The Role of APRIL in Promoting Cell Cycle Progression and Drug Resistance in Multiple Myeloma

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I, John Quinn, declare that the work presented within this thesis is solely my own work and where information from other sources is included, I have indicated this in the text and have provided the appropriate reference.

Signed:

Date:
Abstract

Recent years have seen a significant improvement in survival for patients with multiple myeloma (MM). Novel agent-based induction regimens have resulted in dramatically high overall response rates, however, the overwhelming majority of patients relapse and MM remains incurable. Furthermore, MM shows considerable clinical heterogeneity and patients with “high-risk” MM continue to fare badly even with novel therapies. Thus, identifying the key factors that are responsible for the proliferation of MM cells is a critical step in further improving outcomes for MM patients. It has been extensively shown that bone marrow (BM) microenvironmental factors promote MM cell proliferation, survival and drug resistance. The tumour necrosis factor superfamily member, A Proliferation-Inducing Ligand (APRIL) has recently been shown to be a survival factor for normal plasma cells and to promote drug resistance in IL-6 dependent MM cell lines. Therefore, the overall aim of this work was to examine the role of APRIL in MM and in particular, to determine whether APRIL could induce cell cycle progression in primary MM cells. In summary, APRIL was found to promote cell cycle progression in primary MM cells, but this effect was restricted to those cells expressing cyclin D2 and carrying the t(4;14) and t(14;16) translocations, in contrast to cyclin D1-expressing MM cells that were less responsive. In addition APRIL was found to protect cyclin D2-expressing MM cells from drug-induced apoptosis. Cell surface receptors for APRIL were found to be present on the surfaces of primary MM cells and APRIL was shown to be abundant in the myeloma BM microenvironment. In conclusion, these results show that cell cycle responses to APRIL segregate with respect to D-type cyclin class and translocation status and and pave the way for future mechanistic studies, as well as providing a rationale for designing specific therapies for different genetic subgroups of patients.
Acknowledgements

I would like to sincerely thank my supervisors, Professor Yong and Dr. Glassford for all of their support, guidance and encouragement during the years of research that have led to the completion of this thesis. I wish also to thank my wife Clodagh and children James, Conor and Ailbhe for their patience and understanding without which it would not have been possible to complete this work. I wish to sincerely thank Cancer Research United Kingdom (CRUK) for providing the funding to support this work. Finally, I wish to express my sincere gratitude to all of the multiple myeloma patients who provided bone marrow samples without which this research would have been impossible.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APRIL</td>
<td>A Proliferation Inducing Ligand</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-Cell Activating Factor</td>
</tr>
<tr>
<td>Blimp 1</td>
<td>B-lymphocyte-induced maturation protein 1</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BMCA</td>
<td>B-cell maturation antigen</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone Marrow Stromal Cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin Kinase Inhibitor</td>
</tr>
<tr>
<td>CM</td>
<td>Culture Medium</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorting</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence-In-Situ Hybridisation</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>HMCL</td>
<td>Human Myeloma Cell Line</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan Surface Proteoglycan</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like Growth Factor I</td>
</tr>
<tr>
<td>IgH/TC</td>
<td>Translocation involving the Immunoglobulin Heavy Chain Gene resulting in D-type Cyclin gene overexpression</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple Myeloma</td>
</tr>
<tr>
<td>MNC</td>
<td>Mononuclear Cell</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin Lymphoma</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral Blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>S/G2M</td>
<td>Cells in S and G2M phases of the cell cycle</td>
</tr>
<tr>
<td>TACI</td>
<td>Transmembrane Activator and Calcium-modulator and Cyclophilin Ligand Interactor</td>
</tr>
<tr>
<td>TC</td>
<td>Translocation Cyclin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1 Multiple Myeloma - Introduction

1.1.1 Epidemiology and background

Multiple Myeloma (MM) is a bone marrow-based neoplasm characterized by the clonal accumulation and proliferation of malignant plasma cells, the presence of serum and/or urine monoclonal protein and related end-organ damage (Kyle et al, 2008, see Table 1). More recently, MM has been renamed as “plasma cell myeloma” by the World Health Organisation (WHO), however the term “multiple myeloma” or MM will be used throughout this manuscript owing to its widespread use in the scientific literature and clinical practice (Swerdlow et al, 2008).

As the second most common haematopoietic malignancy, MM accounts for approximately 10-15% of all haematopoietic cancers leading to 15-20% of all deaths from haematopoietic cancer and approximately 2% of all deaths from cancer. There are approximately four thousand new MM cases annually in the United Kingdom and twenty thousand in the United States. MM is primarily a disease of older persons with a median age of onset of 65-70 years. Just 10% of cases are diagnosed in those patients less than 50 years and 2% of cases are diagnosed in patients below 40 years of age (Kyle et al, 2008).

A recent study involving the long-term follow-up of 77,469 volunteers has shown that in the vast majority of cases, MM is preceded by a pre-malignant stage termed “Monoclonal Gammopathy of Undetermined Significance (MGUS)”, which is characterized by the presence of serum and/or urine monoclonal protein (M-protein) without evidence of significant bone marrow infiltration by clonal plasma cells or end-organ damage (Landgren et al, 2009, see Table 1.1). Like MM, the incidence of MGUS
increases with age, being found in approximately 3% of all persons over 70 years, and 5% of those over 85 years. However, the rate of transformation from MGUS to overt MM is low at approximately 1% per year, giving MM an incidence of approximately 4/100,000 (Kyle et al, 2006). Less commonly, MM may also be preceded by a “smouldering phase”, where patients are asymptomatic and there is no evidence of myeloma-related organ damage (see Table 1.1). Although not widely regarded as being a disease with a familial basis, the risk of developing MM is approximately 2-4 times greater in those with first-degree relatives with MM, and first degree relatives of MGUS patients have also been shown to have an increased risk of developing MM suggesting that MGUS is a marker of genetic susceptibility (Kristinsson et al, 2009). Furthermore, a recently published genome wide association study based on single nucleotide polymorphism (SNP) analysis of 1675 MM patients and 5903 control subjects identified risk loci at chromosomes 3p22.1 and 7p15.3 as well as a probable further risk locus at chromosome 2p23.3 (Broderick et al, 2011). Little is known about environmental or occupational factors for MM, but about some studies have identified an association between MM and a history of working in agriculture (Blair et al, 2009).

1.1.2 Multiple myeloma - clinical features and natural history

The cardinal clinical features of MM are hypercalcaemia, renal impairment, anaemia and osteolytic bone lesions (so-called “CRAB” features) and the diagnosis of MM rests on the presence of one of these clinical features in addition to the identification of clonal plasma cells in the bone marrow, with the presence of monoclonal immunoglobulin in the serum or urine (see Table 1.1 for diagnostic criteria). Less common clinical features at presentation include: hyperviscosity, recurrent bacterial infection and extramedullary disease. With currently available drug-combinations almost all MM patients will respond to anti-myeloma therapy, at least initially. However, the disease follows a
typical course of multiple relapses followed by progressively shorter periods of remission until eventually the disease becomes refractory to treatment, heralding the terminal stage of the disease. In fact, MM is still regarded as being incurable for the vast majority of patients, with the exception of a small number of younger patients who are candidates for allogeneic bone marrow transplantation, and a smaller fraction of patients who have achieved longterm survival with standard treatments (Martinez-Lopez et al, 2011). Standard initial treatment for younger patients (typically <65 years) includes remission induction with combination chemotherapy regimens (typically cyclophosphamide, dexamethasone and thalidomide) followed by consolidation with high-dose intravenous melphalan and autologous peripheral blood stem cell transplant. Older, less fit patients (typically >65 years) are treated with alkylating agents (e.g. melphalan, cyclophosphamide) in combination with one of bortezomib, lenalidomide and thalidomide in addition to corticosteroids. As bone disease is found in approximately 80% of MM patients, regular bisphosphonate treatment is also an important component of standard anti-MM treatment (Bird et al, 2011).
### Table 1.1 Diagnostic criteria for multiple myeloma and related disorders (adapted from Kyle *et al*, 2009)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monoclonal gammopathy of undetermined significance (MGUS)</strong></td>
<td>All three criteria must be met:</td>
</tr>
<tr>
<td></td>
<td>1. Serum monoclonal protein &lt;3 g/100 ml</td>
</tr>
<tr>
<td></td>
<td>2. Clonal bone marrow plasma cells &lt;10% and</td>
</tr>
<tr>
<td></td>
<td>3. Absence of end-organ damage such as hypercalcaemia, renal insufficiency, anaemia and bone lesions (“CRAB”) that can be attributed to the plasma cell proliferative disorder</td>
</tr>
<tr>
<td><strong>Smouldering multiple myeloma (also referred to as asymptomatic multiple myeloma)</strong></td>
<td>Both criteria must be met:</td>
</tr>
<tr>
<td></td>
<td>1. Serum monoclonal protein (IgG or IgA) ≥3 g/100 ml and/or clonal bone marrow plasma cells ≥10% and</td>
</tr>
<tr>
<td></td>
<td>2. Absence of end-organ damage such as lytic bone lesions, anaemia, hypercalcaemia or renal failure that can be attributed to a plasma cell proliferative disorder</td>
</tr>
<tr>
<td><strong>Multiple myeloma</strong></td>
<td>All three criteria must be met:</td>
</tr>
<tr>
<td></td>
<td>1. Clonal bone marrow plasma cells ≥ 10%</td>
</tr>
<tr>
<td></td>
<td>2. Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma) and</td>
</tr>
<tr>
<td></td>
<td>3. Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:</td>
</tr>
<tr>
<td></td>
<td>• Hypercalcaemia: serum calcium ≥ 11.5 mg/100 ml or</td>
</tr>
<tr>
<td></td>
<td>• Renal insufficiency: serum creatinine&gt;1.73 mmol/l)</td>
</tr>
<tr>
<td></td>
<td>• Anaemia: normochromic, normocytic with a hemoglobin value of &gt;2 g/100 ml below the lower limit of normal or a hemoglobin value &lt;10 g/100 ml</td>
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<tr>
<td></td>
<td>• Bone lesions: lytic lesions, severe osteopenia or pathologic fractures</td>
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</tbody>
</table>
1.1.3 Multiple myeloma – prognosis and survival

Patients with de novo MM have a median survival of 4-5 years, however the last 15-20 years has seen enormous progress made in our understanding of MM disease biology. Firstly, the identification of recurrent genetic aberrations in MM has provided an insight into the wide clinical heterogeneity observed within MM patients, with some patients having aggressive disease and a shorter survival (<1 year), in contrast to those with more indolent disease and survival of >10 years (Bergasagel et al, 2005b). Secondly, the importance of the BM microenvironment in promoting the survival, growth and drug resistance of MM cells is increasingly recognised (Podar et al, 2009). In parallel with our enhanced understanding of the MM disease biology, the introduction of new anti-myeloma agents such as thalidomide, lenalidomide and bortezomib, which are thought to target some of the key BM microenvironment-MM cell interactions, have dramatically improved response rates and overall survival in MM patients. In fact, it is estimated that younger patients now diagnosed with MM can expect a median survival of approximately 7-8 years (Kumar et al, 2008, Brenner et al, 2008).

