the expression of I κ B α (Dobrzanski et al and Ferreira et al 1998).

It is thought that RelB may exert its transcriptional suppressor function in fibroblasts by modulating the stability of the I κ B α protein (Xia et al 1999). Because RelB is known to have a low affinity for I κ B α , RelB may affect the stability of I κ B α indirectly. It has been suggested by Xia et al (1999) that RelB may affect the stability of I κ B α by affecting IKK activity. This leads to the notion of a hierarchy control of one NF- κ B by another NF- κ B family member may occur in such cells.

Xia et al (1999) have questioned whether RelB is differentially modified in different cells. Perhaps in the cells of patients with AML revert to similar mechanisms of control of I κ B α as in fibroblast cells. AML cells have been found in this study to have lower levels of RelB and I κ B α than in normal CD34+ healthy cells. If the fibroblast mechanism of control of I κ B α was in place in the AML cells studied, it would make sense that I κ B α expression is decreased as a result of decreased expression of RelB. Less RelB expression would mean less RelB present in the nucleus of the AML cells and therefore less induction of I κ B α expression by RelB.

5.7.3 Regulation of I κ B α by C/EBP β

CCAAT-enhancer-binding proteins (C/EBPs) are a family of transcription factors with six members, including C/EBT β . They form homo- and heterodimers and play a role in cellular proliferation/differentiation as well as inflammation and metabolic processes (Ramji and Foka 2002 and Piwien-Pilipuk et al 2002). It has been shown that C/EBP β plays a complex role in NF- κ B-dependent gene regulation (Ramji and Foka 2002) at the promoter level, which lead Cappello et al (2009) to investigate whether C/EBP β also affects NF- κ B-associated signalling upstream of the promoter level.

Cappello et al (2009) demonstrated that the transcriptional protein C/EBP β enhances NF- κ B activation by elevating nuclear levels of p65. Higher cytosolic levels of NF- κ B inhibitor I κ B α were found in C/EBP β knock out cells which suggests that the higher I κ B α levels in the absence of C/EBP β is the reason for the attenuation of the stimulus-induced increase of p65 in the nucleus of knock out cells. C/EBP β is involved in the regulation of I κ B α at the transcriptional level: the I κ B α promoter contains a κ B as well as a C/EBP β binding sites (Chiao et al 1994 and Ito et al 1994). It has also been demonstrated that under certain conditions, C/EBP β negatively affects promoters containing κ B sites (Stein et al 1993), which would lower I κ B α mRNA transcription, resulting in lower protein concentrations. This would in turn enable increased NF- κ B signalling activity as the I κ B presence is lost.

Differentiating pre-monocytic cells dramatically increase their nuclear C/EBP β levels to lower I κ B α levels in order to increase NF- κ B signalling until they become mature monocytes/macrophages (Pham et al 2007). It is perhaps rather likely that in the AML patient sample cells studied in this investigation, C/EBP β may be aberrantly expressed at higher levels than in normal healthy cells. This would lead to the negative regulation of I κ B α

and the reduction of mRNA and protein levels found within the cell, which is exactly what has been found to occur in the AML cells of this study.

The regulation that C/EBP β exerts on the I κ B α promoter under certain conditions has also been reported for transcriptional repressors or activators such as RBP-I and Notch 1 (Oakley et al 2003) and co-activators (SRC-1, SRC-2 and SRC-3) and co-repressors (HDAC1, HDAC3, SMRT and NCoR) (Gao et al 2001). The promoter activity of the I κ B α gene is determined by the combined activities of all I κ B α co-activators and co-repressors (Gao et al 2005).

5.7 4 Regulation of I κ B α by RelA

Rel A is a transcription factor within the NF- κ B family. RelA also has a regulatory function relating to the inhibitory I κ B α protein. Yang et al (2003) have shown that RelA can induce enhanced cytoplasmic retention of I κ B α , thus rendering I κ B α unable to enter the nucleus to inhibit NF- κ B-DNA binding or to transport NF- κ B subunits out of the nucleus. RelA was also shown to cause an increase in I κ B α phosphorylation which therefore lead to an increase in I κ B α degradation. Further *In vivo* analysis demonstrated that the RelA-induced I κ B α /RelA interactions are specific, saturable and phosphorylation-dependent. RelA regulation of NF- κ B-I κ B α complex formation is dependent on the phosphorylation of I κ B α .

RelA in particular was not studied in this investigation however c-Rel was looked at and shares a similar function to RelA. c-Rel mRNA and protein levels appear to remain normal in the AML patient samples used in this study. If RelA levels are also remaining normal in AML patient samples, RelA may be having an influence on the decreased levels of I κ B α found in AML patient cell samples in this study. RelA may be causing the release of NF- κ B inhibition by the same processes as described by previous findings by Yang et al (2003). It would be interesting and useful to further investigate the NF- κ B signalling pathway by looking in particular at the protein and mRNA levels of RelA and its presence within AML cells.