Having outlined above the key epidemiological and clinical features as well as the natural history of MM, this introductory chapter will turn to focus on the biological factors that have been shown to be of greatest importance in the pathogenesis of MM, including the phenotype of MM cells, putative MM precursor cells, as well the BM microenvironment and recurrent genetic aberrations. Finally, the role of the tumour necrosis factor (TNF) superfamily member APRIL (A Proliferation-Inducing Ligand) in normal physiology and plasma cell disorders will be discussed.
1.2 Myeloma Cell Phenotype

1.2.1 Normal plasma cell development

Normal plasma cells are found in the bone marrow (BM) where they comprise approximately 1/100 to 1/400 of BM mononuclear cells and also in the laminae propria of mucosae. Normal plasma cells are terminally differentiated B-cells that are generated in lymph nodes from B-lymphocytes. The first step in B-cell development occurs when early B cells move from the protective niches of the BM microenvironment to the peripheral circulation and lymph nodes where they encounter antigen. Then, B-lymphocytes with high-affinity antigen receptors are selected by antigen through somatic hypermutation of the variable region of immunoglobulin genes. Following this, the B-lymphocytes can then become either memory B-cells or highly proliferative plasmablasts (Klein et al, 2003). These plasmablasts then migrate to the BM where they are supported by a diverse array of survival and differentiation factors such as IL-4, IL-5, IL-6, IL-10 and APRIL to become terminally differentiated, long-lived plasma cells (Cassese et al, 2003). The importance of APRIL in these processes is highlighted by a recent publication that identified APRIL secreting eosinophils as critical cellular factors in maintaining plasma cell survival in murine bone marrow. In this study, it was demonstrated that BM eosinophils secreted large amounts of APRIL and co-localised with Chemokine (C-X-C) Ligand 12 (CXCL12)-expressing BM stromal cells. Furthermore, depletion of eosinophils from the murine BM dramatically reduced plasma cell survival (Chu et al, 2011, Brink et al, 2011). In addition to eosinophils, APRIL-secreting magakaryocytes and bone marrow stromal cells expressing CXCL12 have been shown to be critical in attracting and maintaining the survival of Chemokine (C-X-C) Receptor 4 (CXCR4)-bearing plasma cells (Winter et al, 2010, Togoyoda et al, 2004).
1.2.2 Cell surface proteins and transcription factors expressed by plasma cells

Plasma cells are characterised by their expression of CD138 (also called syndecan-1, an heparan-sulphate proteoglycan) and CD38. Typically, they lack expression of CD19, CD22 and CD45, whilst CD20 and CD56 expression are only found in subsets of MM patients. Plasma cells express the Blimp-1 and XBP-1 transcription factors, which are not expressed by memory B-cells (Klein et al, 2003, Reimold et al, 2001). In contrast, plasma cells do not express the Bcl-6 and Pax-5 transcription factors, both of which are hallmarks of memory B-cells. Importantly, in vitro studies of the early stages of B-lymphocyte differentiation to plasmablasts and plasma cells have shown that the removal of CD40 stimulation from B-lymphocytes leads to the generation of cells with a plasma cell phenotype (Arpin et al, 1995). Other factors that have been shown to play crucial roles in the process of plasma cell generation from B-lymphocytes include IL-6 stimulation and the interaction between IL-10 and CD27 (Agematsu et al, 1998, Tarte et al, 2002, Tarte et al, 2003).

1.2.3 Characteristics of MM cells

Like normal plasma cells, MM cells also show strong expression of both CD138 and CD38. Although CD38 expression is found on virtually all MM cells, it is absent from some HMCLs (Bataille et al, 2006). Importantly, CD138 has been shown to act as a co-receptor for the interaction between MM cells and MM growth factors and the serum level of soluble CD138 has been shown to have prognostic significance in MM, possibly reflecting the tumour cell burden (Seidel et al, 2000, Lovell et al, 2005). MM cells are also characterized by the aberrant expression of other cell surface markers in varying numbers of cases. CD56, an adhesion molecule typically found on NK cells, is expressed in approximately three-quarters of MM cases. In addition, absence of CD56 expression in MM has been associated with extramedullary disease in the peripheral
blood, lambda isotype, lack of osteolytic bone disease and a poorer prognosis and may also be useful in distinguishing between MM and MGUS cases (Van Camp et al, 1990, Ely et al, 2002, Sahara et al, 2002). CD27 expression, a memory B-lymphocyte marker, is found in approximately 50% of MM cases and is associated with a better prognosis (Guikema et al, 2003, Moreau et al, 2006).

1.2.4 Cell surface markers in MM subgroups

CD20 expression is found in 15-20% of MM cases and has been shown to be associated with distinct morphological features (small, round mature plasma cells) and the t(11;14) translocation (Robillard et al, 2003). In contrast, CD20 expression has not been described in MM cases with t(4;14) and only rarely in cases with t(14;16), some of which display plasmablastic morphology and a more aggressive disease course (Mateo et al, 2005, Quinn et al, 2010). Interestingly, aberrant CD117 expression is found in 30% of MM cases and is associated with hyperdiploidy, a better prognosis, and reduced myeloid maturation in the BM resulting in relative neutropenia (Mateo et al, 2008, Schmidt-Hieber et al, 2011).

1.2.5 “Precursor” multiple myeloma cells

The mechanisms that trigger relapse from plateau phase of MM remain poorly understood, thus the cancer stem cell (CSC) hypothesis, which suggests that minor subpopulations of tumour cells possessing the stem cell properties of self-renewal and differentiation along with drug-resistance are ultimately responsible for MM-relapse is an understandably appealing one. Such cells have been identified in acute myeloid leukaemia (AML), breast cancer, prostate cancer and melanoma and in recent years much effort has focused on the identification and characterisation of MM stem cells (MMSCs) (Dean et al, 2005). Several independent groups have now identified MM cells with some or all of these features of CSCs, however the exact phenotype and the
frequency of such cells in MM remains controversial and is the subject of ongoing debate (Davies et al, 2000, Brennan et al, 2009).

The possibility that a distinct subpopulation of cells, other than terminally differentiated plasma cells, might be part of the “MM clone” was proposed when it was demonstrated by polymerase chain reaction (PCR) that MM cells possess immunoglobulin heavy chain genes that are somatically hypermutated and remain constant throughout the disease course, in keeping with the disease having arisen from a post-germinal centre B-cell (Bakkus et al, 1992). This finding led to investigations to determine if cells with a different surface phenotype were clonally related to the main tumour bulk, and it was subsequently shown by several groups using PCR-based assays, in samples taken from MM patients that small populations of B-lymphocytes contained identical immunoglobulin gene rearrangements to the malignant plasma cells comprising the main tumour bulk (i.e. clonotypic, Billadeau et al, 1993, Bergsagel et al, 1995, Rasmussen et al, 2000). Naturally, these findings were followed by studies to characterise these cells from a functional perspective and also to determine if these clonotypic B-cells were capable of giving rise to malignant plasma cells and several studies have shown that clonotypic B-cells were capable of myelomagenesis upon transplantation into non-obese, diabetic, severe combined immunodeficient (NOD/SCID) mice (Pilarski et al, 2000, 2002, Matsui et al, 2004).

Subsequently, the findings of Matsui et al (Matsui 2004) raised the possibility that clonogenic MM cells lacked expression of CD138. These authors identified small populations (2-5%) of CD138- cells in the H929 and RPMI8226 MM cell lines and found that these CD138- cells showed increased expression of CD19, CD20 and Ki67
relative to the CD138+ fraction. They went on to show that CD138- cells underwent significantly greater clonal expansion than CD138+ cells during serial re-plating experiments and further, that CD138+/CD34- MM patient cells were unable to form colonies unlike CD138-/CD34- cells that were shown to generate colonies containing mature plasma cells. In addition, CD138-/CD34- cells from a single MM patient were injected intravenously into NOD/SCID mice and found to result in engraftment as shown by the presence of light-chain restricted, CD138+ cells in the murine BM. In contrast, the injection of CD138+ cells did not result in engraftment. Importantly, depletion of cells expressing CD45, CD22 and CD19 significantly reduced the growth of MM colonies and furthermore that pre-treatment of MM BM with rituximab led to a significant reduction in MM colony formation, suggesting that MM progenitors have a B-cell phenotype (Matsui et al, 2004). In a follow-up study from the same group it was shown that the CD138- cells from the RPMI8226 and H929 cell lines displayed greater drug resistance than CD138+ cells (Matsui et al, 2008).

However, more recently, evidence has accumulated that suggests CD138+ MM cells are capable of colony formation and display CSC characteristics. Yata et al (2004) injected CD138-selected cells from MM patients directly into rabbit bones implanted in SCID mice (SCID-rab model) and found successful engraftment of such cells in 81% of cases along with MM disease characteristics such as paraproteinaemia and lytic bone disease. Other researchers have sought to characterise the MM “side population” (SP) cells. SP cells demonstrate a distinctive low-staining pattern with the Hoechst 33342 DNA-binding dye that is effluxed via the ABCG2/BCRP transporter, and have been shown to be drug-resistant, tumorigenic and capable of differentiation (Ho et al, 2007, Li et al, 2007). Jakubikova et al (2011) sought to fully characterise MM SP cells and working
with 18 MM cell lines, these authors identified 3 populations of MM cells based on CD138 expression (CD138+, CD138low+ and CD138-) and found SP cells to be predominantly found amongst CD138+ or CD138low+ MM cells. However, within primary MM samples SP cells were found to be heterogeneous with regard to CD138-expression. When compared with main population cells, SP cells were found to have a higher rate of proliferation, a significantly increased rate of colony formation, enhanced tumorigenic potential in an immunodeficient murine model and led to repopulation of the main population cell bulk during in-vitro culture. In keeping with these findings, it was subsequently shown that purified CD138+ cells from patients with plasma cell leukaemia (PCL) were capable of colony formation, in contrast to the CD138- cells, which were not (Chiron et al, 2011). Another group purified both CD138+ and CD138- cells from 5T33MM immunocompetent mice, and found that the CD138- cells, accounting for 6-15% of cells, showed greater expression of Bcl-6 in contrast to the CD138+ cells which showed greater expression of plasma cell-associated transcription factors XBP-1, IRF-4 and BLIMP-1 (Van Valckenborgh et al, 2012). However, whilst CD138+ cells showed greater colony-forming potential than CD138- cells, both CD138+ and CD138- cells were found to be tumorigenic in vivo. CD138- cells showed slower engraftment but were also found to be significantly more resistant to bortezomib treatment.

Thus, there is evidence from several groups that small populations of MM cells possess some CSC characteristics but the phenotype and the functional relevance of these cells remains to be fully defined. Divergent results have emerged from different studies, however this may be due to differences in culture methods, animal models and the heterogeneity of primary MM samples. With studies showing evidence for the
tumorigenic and colony-forming potential of both CD138+ and CD138- MM cells as well as MM cells with and without a B-cell phenotype (CD20, CD27), it would appear that future attempts to further characterise these cells will not be based on cell surface immunophenotype alone. In fact, there is now emerging evidence of “plasticity” amongst CSCs that might also give some insight into why divergent results have been achieved by different groups (Matsui et al, 2012, He et al, 2011).