5.8 How can I κ B α be increased?

It has become obvious from this investigation that the levels of I κ B α are reduced in the AML cell samples studied and that this has an effect on the cell such that it leads to increased NF- κ B signalling which in turn provides cancerous cells with the ability to overcome cell death. In

AML cells it appears it would be beneficial to the patient if levels of I κ B α were brought back up to normal healthy levels to allow apoptosis to occur. There are a number of factors that can cause an increase in the amount of active I κ B α present within a cell.

Vitamin D

Vitamin D can decrease NF- κ B activity by increasing levels of I κ B α . In cells treated with vitamin D, Cohen-Lehav et al (2006) found that there were decreased levels of NF- κ B p65 in the nucleus and increased NF- κ B p65 in the cytosol. There were no changes however in the total p65 protein and mRNA levels, the change is that the p65 is simply being transported to different areas within the cell. Vitamin D addition did induce a significant increase in mRNA and protein levels of I κ B α . These elevated levels of I κ B α can be explained by vitamin D-induced prolongation of the half-life of I κ B α thus increasing the stability of the I κ B α molecule, and also by a decrease in phosphorylation events occurring on I κ B α molecules. A vitamin D-induced increase in I κ B α levels will lead to decreased nuclear translocation of NF- κ B therefore a decrease in its activity.

IL-10 and IL-13

IL-10 and IL-13 are powerful anti-inflammatory cytokines which suppress the nuclear localisation of NF- κ B as a result of preserved protein expression of I κ B α in cells (Lentsch et al 1997).

Glucocorticoids

Glucocorticoids are immune-suppressive agents that inhibit NF- κ B activation and mediate this by induction of I κ B α protein synthesis (Auphan et al 1995). In monocytic and lymphocytic cells, I κ B α mRNA and protein increases as a result of treatment with glucocorticoids (Brostjan et al 1996). Dexamethasone in particular induces synthesis of I κ B α mRNA and protein in monocytic cell lines which leads to an increase in I κ B α levels present in the cytosol of cells. This presence of I κ B α prevents the release and activation of NF- κ B signalling (Brostjan et al 1996).

Cytokine IL-8

Minekawa et al (2004) have studied Neonatal necrotizing enterocolitis (NEC) in which the pro-inflammatory cytokine IL-8 plays an important role in the pathophysiology of the disease.

Another cytokine IL-1 β activates the IL-8 gene by regulating the NF- κ B signalling pathways in intestinal cells. Human breast milk was found to suppress the IL-1 β -induced activation of the IL-8 gene promoter by inhibiting the activation pathway of NF- κ B. This induces production of I κ B α and regulates its phosphorylation. Minekawa et al (2004) concluded that breastmilk can increase the basal levels of I κ B α which leads to more NF- κ B-I κ B α interactions, preventing NF- κ B signalling. Breastmilk was also found to directly suppress the phosphorylation of I κ B α , preventing its degradation and promoting its stability.

5.9 Areas for possible further investigation or improvement

Several conclusions have been drawn from the data presented in Figures 1 and 2, however much of the data presents large amounts of error represented by the error bars shown on the figures. To be certain about the conclusions drawn from these results, they perhaps could be repeated further to reduce the error. Error was possibly caused by using different mRNA preparations for individual patient samples therefore this should be avoided.

Western blots should also be run to investigate the levels of protein for NF- κ B components present in the cytoplasm and in the nucleus. Detecting levels of protein by western blotting would further support the present protein expression data which used immunofluorescence to detect the presence of NF- κ B proteins within the AML cells.

AML cell lines were treated with anti-TNF to observe the effects of the treatment on levels of I κ B α mRNA within the cells (Fig 5). It was observed that each cell line had a peak in I κ B α mRNA levels at either four hours (THP-1) or eight hours (HL60 and U937) following anti-TNF treatment. Following this peak, I κ B α mRNA levels decreased again but not to levels prior to anti-TNF treatment. This is an interesting observation and is worth investigating to see whether it holds any significance in understanding the signalling process relating to I κ B α and NF- κ B.

The effect of treating AML cells with anti-TNF on mRNA levels of I κ B α was investigated using the cell lines HL60, U937 and THP-1. As previously mentioned, these cell lines are

good indicators of what is likely to occur in the cells of individuals suffering from AML. To learn more about the effect that anti-TNF treatment is likely to have on AML cells *in vivo*, it would be necessary to do anti-TNF treatment investigations using actual AML patient cell samples. The same use of AML patient cell samples would also be useful when investigating the effects of anti-TNF on cell survival and also investigating mRNA levels of I κ B α following treatment of anti-TNF and with the chemotherapeutic drugs daunorubicin and cytarabine.

Finally, it would be useful to investigate the protein and mRNA levels of RelA and in particular, its distribution between the cytoplasm and nucleus of AML patient cell samples. RelA has not been investigated in particular in this study however cRel has been and the two are known to share similar functions. On the other hand, RelA in particular (and not cRel) has been shown to be able to enhance cytoplasmic retention of I κ B α and also increase I κ B α phosphorylation which leads to its destabilisation and degradation.

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