This section has outlined the phenotype of normal and malignant plasma cells. The next section will focus on the dysregulation of the cell cycle in MM and the other factors that permit cell cycle progression and proliferation of MM cells.

1.3 Cell cycle dysregulation and a genetic classification in multiple myeloma

1.3.1 Background: Cell-cycle control

The mammalian cell cycle is tightly regulated through “checkpoints” to ensure that the cell enters S-phase undamaged. The transition between G1 and S-phase is regulated by the interaction between the major G1 cyclins – the D-type cyclins and the cyclin-dependent kinases (CDKs). D-type cyclins (Cyclin D1, D2 and D3) control entry to the cell cycle and are usually induced in response to mitogens i.e. micro-environmental cytokines. D-type cyclins then bind to and activate CDK-4 and -6 and these complexes phosphorylate retinoblastoma protein (phospho-pRb), thus allowing S-phase entry by releasing the E2F transcription factors which regulate genes (Cyclin E, Cyclin A2) controlling DNA synthesis (Sherr et al, 1995, Dyson et al, 1998, Grana et al, 1998, see Figure 1.1). The interaction between CDK2 and cyclin E also contributes to G1-S progression by phosphorylating pRb and the cyclin E/CDK2 complex is activated once the cyclin D-CDK4/6 complexes bind p21Cip1 and p27Kip1 (Cheng et al, 1999, Meloche et al, 2007). G1-S progression is subject to further regulation by the cyclin-kinase
inhibitor proteins (CKIs), which inhibit the actions of the CDKs. The INK4 family of proteins (p15\textsuperscript{INK4a}, p16\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, p19\textsuperscript{INK4d}) negatively regulates the effects of the cyclin-D/CDK complexes, whilst the WAF/CIP family of CKIs (p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}) regulates the late G1 phase actions of cyclin-E and CDK2 (Sherr et al, 1995).

In contrast, CKIs may also act as positive cell cycle regulators by stabilizing the cyclin-CDK complexes (Sherr et al, 1999).

**Figure 1.1**

**Figure 1.1: Control of G1-to-S phase cell cycle progression.** In early G1, D-type cyclins are induced in response to mitogens to bind to and activate CDKs 4/6. These complexes then phosphorylate pRb allowing S-phase entry through the release of E2F transcription factors. The INK4 and WAF/CIP families of CKIs inhibit the action of the CDKs as shown (adapted from “Biology of plasma cells” by Selina Chen Kiang, Best Practice and Research Clinical Haematology, Volume 18, Issue 4, pages 493-507, December 2005).
1.3.2 Cell cycle dysregulation in multiple myeloma

MM is a tumour with a low proliferative rate, thus it was surprising when data from gene-expression profiling experiments involving 2 large groups of MM patients showed that D-type cyclin expression was aberrantly increased in nearly all cases of MM (Bergsagel et al, 2005a, Zhan et al, 2006). These findings are partially explained by the fact that one of 5 recurrent translocations involving the IgH locus on 14q32 (IgH/TCs) is found in 40-50% of MM cases (Kuehl et al, 2005). It is thought that these errors occur during IgH switch recombination during B-cell maturation in germinal centres (Bergsagel et al, 2003). These translocations result in the dysregulation or increased expression of a D-type cyclin gene that is positioned near one of the strong immunoglobulin enhancers. The partner genes in these translocations include the cyclin D1 or D3 genes (11q13 or 6p21 respectively), those encoding the c-Maf (16q23) and B-Maf (20q11) transcription factors that target the cyclin D2 gene, or FGFR3/MMSET (4p16), also targeting the cyclin D2 gene via an unknown mechanism. More recently, translocations involving the cyclin D2 gene on chromosome 12p13 and the MAF-A locus on chromosome 8q24.3 have been described, suggesting further mechanisms may exist that contribute to the overexpression of D-type cyclins (Chng et al, 2007). A schematic representation of how these IgH/TCs increase D-type cyclin expression and subsequently how they might promote cell cycle progression in the G1 phase is shown in Figure 1.2. In clinical practice, these TCs are detected by fluorescence in-situ hybridization (FISH) and have been shown to increase in prevalence with advancing disease stage, with rates of 85% in plasma cell leukaemia (PCL). Because these mechanisms are not a feature of normal plasma cell biology, it has been widely suggested that these translocations represent tumour-initiating or oncogenic events in
normal B cells as they pass through the germinal centres of lymph node tissue (Kuehl et al, 2005).

Figure 1.2

![Diagram of G1-S progression in myeloma](image)

**Figure 1.2: Impact of different TC groups on G1-S progression in myeloma.** In MM, one of the 3 D-type cyclins is dyregulated via a translocation involving the IgH locus on chromosome 14 or through an unknown mechanism in the presence of hyperdiploidy. D-type cyclins form complexes with CDK4 and -6 to phosphorylate retinoblastoma protein, thus allowing the release of E2F transcription factors and progression through to S-phase. (From “Early genetic events provide the basis for a clinical classification of multiple myeloma” Kuehl WM, Bergsagel PL. Hematology Am Soc Hematol Educ Program. 2005:346-52)

1.3.3 Functional consequences of IgH/TCs in myeloma cells

Whether these IgH translocations are necessary for progression of MM has not been fully resolved, although there is now increasing evidence that this may be the case. For example, when KMS12BM MM cells were transfected to overexpress c-Maf, this led to
increased expression of the cyclin D2 and β-7-integrin genes suggesting a mechanism whereby overexpression of c-Maf leads to increased MM cell proliferation and also alters integrin-mediated interactions between MM cells and microenvironmental factors (Hurt et al., 2004). These findings were recently confirmed in a transgenic murine model of MM with t(14;16), where increased expression of the cyclin D2 and β-7-integrin genes was observed (Morito et al., 2011). In addition, SiRNA-mediated knockdown of the cyclin D1 gene lead to cell cycle arrest in KMS12BM cells which carry a t(11;14) translocation, providing evidence that the cyclin D1 protein plays an important functional role in MM cells with this translocation (Glassford et al., 2007). It has also been shown in transgenic mice that cyclin D1 overexpression does not lead to increased proliferation or to the development of lymphoid tumours suggesting that cooperation between cyclin D1 and other factors such as Myc or Ras may be necessary to drive oncogenesis (Lesage et al., 2005). In keeping with this, the percentage of S-phase cells amongst those MM cells expressing t(11;14) was lower than that of other MM cells, again suggesting that cyclin D1 overexpression in isolation is insufficient to stimulate proliferation, and furthermore that MM cells with t(11;14) may constitute a biologically defined subset of MM cases (Fonseca et al., 2002). There is also evidence that cyclin D1 plays a role beyond its key part in the G1 phase of the cell cycle, by regulating the transcription of STAT-3 and thyroid receptors (Lesage et al., 2005). From a molecular perspective, the translocation breakpoints in MM cases of t(11;14) and t(14;16) are found in the switch region near the powerful 3′ enhancer, or alternatively near the Variable, Diversity, Joining (VDJ) region of the IgH locus. In contrast, the translocation breakpoints in t(4;14) cases are only found near switch regions (Bergsagel et al., 2001, Chng et al., 2007).
The functional consequences of the t(4;14) translocation, which result in the overexpression of the Multiple Myeloma SET domain (MMSET) protein have, until recently, been poorly understood. Found in approximately 15% of MM cases, this translocation results in fusion of the MMSET and Fibroblast Growth Factor Receptor-3 (FGFR3) genes resulting in their overexpression. However, in 30% of cases with t(4;14), MMSET overexpression is found without increased expression of FGFR3, suggesting a pivotal role for the MMSET gene in MM pathogenesis. Furthermore, a recent study demonstrated an epigenetic role for MMSET by showing that MMSET functions as a histone methyltransferase. In this study, loss of MMSET was shown to result in apoptosis of MM cells as well as alterations in their interaction in the BM microenvironment (Martinez-Garcia et al., 2011). Furthermore, it has also been shown that MMSET accumulates at the site of DNA damage and that knockdown of MMSET resulted in enhanced sensitivity to radiation, which may explain to some degree why MM patients with t(4;14) are less responsive to alkylator therapy (Pei et al., 2011). On the other hand, the importance of FGFR3 in MM tumour initiation is emphasised by a study which showed that FFGFR3 cooperated with MYC to promote B-cell tumour development in mice over-expressing both FGFR3 and MYC (Zingone et al., 2010).

Owing to their rarity, the functional consequences of the t(14;20) and t(6;14) translocations are less well studied. One study identified target genes of maf-B upregulation in the presence of t(14;20) and found that several anti-apoptotic genes were upregulated along with the NOTCH2 gene (Van Stralen et al., 2009). The functional effects of the t(6;14) translocation which leads to cyclin D3 over-expression have not been extensively studied.
1.3.4 The translocation/cyclin-D (TC) classification system

The demonstration of recurrent genetic abnormalities in MM cells by several groups of researchers led to attempts to correlate these genetic aberrations with prognosis and response to anti-MM therapies. The almost universal finding of increased D-type cyclin expression in MM led to the establishment of the TC (Translocation/Cyclin) classification (Bergsagel et al., 2005b, see Table 1.2). Combining gene expression profiling data from two independent groups, this classification system was based on the over-expression of D-type cyclin genes in purified plasma cells from 231 untreated and 30 relapsed MM cases, in comparison with the expression of D-type cyclin genes in normal BM plasma cells. The patterns of D-type cyclin overexpression permitted the division of the MM cases into 8 groups, based on the overexpression of one of the 3 D-type cyclins with or without an IgH/TC. Those with an IgH/TC were designated 4p16, 11q13, 6p21 and Maf, and those with increased expression of a D-type cyclin gene in the absence of an IgH/TC were named D1, D2, and those with co-expression of D1 and D2; D1+D2, with a final group, accounting for 1% of cases, comprising MM cases where there is no increased expression of D-type cyclin genes (see Table 1.2). The greatest expression of cyclin D1 was observed in the 11q13 group, with the highest expression of cyclin D2 observed in the maf group, whilst the overexpression of cyclin D3 was not seen outside the 6p21 group. In a further 1% of cases the BM plasma cells had a polyclonal signature. Overall, in this study, IgH/TCs were found in approximately 40% of MM cases and this group was also characterized by the absence of a hyperdiploid karyotype. In contrast, the MM cases lacking an IgH/TC displayed features of hyperdiploidy, usually with trisomies of the odd chromosome numbers (i.e. 3, 5, 7, 9, 11, 15, 19, 21 see Figure 1.3). Overall, the authors of this study proposed a model (see Figure 1.2) of MM disease initiation and progression, with the
overexpression of a D-type cyclin gene as the key “oncogenic” event. In this hypothesis, upregulation of a D-type cyclin promotes cell cycle progression by forming complexes with CDK-4/6 that can phosphorylate pRb permitting S-phase entry and ultimately MM cell proliferation (Kuehl et al., 2005).

Table 1.2 The translocation/cyclin-D (TC) classification system and clinical correlates

<table>
<thead>
<tr>
<th>Group</th>
<th>Translocation</th>
<th>Gene</th>
<th>Cyclin</th>
<th>Ploidy</th>
<th>Proliferation</th>
<th>Bone Disease</th>
<th>Frequency</th>
<th>Prognosis</th>
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</thead>
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<tr>
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<td>Good</td>
</tr>
<tr>
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<td>11q13</td>
<td>CCND1</td>
<td>D1</td>
<td>D = NH</td>
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<td>16</td>
<td>Good</td>
</tr>
<tr>
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<td>Good</td>
</tr>
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<td>None</td>
<td>D1 and D2</td>
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<td>2</td>
<td>? Good</td>
</tr>
<tr>
<td>4p16</td>
<td>4p16</td>
<td>FGFR3/MMSET</td>
<td>D2</td>
<td>NH &gt; H</td>
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<tr>
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<td>c-Maf/mafB</td>
<td>D2</td>
<td>NH</td>
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<td>55</td>
<td>5</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Key: H=hyperdiploidy, NH=non-hyperdiploidy, D=diploid, Bone disease=% with bone disease on magnetic resonance imaging (MRI). Adapted from “Early genetic events provide the basis for a clinical classification of multiple myeloma” Kuehl WM, Bergsagel PL. Hematology Am Soc Hematol Educ Program. 2005:346-52
1.3.5 Clinical associations of the TC groups

Within the patient group on which the TC classification was based, the presence or absence of lytic bone disease was determined by magnetic resonance imaging (MRI) of 172 untreated patients. Although present in 79% of cases overall, the rate of lytic bony disease was significantly lower in the maf and 4p16 groups (57% and 55% respectively, p<0.05 both). In contrast, lytic bone disease was more prevalent in the 11q13 group (94%) and the D1 group (86%), although these differences did not reach statistical significance. In this study, a proliferation index (PI) was calculated based on the median expression values of 12 genes known to be associated with proliferation (TYMS, TK1, CCNB1, MKI67, KIAA101, KIAA0186, CKS1B, TOP2A, UBE2C, ZWINT, TRIP13, KIF11). This expression based PI had previously been shown to correlate strongly (r=0.73) with the plasma cell labeling index. Within the TC groups, a significantly lower PI was found within the D1 group, a significantly higher PI within the maf group and also the D1+D2 group. However, overall there was no correlation between D-type cyclin expression and PI. The expression of D-type cyclins within 12 MGUS cases and 32 HMCLs was also determined as part of this study. Within the limited number of MGUS cases, 5 of the TC groups were represented with no cases of 4p16, 6p21 or none (no D-type cyclin) found. Unsurprisingly, the maf and 4p16 groups were over-represented within the HMCLs being found in 31% and 28% respectively (Bergsagel et al, 2005b). In fact more recent work from our laboratory reinforces the current evidence that different patterns of MM bone disease may exist in MM patients with different IgH/TCs. Two separate murine models of MM bone disease, one bearing MM1S cells with t(14;16) and the other bearing KMS12BM cells with t(11;14) showed significantly different patterns in bone disease with little evidence of osteolytic disease found in the MM1S murine model (Kassen et al, 2010).
Figure 1.3: Genetic classification of multiple myeloma. Based on metaphase cytogenetics, MM patients can be divided into 2 broad categories with a small degree of overlap; those with hyperdiploidy (HD, 48-74 chromosomes) and those without hyperdiploidy (nHD). The nHD group is characterized by a high frequency of IgH/TCs in contrast to the HD group where IgH/TCs are not usually present. The HD group is characterized by the presence of multiple trisomies as shown.

1.3.6 University of Arkansas (UAMS) classification of MM

Another classification system, which closely resembles the TC classification system subdivides MM cases into 7 groups (Zhan et al, 2006).

(i) MMSET/FGFR3 (defined by the overexpression of either of these genes),
(ii) MF - Characterized by upregulation of either of the MAF genes
(iii) CD-1 - consisting of MM cases with upregulation of either t(6;14) or t(11;14)
(iv) CD-2 - consisting of similar MM cases to the CD1 group but with associated expression of CD20
This classification system was recently partially validated by an independent study which also confirmed the existence of six out of the seven patient “clusters” apart from the “low-bone disease” cluster which could not be confirmed (Broyl et al, 2010). This more recent study also identified two additional groups: one characterized by the overexpression of positive regulators of the NFκB pathway and another identified by the overexpression of cancer testis antigen genes. The UAMS classification has prognostic significance, with those patients in the groups overexpressing MMSET/FGFR3 and MAF having a poorer outcome in contrast to those patients in the CD1 and CD2 groups who have a better or neutral prognosis (Zhan et al, 2006).

1.3.7 Response to anti-myeloma treatment according to IgH/TC

Taken together, these classification systems confirm that marked heterogeneity exists amongst MM cases at the molecular level, but that it is also possible to define different MM subgroups that may have different biological characteristics. The heterogeneity observed during gene expression profiling experiments is underscored by the differential responses to some anti-myeloma therapies amongst MM patients baring different IgH/TCs. For example, amongst MM patients undergoing high-dose chemotherapy and autologous stem-cell transplantation, the t(4;14) translocation was associated with a reduced overall survival, in contrast to those with t(11;14) who fared better (Moreau et al, 2002, Gertz et al, 2005). Other studies have also noted the poor outcome in those MM cases with t(4;14) with one study suggesting that, although these patients respond rapidly to treatment, they suffer relapse quickly and display resistance
to alkylator treatment (Jaksic et al, 2005, Cavo et al, 2006). In a recent study, which compared the proteasome inhibitor bortezomib and dexamethasone (BzD) with vincristine-adriamycin-dexamethasone (VAD) as induction therapy for MM patients with de novo disease, the outcome for patients carrying t(4;14) was significantly better for those patients treated with BzD compared with those treated VAD. These results suggest that patients with t(4;14) may be more sensitive to effects of proteasome inhibition, although in this study, the presence of t(4;14) still remained a poor prognostic factor even in those patients treated with BzD (Avet-Loiseau et al, 2010).

The presence of t(14;16) in de novo MM has also been associated with a poorer outcome whether detected by metaphase cytogenetics or FISH analysis (Dewald et al, 2005, Chiecchio et al, 2006). However, a more recent retrospective study of more than one thousand patients questions the prognostic significance of this IgH/TC, whilst acknowledging important differences in anti-MM treatment between the different studies (Avet-Loiseau et al, 2011). An analysis of MM patients with t(14;20) confirmed that this IgH/TC is also associated with a worse outcome suggesting that overexpression of a maf gene is a negative prognostic indicator (Ross et al, 2010). It is not yet known if the poor outcome associated with Maf overexpression can be overcome with novel treatments.

The vast majority of MM patients without IgH/TCs are found to have a hyperdiploid karyotype (Figure 1.3) associated with overexpression of either cyclins D1, D2 or D3 through an unknown mechanism. By gene expression profiling the expression of D-type cyclins in the context of hyperdiploidy is lower than that which occurs in the setting of an IgH/TC (Bergsagel et al, 2005b). Overall, the finding of hyperdiploidy has been shown to be associated with a better outcome, with hypodiploidy shown to be a poor
prognostic factor (Smadja et al, 2001). For example cyclin D2 expression in the absence of an IgH/TC has been shown in one analysis to confer a neutral prognosis (Hanamura et al, 2006). However, the hyperdiploid group has been shown to display considerable heterogeneity with one group detecting 4 distinct hyperdiploid subgroups based on gene expression profiling, including one group with a more proliferative profile that was found to overexpress cancer testis antigen genes and to have a poorer outcome (Chng et al, 2007). In isolation, overexpression of cyclin D1 has been associated with a better prognosis but it is evident that there is heterogeneity within this group (Cook et al, 2006).

Overall, when the gene expression profiling data (TC classification and UAMS classification) is viewed in the context of the distinct clinical features and the varying responses to anti-myeloma treatments, there is convincing evidence that the sub-groups displaying differing D-type cyclin overexpression via multiple mechanisms provide a basis for the biological and clinical heterogeneity observed in MM patients.

1.3.8 Dysregulation of cyclin kinase inhibitors in myeloma

Although much focus has been placed on the increased expression of the “positive” regulators of the cell cycle i.e. D-type cyclins in MM, a parallel examination of the functional role of the “negative “ cell cycle regulators in MM has also taken place. Several studies have highlighted dysregulation in the genes encoding CKIs as well these abnormalities are outlined below.

**p18 (INK4c):** Deletions of chromosome 1p are found in up to 40% of MM cases. Importantly the CDKN2C gene encoding p18, a member of the INK family of cyclin kinase inhibitors, is found at 1p32.3 and is a possible target of this deletion. A recent
study, using single nucleotide polymorphism (SNP) analysis, found that the CDKN2C gene was deleted in 4.5% MGUS cases and 15% of MM cases. Furthermore, deletion of CDKN2C was associated with an adverse outcome, suggesting that absence of p18 is an important factor in inhibiting cell cycle progression in MM cells (Leone et al., 2008). In addition, biallelic deletion of p18 is found in approximately 40% of HMCLs suggesting that this finding is associated with disease progression and increasing proliferative potential (Kulkarni et al., 2002). However, a separate study found a paradoxical increase in p18 expression in 60% of MM cases with a high proliferation index, perhaps suggesting that some MM cases may become less responsive to the anti-proliferative effects of p18 (Dib et al., 2006).

In addition, inactivation of the p16 (INK4a/CDKN2A) gene, which is found at chromosome 9p21, has been described in several solid malignancies. Furthermore, the gene that encode the Alternative Reading Frame (ARF) tumour suppressor protein is also transcribed from the p16/INK4a locus. Although the ARF and p16 genes have different first exons, they have identical second and third exons and encode entirely different proteins (Kim et al., 2006). ARF is upregulated in response to mitogenic stimulation and has been shown to act as a tumour suppressor protein by binding to and inactivating the MDM2 (Mouse double minute 2 homolog) protein which then leads to p53-mediated cell cycle arrest (Chene et al., 2003). Gonzalez-Paz et al (2007) recently found the p16 gene to be methylated in approximately 40% of MM cases whilst deletion of p16 was found in just 10% of cases. However, p16 methylation was not associated with a statistically significant adverse clinical outcome. Despite this, and although Taniguchi et al (1999) did not observe loss of ARF expression in myeloma patients, it remains possible that p16/INK4a inactivation may render MM cells more susceptible to
proliferative stimuli as result of dysregulated expression of ARF and ultimately leading to cell cycle progression (Taniguchi et al, 1999).

**p27 and p21 (p27Kip1, p21Waf/Cip1):** Both proteins are members of the families of CKIs that interact with complexes containing cyclins D, E and A and regulate CDK activity during G1-S phase transition (Figure 1.1). One study showed an inverse relationship between p27 expression and survival in MM patients, suggesting that low p27 expression is an independent poor prognostic factor. This finding would suggest that p27 expression is important, perhaps for inhibiting G1-S progression in MM. However, in the same study no correlation was found between Ki67 expression and p27 expression, suggesting that increased p27 expression alone is insufficient to inhibit G1-S progression, or that p27 may play other biological roles apart from cell cycle regulation in MM cells (Filipits et al, 2003). In fact, the role of p27 may depend on the cellular context of individual MM cells within the BM microenvironment. For example, the adhesion of MM cells to extracellular matrix proteins such as fibronectin induces cell adhesion mediated drug resistance (CAM-DR) and such adhesion has been shown to increase p27 expression, suggesting that the induction of cell cycle quiescence reduces drug sensitivity (Damiano et al, 1999, Hazlehurst et al, 2000). The poor overall survival of MM patients with 17pdel or p53 mutations suggests an important role for p21 in MM cells, as the expression of this protein is regulated by p53 expression. A recent study has shown a functional role for p21 in inhibiting G1-S progression in the setting of a novel anti-MM agent (galectin-3 antagonist) whereas p27 expression was unaltered (Streetly et al, 2010). Induction of p21 expression has also been observed in the setting of treatment of MM cells with histone-deacetylase inhibitors (HDACi)
suggesting an important role for p21 in inhibiting MM cell proliferation (Mandl-Weber et al., 2010).

1.3.9 1q gain/CKS1B overexpression

Amplification of the chromosomal region 1q21 (1q21 gain) is found in approximately 30-50% of newly diagnosed MM cases and has been shown to be a poor prognostic factor (Zhan et al., 2007). Importantly, 1q21 gain is not seen in MGUS and but is present in a proportion of smouldering MM cases where it is a risk factor for early disease progression (Hanamura et al., 2006). The CKS1B gene encodes the “Cyclin-dependent kinases regulatory subunit 1 protein” that is a member of the Cks/Suc1 family of proteins that bind to the catalytic subunit of CDKs and regulates their function whilst also playing a role in protein catabolism as an accessory protein for the Skp-Cul-F-box protein-SKP2 (SCF^{Skp2}) ubiquitin ligase. It has been suggested that increased expression of CKS1B may accelerate the degradation of p27, thus reducing negative regulation of the G1-S phase and overall promoting cell cycle progression. Consistent with this hypothesis, it has recently been shown that knockdown of CKS1B in 3 different HMCLs led to stabilization of the p27 protein, cell cycle arrest and apoptosis (Hanamura et al., 2006).

MM is characterized by a wide array of mutations in the genes encoding both positive and negative regulators of the cell cycle. Crucially, D-type cyclins are upregulated in almost all MM patients and different patterns of D-type cyclin overexpression have been shown to correlate with different clinical characteristics and outcome. The dysregulated cell cycle “machinery” within MM cells creates a platform for MM cells to survive and proliferate and the next section will examine the role of the BM
microenvironment in MM and specifically how BM microenvironmental factors interact with MM cells to promote their growth and survival of MM.

1.4 The role of the bone marrow microenvironment in MM

1.4.1 Background

There is now extensive evidence that BM microenvironment plays a critical role in promoting the growth, survival and drug resistance of MM cells (Hideshima et al, 2007, Podar et al, 2009). The MM BM microenvironment consists of a complex interplay between MM cells and BM cytokines (soluble factors), cellular factors and extracellular matrix proteins (see Figure 1.4). The key cytokines known to promote MM cell survival include interleukin-6 (IL-6), Insulin-like growth factor-I (IGF-I), Vascular endothelial growth factor (VEGF), B-cell activating factor (BAFF), A Proliferation-Inducing Ligand (APRIL), hepatocyte growth factor (HGF) and CXCL-12. The most important cellular factors in this process are osteoclasts, osteoblasts, bone marrow stromal cells and cells with an immune role such as plasmacytoid dendritic cells whereas the key extracellular matrix proteins involved are fibronectin and collagen. The interaction between these factors and MM cells occurs via autocrine and paracrine loops and leads not only to the growth and survival of MM cells but also to the development of both cytokine-mediated drug resistance (CM-DR) and cell-adhesion mediated drug resistance (CAM-DR). The expression of adhesion molecules by MM cells such as integrins, syndecans and CXCR4 have been shown to be important in CAM-DR in particular (Katz et al, 2010). The multiple interactions within the MM microenvironment are also believed to be important in the pathogenesis of myeloma bone disease, stimulating osteoclastic activity and inhibiting osteoblast activity. As multiple BM microenvironmental factors promoting MM cell growth have been described, the following section will focus only on the microenvironmental factors that to date have
been shown to be the most critical in MM cell growth and survival. The key BM microenvironment components and their interactions are depicted in Figure 1.4.

1.4.2 Role of cytokines in the myeloma bone marrow microenvironment

The following soluble factors have been shown to be particularly important in the pathogenesis of MM on the basis of multiple in vitro studies demonstrating their abilities to promote MM cell survival. In addition, several studies exist showing increased levels of these cytokines in MM patients’ sera, along with expression of cytokine receptors by MM cells. Furthermore, blocking the actions of these cytokines has been shown to have an anti-MM effect in vitro and in some cases, in vivo.

Figure 1.4

Figure 1.4: The multiple myeloma bone marrow microenvironment. The key interactions in the bone marrow microenvironment in multiple myeloma: Paracrine and autocrine loops involving MM cells, osteoclasts, osteoblast, BM stromal cells and growth factors: IL-6, IGF-I, BAFF and APRIL protect MM cells from apoptosis and promote drug resistance.
IL-6: Often regarded as the most important MM growth factor, IL-6 was one of the first myeloma growth factors described and since then many studies have confirmed a key role for IL-6 in MM pathogenesis (Klein et al, 1995). IL-6 binds to a specific receptor (IL-6R) and this interaction leads to the homodimerisation of the gp130/IL-6 transducer (Nishimoto et al, 1994). The role of IL-6 as a MM growth factor is emphasised by the development of many HMCLs that are IL-6 dependent and IL-6 deficient mice were shown not to develop MM (Zhang et al, 1994, Hilbert et al, 1995). Although initially shown to be produced in an autocrine fashion, IL-6 has also been shown to be produced in a paracrine fashion by monocytes and bone marrow stromal cells (Klein et al, 1989, Chaughan et al, 1996). IL-6 has been shown to promote the survival and growth of MM cells by activating the Januse Kinase and signal transducers and activation of transcription 3 (JAK/STAT3), extracellular signal-related kinase 1/2 (ERK 1/2) and Phosphatidyl-inositol-3-kinase/AKT (PI3K/AKT) signalling pathways (Hideshima et al, 2001, Brocke-Heidrich et al, 2004, Ogata et al, 1997). IL-6 also inhibits dexamethasone-induced apoptosis in MM cells in a dose dependent-fashion and interacts with fibronectin in the BM microenvironment to promote drug-resistance (Hardin et al, 1994, Shain et al, 2009).

From a clinical perspective, it has been shown that MM patients have elevated serum levels of IL-6 and IL-6R, which are features associated with a poor prognosis (Bataille et al, 1989, Pulkki et al, 1996). Given its prominent role in MM pathogenesis, much effort as been directed at targeting IL-6 as an anti-MM strategy. Although anti-IL-6 antibodies have been shown to inhibit myeloma cell proliferation in-vitro, and also in a MM patient with end-stage disease, a significant anti-MM effect was not seen in early phase clinical trials in MM patients (Klein et al, 1991, Trikha et al, 2003). Nevertheless,
this cytokine remains an attractive target, and a recent study has suggested that the anti-IL-6R monoclonal antibody, silituximab, may enhance melphalan-induced cytotoxicity in MM cells (Hunsucker et al, 2011). Intriguingly, it has also been shown that myeloid engraftment following high-dose melphalan and autologous stem cell transplantation results in the increased concentration of IL-6 in MM bone marrow microenvironment, which may promote the survival of drug resistant MM cells, thus presenting a possible therapeutic window. Accordingly, treating patients with IL-6 blocking agents is currently being investigated in this setting (Condomines et al, 2010).

**IGF-I:** Along with IL-6, IGF-I is felt to be one of the two most critical MM growth factors. In fact, it has recently been shown that IGF-I is perhaps the most important growth factor for HMCLs, as IGF-I stimulation of HMCLs led to a growth effect in all HMCLs treated, in contrast to cytokines such as APRIL and HGF which induced a growth response in less than one third of cell lines treated (Sprynski et al, 2009). Furthermore, recent work from our laboratory has shown that IGF-I promotes cell cycle progression in HMCLs and MM cells bearing t(4;14) and t(4;16) with increased expression of cyclin D2 protein (Glassford et al, 2007). IGF-I predominantly activates both the PI3K/AKT and MAPK signalling pathways but would appear not to activate the JAK/STAT pathway, in contrast to IL-6 (Qiang et al, 2002). Activation of the NFkB pathway by IGF-I in MM cells has been demonstrated in one study to date (Mitsiades et al, 2002). IGF-I attenuates dexamethasone-induced apoptosis in HCMLs and cooperates with IL-6 to enhance the growth and survival of MM cells (Xu et al, 1997, Abroun et al, 2004).

In MM patients, expression of the IGF-I receptor (IGF-IR) is found in 30-50% of cases and is associated with a poor prognosis, particularly when found to co-exist with the
t(4;14) translocation in MM patients (Sprynski et al, 2009). Furthermore, although the mean serum level of IGF-I was not found to be elevated in the sera of MM patients when compared with controls, elevated individual IGF-I serum levels in MM patients were found to be predictive of a poor outcome (Standal et al, 2002). The abundant in-vitro evidence demonstrating a key role for IGF-I in promoting MM cell growth and survival has prompted investigators to attempt to block its actions in vivo. To date, a small molecule IGF-IR tyrosine kinase inhibitor (NVP-ADW742) has shown promising pre-clinical activity and the results of clinical studies are awaited (Mitsiades et al, 2004).

VEGF: There is strong evidence that angiogenesis plays an important role in the growth and survival of MM cells (Hose et al, 2009, Rajkumar et al, 2001). In fact, angiogenesis appears to be associated with disease activity as one study showed that BM angiogenesis was correlated with plasma cell labeling index (PCLI), whilst another showed that increased serum levels of VEGF portended a poor prognosis when combined with HGF levels in MM patients (Vacca et al, 1994, Iwasaki et al, 2003). In the MM BM microenvironment VEGF is produced not only by bone marrow stromal cell (BMSCs) but also by MM cells. VEGF has been shown to interact with several other BM factors such as IGF-I, IL-6, stromal derived factor-1 (SDF-1) and fibronectin (FN) in paracrine loops to promote MM cell survival, as well as attenuating MM cell apoptosis through increased expression of Mcl-1 in MM cells (Gupta et al, 2001, Dankbar et al, 2000, Le Gouill et al, 2004).

Hepatocyte Growth Factor (HGF): Like VEGF, HGF is secreted in the MM BM microenvironment by BM stromal cells and also by MM cells (Takai et al, 1997, Borset et al, 1996). In addition, HGF is found at increased concentrations in the sera of MM
patients with the highest levels being associated with a poor prognosis (Seidel et al., 1998). HGF mediates its growth factor activity by signalling through its receptor c-Met and it has recently been shown that HGF co-operates with IL-6 to promote MM cell growth (Hov et al., 2009). In addition, HGF also has a growth-promoting effect on BM stromal cells and has also been shown to stimulate the migration of MM cells (Holt et al., 2008). Importantly, HGF activity also appears to rely to some degree on syndecan-1 (CD138) to signal through c-Met and it is possible that this interaction may be inhibited by heparitinase which promotes syndecan-1 shedding thus offering a potential therapeutic target (Seidel et al., 2000).

In addition to the cytokines discussed above, multiple other cytokines have been shown to be present at increased concentrations in the MM bone marrow microenvironment and to promote the survival of MM cells such as TNFα, IL-4, IL-10, Transforming Growth Factor-β (TGF-β) and Fibroblast Growth Factor (FGF) but it is beyond the scope of this thesis to discuss all of them in detail (Klein et al., 2011). Establishing a hierarchy of MM cytokines is difficult but IGF-I and IL-6 are thought to be among the most critical in promoting MM pathogenesis. The TNF superfamily members BAFF and APRIL will be discussed in a subsequent section.

1.4.3 Role of cellular factors in the myeloma bone marrow microenvironment

Interactions between MM cells and the other cellular components of the MM bone marrow microenvironment have been increasingly shown to be critical in sustaining MM cell survival, promoting drug resistance and in the case of MM cell-osteoclast interaction promoting MM bone disease.

**Osteoclasts:** Osteoclast-MM cell interactions have been shown to be crucial in MM pathogenesis. For example, co-culturing primary MM cells with osteoclast precursors
has been shown on one hand to promote osteoclast differentiation to multinucleated cells, whilst in turn the direct contact between MM cells and osteoclasts significantly improved the viability of MM cells in comparison with control samples as well as promoting cell cycle progression as shown by increased uptake of bromodeoxyuridine (BrdU) in the MM cells (Yaccoby et al, 2004). A more recent study focusing on the expression of growth factors by osteoclasts from MM patients has shown that these osteoclasts express APRIL, IGF-I and IL-10 in large amounts but show lesser expression of BAFF and IL-6, whilst also showing that MM cell-osteoclast co-culture dramatically increased MM cell viability in comparison with the effects of IL-6. Furthermore, this study demonstrated an important role for the chemo-attractant receptor chemokine C-C motif receptor 2 (CCR2) as an anti-CCR2 monoclonal antibody blocked osteoclast chemo-attractant activity for MM cells (Moreaux et al, 2011). The importance of osteoclast activity in MM is underscored by the results of a recently published large randomized trial which demonstrated that treatment of MM patients with the bisphosphonate zoledronic acid led to improved overall survival (Morgan et al, 2010).

**Bone Marrow Stromal Cells:** Multiple studies have confirmed that co-culture of MM cells with bone marrow stromal cells (BMSCs) promotes survival and, in particular, drug resistance in MM cells (Klein et al, 2011). Drug resistance may be secondary to the autocrine and paracrine secretion of growth factors such as IL-6, IGF-I and VEGF that are stimulated by the MM cell-BMSC interaction (Abe et al, 2011). Alternatively, direct contact between MM cells and BMSCs via “very late antigen-4 or -5” (VLA-4 or VLA-5) may induce MM cell “quiescence” thus rendering MM cells less chemosensitive (Dalton et al, 2003). Interrupting the BMSC-MM cell interaction is an attractive therapeutic target, and a recent study suggests that blocking the interaction
between the chemokine CXCL-12 and its receptor CXCR4 increases the sensitivity of MM cells to anti-MM agents in vitro (Azab et al, 2009).

1.4.4 Plasmacytoid dendritic cells and macrophages

More recently the number of cellular components within the BM microenvironment shown to be important in promoting MM cell survival has expanded to include accessory cells such as macrophages and plasmacytoid dendritic cells (pDCs). One recent study showed that the immune function of pDCs was defective in MM and that the interaction between MM cells and pDCs stimulated MM cell growth and drug resistance. Furthermore, this study showed that although pDCs were relatively resistant to novel anti-myeloma agents, targeting toll-like receptors abrogated the growth effect of pDCs on MM cells and also restored the immune function of pDCs (Chauhan et al, 2009). In another recent study, macrophages were shown to be more plentiful in the MM BM microenvironment in comparison with the BM from healthy controls. In addition, the interaction between MM cells and macrophages stimulated increased the secretion of IL-6 and also attenuated drug-induced apoptosis of MM cells (Zheng et al, 2009).

1.4.5 Role of extracellular matrix proteins in the myeloma bone marrow microenvironment

It has been shown that the predominant role of extracellular matrix proteins such as fibronectin in the myeloma BM microenvirment is to promote drug resistance. MM cells have been shown to express integrins such as VLA-4 and integrin-fibronectin interactions have been shown to be important in protecting MM cells from drug-induced apoptosis. One study showed that MM cells co-cultured with fibronectin were less sensitive to the cytotoxic effects of melphalan and doxorubicin in comparison to MM
cells grown in suspension (Damiano et al, 1999). In a further study from the same group, it was shown that this effect was mediated through the upregulation of p27kip1 expression, thus inducing cell cycle arrest and chemo-resistance (Hazlehurst et al, 2000).

In summary, the myeloma BM microenvironment is the site of complex interplay between cellular factors, soluble factors and extracellular matrix proteins which promote the survival of MM cells through a wide array autocrine and paracrine pathways and also through adhesion molecule direct contact mechanisms. The next section focuses on one of the more recently described MM growth factors, APRIL, which is the focus of this thesis.

1.5 A Proliferation Inducing Ligand (APRIL) and its role in MM

1.5.1 Background

APRIL was originally described as a cytokine that was capable of stimulating the proliferation of the Jurkat T-cell leukaemia cell line (Hahne et al, 1998). Since then, APRIL (also referred to as TNFSF-13) and the closely related B-cell activating factor (BAFF), both of whom are members of the tumour necrosis factor (TNF) superfamily of cytokines have been shown to play key roles in normal B-cell and plasma cell development as well as in an increasing number of autoimmune disorders and malignant diseases (Roosnek et al, 2009, Rickert et al, 2011). The importance of APRIL in the development of normal immune function is underscored by the demonstration that APRIL-deficient (-/-) mice were found to have significantly reduced IgA levels despite having normal B and T-lymphocyte development (Castigli et al, 2004). In contrast, transgenic mice overexpressing APRIL developed lymph node hyperplasia and lymphoid infiltration of kidneys and liver (Planalles et al, 2004). Subsequently, it was
shown that APRIL is important survival factor for BM plasmablasts (Belnoue et al., 2008). APRIL is a type II membrane protein that is released by proteolytic cleavage in the Golgi apparatus to form active soluble homo-trimers, which prevents its expression on the cell surface (Lopez-Fraga et al., 2001).

1.5.2 APRIL receptors

APRIL and BAFF share two common receptors: B-cell maturation antigen (BCMA) and transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI). In addition APRIL also binds to heparan sulphate proteoglycans (HSPGs), whilst BAFF (but not APRIL) binds to BAFF-receptor (BAFF-R) (see Figure 1.5) (Rickert et al., 2011).

**BCMA (B-cell maturation antigen):** BCMA expression is restricted to B-lineage cells unlike TACI, which is also expressed by T-cells (Gross et al., 2000). The role of BCMA in normal physiology has been the subject of debate. In an initial study BCMA (-/-) knockout mice were shown to have normal B cell development, normal T-cell dependent immune responses and a normal lifespan in comparison to wild type B-lymphocytes (Xu et al., 2001). However, a more recent analysis of BCMA (-/-) mice showed a significant reduction in the numbers of long-lived plasma cells, suggesting that BCMA is critical for the survival of plasma cells (O’Connor et al., 2004). From a structural perspective BCMA contains only one cysteine-rich domain and also binds APRIL with significantly higher affinity than it binds BAFF (Hymowitz et al., 2005).

**TACI (Transmembrane activator and calcium-modulator and cyclophilin ligand interactor):** As with BCMA the precise role of TACI has been the subject of confusion with knockout murine models providing inconsistent findings. Initially, TACI (-/-) mice
were surprisingly found to show increased numbers of circulating B-cells and defective T-cell humoral responses (Von Bulow et al, 2001). Two subsequent studies found that TACI (-/-) mice again developed B-cell hyperplasia but also increased levels of circulating autoantibodies (Yan et al, 2001, Seshasayee et al, 2003). It was then proposed that TACI had two functions: inhibiting B-cell proliferation and stimulating plasma cell differentiation which would provide a rationale for the observed features of lymphoproliferation, hypogammaglobulinaemia and autoimmunity in TACI (-/-) mice (Mantchev et al, 2007). In fact, more recently, researchers in the same laboratory have shown that TACI deficiency leads to reduced Blimp-1 (B-lymphocyte-induced maturation protein 1) expression in B cells and therefore reduced numbers of long-lived plasma cells, providing a rationale for the observed hypogammaglobulinaemia (Tsujii et al, 2011). Unlike BCMA, TACI has two cysteine-rich domains but it has been shown that APRIL binds both TACI and BCMA with equivalent affinity (Hymowitz et al, 2005). APRIL also interacts with HSPGs in a process that has been shown to promote tumour cell proliferation (Ingold et al, 2005, Hendriks et al, 2005). The interaction between APRIL, HSPGs and TACI is also important in production of IgA suggesting that APRIL-receptor interactions may be complex and not yet fully understood (Sakurai et al, 2007).

1.5.3 Cellular expression of APRIL

An early report based on Northern Blotting studies suggested that APRIL is widely expressed in many tissues, being particularly high in the peripheral blood with lesser expression in the pancreas, small intestine, prostate and ovaries (Roosnek et al, 2009, Kelly et al, 2000). Subsequent reports demonstrated APRIL mRNA in myeloid cells, megakaryocytes and osteoclasts (Roosnek et al, 2009). Since then, a number of reports have emphasised that myeloid cells are a particularly rich source of APRIL. By using
an antibody that detects cells bearing the intracellular “Stalk” fragment of APRIL (remaining after intracellular processing), it was observed that APRIL-secreting neutrophils promote plasma cell survival in mucosa-associated lymphoid tissue (MALT) (Huard et al, 2008). A study of the individual myeloid compartments within the BM found that myeloid precursors displayed the highest levels of APRIL (Matthes et al, 2011). Following on from this, eosinophils in the murine BM were also shown to express significant amounts of APRIL (Chu et al, 2011). Although this finding was based on a murine model, it was previously noted that a patient with severe eosinopenia had marked reduction in BM plasma cell numbers (Juhlin et al, 1977).

1.5.4 Expression of APRIL in cancer

APRIL mRNA has also been detected in a variety of tumour cells including solid tumours and B-cell malignancies including Hodgkin (HL), Non-Hodgkin Lymphoma (NHL) tissues and also MM cells (Roosnek et al, 2009, Moreaux et al, 2009). Although some of these studies suggested that APRIL is produced in an autocrine fashion by tumour cells, more recent immunohistochemical studies have shown that APRIL is almost exclusively produced in a paracrine fashion in the tumour microenvironment (Mhawech-Faucegilia et al, 2006). APRIL expression was shown in 46% of Diffuse Large B-Cell Lymphoma and 20% of Burkitt Lymphoma tumour specimens and neutrophils were shown to be the predominant source of APRIL in this study with the same group observing similar findings in Hodgkin Lymphoma specimens (Schwaller et al, 2007a, Schwaller et al, 2007b). In keeping with these findings, it has recently been shown that gastric MALT-lymphoma tumour-associated macrophages express abundant APRIL suggesting a prominent role for APRIL in the pathogenesis of this lymphoma (Munari et al, 2011).
1.5.5 Role of APRIL in Multiple Myeloma

A small number of studies to date have demonstrated a role for APRIL in MM pathogenesis. Firstly, HMCLs and primary MM cells were shown to express BCMA, TACI, and BAFF-R (Novak et al, 2004). Following this study an independent group confirmed the expression of APRIL receptors by MM cells and furthermore showed that APRIL protected IL-6 dependent MM cell lines from dexamethasone-induced apoptosis, rescued IL-6 dependent MM cell lines from IL-6 withdrawal whilst also demonstrating that MM patients have higher circulating serum levels of APRIL in comparison to normal controls (Moreaux et al, 2004). In common with other lymphoid malignancies, the main source of APRIL in the MM tumour microenvironment is myeloid cells, in particular monocytes and osteoclasts although one study suggested that some HMCLs produce low levels of APRIL in an autocrine manner (Moreaux et al, 2004, Moreux et al, 2005). In MM cells, APRIL has been shown to activate the MAPK, PI3K/AKT and NFκB signalling pathways and more recently, APRIL has been shown to upregulate the expression of the anti-apoptotic protein PIM-2, particularly in cooperation with IL-6, via activation of the NFκB signalling pathway in HMCLs (Moreaux et al, 2004, Asano et al, 2011).

1.5.6 Expression of APRIL-receptors by MM cells

Several studies have now confirmed the expression of the BCMA and TACI by HMCLs and also primary MM cells. In one study, where CD138+ cells from 36 MM patients were subjected to microarray analysis, BCMA expression was found to be greater than TACI-expression, with BAFF-R showing very low levels of expression (Tai et al, 2006). Another group examined sixteen MM cell lines by flow cytometry and found BCMA expression in all cases. In contrast, TACI expression was observed in eight of sixteen cell lines whilst BAFF-R was not significantly expressed by any of the cells. In
the same study, twenty-two primary samples were analysed and BCMA expression was found in all but two samples, whilst TACI was expressed in fifteen cases with BAFF-R surprisingly present in seventeen cases (Moreaux et al., 2009). Interestingly, the same authors have demonstrated that the expression of TACI is bimodal, with those HMCLS with “high” expression of TACI (TACI^{high}) having a BM microenvironment-dependent signature, with TACI^{low} HMCLs having a more plasmablastic signature (Moreaux et al., 2005). The signature was derived from gene expression profiling of genes associated with the BM microenvironment. Furthermore, TACI expression was found to correlate with cMaf expression in MM cell lines suggesting a mechanism through which APRIL-TACI interaction might promote MM cell growth (Moreaux et al., 2007). APRIL also binds HSPGs, and the findings of a recent study suggests that this interaction might also mediate MM cell growth as APRIL-mediated growth in the TACI-negative XG-1 cell line was shown to be overcome by the addition of heparin to cultures (Moreaux et al., 2009). In view of this, the key interactions between APRIL and its receptors that promote growth and survival in MM cells may be complex and are yet to be fully elucidated.

1.5.7 APRIL-inhibition as an anticancer strategy

The therapeutic potential of interrupting the binding of APRIL to its receptors has been demonstrated both in vitro and in vivo, employing TACI-Fc a fusion protein consisting of the extracellular domain of TACI fused with human IgG1. In vitro, TACI-Fc has been shown to overcome the anti-apoptotic effect of APRIL in the presence of dexamethasone and also to inhibit the growth of TACI^{high} MM cells in a murine model (Moreaux et al., 2004, Yaccoby et al., 2007). The importance of APRIL-TACI interaction was also confirmed in a recent study of follicular lymphoma cells, where siRNA mediated knockdown of TACI inhibited APRIL-mediated activation of the
PI3K/AKT pathway leading to downregulation of cyclin D1, unlike heparin treatment or BCMA-silencing which were both ineffective (Gupta et al, 2009). Furthermore, TACI-Fc was shown to inhibit the dendritic cell-mediated clonogenic growth of HMCLs (Kukreja et al, 2006).

Figure 1.5

**Figure 1.5: APRIL and BAFF in the multiple myeloma bone marrow microenvironment.** Schematic representation of the roles of BAFF and APRIL in the MM bone marrow microenvironment. BAFF, produced mainly by bone marrow stromal cells and APRIL, produced by osteoclasts and myeloid cells interact with BAFF-R, BCMA, TACI and HSPGs as shown. These interactions activate MAPK, NFκB and PI3K/AKT signalling pathways leading to the upregulation of genes controlling MM cell survival and drug resistance.
Recently, Atacicept was used to treat patients with MM and Waldenstrom’s Macroglobulinaemia in a Phase I clinical trial. In this study Atacicept treatment was well tolerated and led to disease stabilization in 5 out of 25 patients treated (Rossi et al., 2009). More recently, another group has developed 2 novel specific anti-APRIL monoclonal antibodies both of which have been shown to inhibit the in vitro growth of chronic lymphocytic leukaemia (CLL) cells. Importantly, this approach has the advantage of inhibiting the effect of APRIL but not BAFF. BAFF has been shown to play a critical role in normal B-cell development, and it has been suggested that simultaneously targeting BAFF may lead a significant depletion of the B-cell compartment with resulting toxicity (Guadagnoli et al., 2011).

1.5.8 Summary

The role of APRIL in normal B-cell physiology is well established and increasing evidence supports its place as an important factor in several cancers but in B-cell malignancies in particular. Although just a handful of papers examine its role in MM, there is evidence that MM cells express APRIL receptors and that APRIL protects MM cells from drug-induced apoptosis. However, there is a paucity of published data regarding the ability of APRIL to stimulate growth and proliferation in MM cells, either MM cell lines or primary MM cells. Furthermore, although it has been shown that APRIL upregulates anti-apoptotic proteins in MM cells, little is known about its ability to promote cell cycle progression in primary MM cells.

1.6 Summary

The last fifteen to twenty years have seen enormous progress made in our understanding of the pathogenesis of MM. In particular, there is abundant evidence of the crucial role played by the bone marrow microenvironment in promoting the MM cell survival and drug resistance. Furthermore, the almost universal overexpression of one of the three D-
type cyclins in MM cells suggest that this is a critical event in the oncogenesis of MM cells. The demonstration that one of five recurrent IgH/TCs, which target different D-type cyclin genes is found in 40-50% of MM patients provides a clear basis for the upregulation of D-type cyclins in these patients. Several models describing the pathogenesis of myeloma have been developed, taking into account these important findings. The model depicted in Figure 1.6 proposes that during the earlier phases of MM, malignant plasma cells are dependent upon the BM microenvironment for growth and survival signals (Kuehl et al, 2002). However, as disease progresses to an extramedullary or leukemic phase, often during the acquisition of additional genetic abnormalities such as 17p deletion and NFkB or RAS mutations, the MM cells are no longer dependent on the BM microenvironment for survival. This phase is usually characterized by a more proliferative plasma cell phenotype, poor response to anti-myeloma therapy and clinical deterioration.

Despite these major findings, many questions remain. The complexity of the interplay between multiple cytokines, cellular factors and extracellular matrix proteins has made it difficult to establish which cytokine or paracrine loop is the most critical in sustaining MM cell survival. Neither do we know what the precise factors are that mediate cell cycle progression. The heterogeneity that is observed at a clinical level may be linked to how MM cells with different genetic characteristics interact with different cytokines or stromal factors in the BM microenvironment, with some micro-environmental factors being more relevant in different “types” of MM.
**Figure 1.6: Molecular pathogenesis of myeloma.** Primary IgH/TCs arise due to errors in switch recombination or somatic hypermutation in germinal centre B-cells and provide initiating oncogenic effects in approximately 50% of MM cases. As disease progresses there is less dependence on the bone marrow microenvironment for MM cell survival and an increased frequency of secondary IgH/TCs with activation of c-MYC as well as an increased frequency of RAS mutations, NFκB mutations and the acquisition of a more proliferative phenotype (adapted from “Multiple Myeloma: evolving genetic events and host interactions” by Kuehl WM, Nat Rev Cancer. 2002. Mar; 2(3);175-87)

APRIL is a relatively recently described TNF-family member and whilst it has been shown to attenuate dexamethasone induced apoptosis in HMCLs and primary myeloma cells, its effects on cell cycle progression in MM cells are relatively unknown. In particular, there is a lack of data relating to the effect of APRIL on primary MM cells which may in part relate to the widely acknowledged tendency of primary MM cells to survive poorly in vitro with standard culture media.
1.7 Aims and Hypothesis

**Hypothesis:** The hypothesis at the heart of this project is that aberrant D-type cyclin expression provides the molecular basis for cell cycle entry and self-renewal in MM cells responding to extrinsic signals within the BM (i.e. APRIL or BAFF stimulation). Furthermore this hypothesis also proposes that MM cells of different D-type cyclin class and translocation status (TC group) differ in their cell-cycle specific responses to APRIL and BAFF.

**Aims:** This work aims to answer the following questions:

- Do primary MM cells undergo cell cycle progression in response to APRIL?
- Is APRIL expressed in the MM BM microenvironment?
- Do primary MM cells express APRIL receptors?
- Do primary MM cells express cell cycle regulatory proteins?
- Is the cell cycle response of primary MM cells to APRIL influenced by underlying D-type cyclin group and translocation class?
- Do APRIL and BAFF attenuate drug-induced apoptosis in primary MM cells and is this also determined by D-type cyclin group and translocation class?

The overall aim of this work is to identify a BM micro-environmental factor upon which MM cells of a specific TC group are particularly dependent, with the ultimate goal of designing specific anti-myeloma therapies tailored to different TC groups.
Chapter 2. Materials and Methods

2.1 Cell Culture

2.1.1 Human Myeloma Cell Lines

The following autonomously growing human myeloma cell lines (HMCLs) were routinely cultured in 75 cm² flasks in RPMI1640 supplemented with 10% foetal calf serum and 1% penicillin/streptomycin (hereafter referred to as RPMI/10%FCS). Typically, cells were passaged every 48 hours. The IgH/TCs found within each of these HMCLs are detailed in Table 2.1.

- NCI-H929
- JJN3
- KMM1
- KMS12BM
- KMS21BM
- KMS27PE
- LP-1
- JIM-1
- RPMI8226
- OPM2
- MM1S
- U266
Table 2.1: Human myeloma cell lines (HMCL) with D-type cyclin and genetic lesions

<table>
<thead>
<tr>
<th>Predominant D-type cyclin expressed and IgH/TC</th>
<th>HMCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1 and t(11;14)</td>
<td>KMS-12-BM/PE, KMS-21-BM/PE, KMS-27, U266, XG1</td>
</tr>
<tr>
<td>Cyclin D2 and t(4;14)</td>
<td>KMS-28-BM/PE, NCI-H929, JIM1, JIM3, OPM2, KMS-34, KMS-18</td>
</tr>
<tr>
<td>Cyclin D2 and t(14;16)</td>
<td>MM1S, MM1R, JJN3, KMS11*</td>
</tr>
<tr>
<td>Cyclin D2 and t(16;22)</td>
<td>RPMI 8226</td>
</tr>
</tbody>
</table>
* also has t(4;14)

2.1.2 Cell culture reagents

- Hank’s BSS (HBSS, PAA, Pasching, Austria)
- Foetal Calf Serum (FCS, non heat-inactivated, PAA, Pasching, Austria)
- Phosphate Buffered Saline (PBS, PAA, Pasching, Austria)
- RPMI1640 (with L-Glutamine, PAA, Pasching, Austria)
- Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, UK)
- Penicillin/Streptomycin (PAA, Pasching, Austria)
- 0.4% Trypan blue (Sigma Aldrich, Poole, UK)
- Automacs Running Buffer (Miltenyi Biotech, Surrey, UK)
- CD138 MicroBeads (Miltenyi Biotech, Surrey, UK)
- Rosette-Sep myeloma cell purification cocktail (StemCell Technologies, USA)
- FACS (Fluorescence activated cell-sorting) buffer – PBS with 0.1% Bovine Serum Albumin (PBS/0.1%BSA)
• Human serum albumin (HAS, 10% solution, Sigma Aldrich, Poole, UK)
• X-VIVO 10 medium (Cambrex, Bath, UK)

2.1.3 Cell Culture Plastics
• 5ml, 10ml and 25ml pipettes (VWR International, Lutterworth, UK)
• 25cm$^2$, 75 cm$^2$, 175 cm$^2$ tissue culture flasks (VWR International, Lutterworth, UK)
• 6, 12, 24 and 96 well plates (VWR International, Lutterworth, UK)
• 15ml, 25ml and 50ml universal containers (BD Biosciences, Oxford, UK)
• LS columns and pre-separation filters (Miltenyi Biotec, Surrey, UK)
• 20-40µm cell strainers (BD biosciences, Oxford, UK)

2.1.4 Recombinant Growth Factors
• APRIL (TNFSF13, 884-AP, R & D Systems, Abingdon, UK)
• BAFF (TNFSF13B, 2149-BF, R & D Systems, Abingdon, UK)
• IGF-I (Peprotech, London, UK)
• IL-6 (Peprotech, London, UK)
• TNFα (R & D Systems, Abingdon, UK)

2.1.5 Purification of MM cells by CD138+ cell selection
• Bone marrow samples were obtained from MM patients with full informed consent and collected in EDTA-lines vacutainer tubes at the bedside.
• Whole bone marrow was first washed through a 40µm cell strainer with HBSS and made up to 25mls total solution with HBSS.
• This solution was then carefully layered onto 25mls of FicollPaque and centrifuged at 1900rpm (revolutions per minute) for 30 minutes after which the
mononuclear layer (MNCs) was carefully removed and re-suspended in 20mls of HBSS/5% FCS before centrifugation at 1800rpm for a further 10 minutes.

- The resulting cell pellet was then re-suspended in 5mls of Automacs running buffer.
- The number of MNCs was calculated by Trypan Blue exclusion (see below). The cell suspension was then centrifuged at 1600rpm for 10 minutes and the cell pellet was resuspended in Automacs running buffer and CD138 MicroBeads.
- The volume of running buffer and CD138 MicroBeads required was calculated based on the number of MNCs harvested: for every $10 \times 10^6$ MNCs, 20µl of CD138 MicroBeads was added to 180µl of running buffer.
- This solution was mixed thoroughly and incubated for 20 minutes at 4°C.
- The cells were then washed in 10mls of running buffer and centrifuged at 1600rpm for 10 minutes.
- The cell pellet was then re-suspended in 1ml of running buffer before being passed over an LS column, which had been primed with 3mls of running buffer.
- The column was then removed from the magnetic field and CD138+ cells were washed from the column with 1ml running buffer.
- The number of CD138+ cells was counted using trypan blue exclusion and plasma cell purity was assessed by MGG-stained slides ± flow cytometry.

2.1.6 Purification of MM cells by negative selection

- This method purifies MM cells from patient bone marrow aspirates by “negative” selection using RosetteSep which is an antibody cocktail designed to isolate MM cells by removing all other BM cells.
• These cells are removed by the formation of antibody complexes targeting CD2, CD14, CD33, CD41, CD45RA, and CD66b on leucocytes and glycophorin-A on red cells.
• Following centrifugation the unwanted cells form a pellet leaving the purified MM cells present as a layer at the interface between the plasma and the ficoll.

Method:
• 2-5mls of whole BM was collected in EDTA-lined vacutainers.
• For every 1 ml of BM, 50µl of RosetteSep cocktail was added and mixed thoroughly with whole BM in a 25ml universal container.
• The cell suspension was then incubated at room temperature for 20 minutes after which it was diluted in an equal volume of PBS/2%FCS.
• The cell suspension was then carefully layered onto an equal volume of ficoll in a 15ml universal container and centrifuged at 1200g for 20mins.
• The “MM cell layer” was then carefully aspirated and resuspended in PBS/2%FCS before centrifugation at 300g for 7 minutes. This step was repeated before the cell pellet was resuspended in 1ml of PBS/2%FCS.
• The number of MM cells was then counted cells and purity was assessed by MGG-stained slides ± flow cytometry.

2.1.7 Trypan blue exclusion
• This assay determines the number of viable cells in a cell suspension on the basis that viable cells with intact cell membranes exclude the blue dye, unlike dead cells where the cytoplasm appears blue.
• A known volume of cell suspension was mixed with an equal volume of 0.4% trypan blue for 2 to 5 minutes
• The trypan blue cell suspension was then applied to a haemocytometer
• The number of viable cells was determined by counting the live cells in each of the 4 corners of the chamber (which contain 16 smaller squares each) by light microscopy.
• This number was then divided by 4 and adjusted for the dilution factor, before the final number of viable cells was determined (x $10^4$/ml).

2.1.8 Preparation of culture medium containing 20% plasma pooled from MM patients

• Plasma samples were collected from MM patients (not on any active anti-MM treatment) in lithium-heparinised vacutainers and centrifuged at 3000rpm for 15 minutes.
• The plasma was then carefully aspirated and pooled before freezing at -80°C.
• Upon thawing at 37°C, MM plasma was added to RPMI1640 at a concentration of 20%.
• This medium was then passed through a 20µm filter before being immediately used in cell culture experiments
• An identical method was used to prepare culture media containing pooled serum or plasma from normal donors and pooled serum from MM patients.
2.1.9 Cryopreservation of primary myeloma cells

- Cells were centrifuged at 300g for 5 minutes and resuspended in 100% FCS on ice (volume dependent on cell number i.e. 5 x 10^6 cells in 1 ml).

- An equal volume of “Freezing Medium” (80%FCS/20%DMSO) was added “drop by drop” into the cell suspension over 5 minutes on ice.

- The final volume of the cell suspension was dependent on the cell number i.e. 5 x 10^6 in 2 mls.

2.1.10 Anti-Myeloma Agents

- U0126 10µM (MAPK 1/2 Inhibitor, Cell Signalling Technology, Hitchin, UK)
- Dexamethasone 0.01-1µM (Sigma Aldrich, Poole, UK)
- Lenalidomide 50µM (From unused clinical stock)
- Bortezomib 10-20nM (From unused clinical stock)
- Melphalan 50nM (From unused clinical stock)
- TACI-Fc 10µM (Alexis, Carlsbad, CA, USA)

2.2 Cell cycle analysis

All flow cytometry analysis was performed using a Cyan ADP flow cytometer (Dako, Cambridgeshire, UK)

2.2.1 Materials for cell cycle analysis

- 5ml polyethylene FACS tubes (VWR, Lutterworth, UK)
- FACS buffer (PBS/0.1% BSA)
- APC-conjugated anti-CD138 (Miltenyi Biotech, Surrey, UK)
- APC-conjugated anti-IgG1 Isotype control (Miltenyi Biotech, Surrey, UK)
- FITC-conjugated anti-Ki67 (BD biosciences, Oxford, UK)
• FITC-conjugated anti-IgG1 (BD biosciences, Oxford, UK)
• FITC-conjugated anti-BrdU (BD Biosciences, Oxford, UK)
• PermWash, FixPerm and FixPerm-Plus buffers (BD biosciences, Oxford, UK)
• Propidium iodide (Sigma Aldrich, Poole, UK)
• 7-Aminoactinomycin D (7-AAD, BD biosciences, biosciences, UK)
• 4% paraformaldehyde (Electron Microscopy Sciences, USA)
• Triton-X-100 (Sigma Aldrich, Poole, UK)
• RNAase (Sigma Aldrich, Poole, UK)
• [3H]-Thymidine (GE Healthcare, Buckinghamshire, UK)
• Filtermats A (Meltilex; Wallac, Turku, Finland)
• MeltiLex A solid scintillant sheets (Meltilex; Wallac, Turku, Finland)
• Sample Bags (PerkinElmer, USA)

2.2.3 Flow Cytometric Assay of Bromodeoxyuridine Uptake

Bromodeoxyuridine (BrdU) is a nucleoside analogue that is incorporated into cells in the DNA synthesis phase of the cell cycle (S-phase). This assay determines the S/G2M fraction by pulsing cells with bromodeoxyuridine and then incubating the cells with FITC-conjugated anti-BrdU and 7-AAD, a DNA-intercalating agent (see Figure 4.4B for representative example).

Method:

• 0.5-1 x 10^6 cells in 1ml of medium were pulsed with 10µl of a 1mM BrdU solution and incubated for 2 hours at 37°C.

• The cells were then washed in FACS buffer, divided equally and then re-suspended in 50µl of FACS buffer in each of two FACS tubes (A and B).
• 5µl of APC-conjugated anti-CD138 was added to one tube and 5µl of APC-conjugated anti-IgG1 isotype control to the other before the samples were incubated for 30 mins on ice.

• The cells were then washed in FACS buffer (1500 rpm for 5 minutes) and then resuspended in 100µl of “Cytofix/Cytoperm” per tube before incubation for 15 minutes on ice.

• The cells were then washed with 1ml of “PermWash” Buffer (1500 rpm for 5 minutes) and then resuspended in 100µl of “Cytoperm Plus” buffer and incubated on ice for 10 minutes before washing again with 1ml of “PermWash” per FACS tube.

• The cells were then re-suspended in 100µl of “Cytofix/Cytoperm” buffer and incubated for 5 mins on ice before washing in 1ml of “PermWash” and being re-suspended in 100µl of RNAase per tube at a concentration of 300µg/ml per tube and incubated for 1 hour at 37º.

• The cells were then washed again in 1ml of “PermWash” buffer and re-suspended in 50µl of PermWash buffer with 1µl of FITC-conjugated anti-BrdU (or FITC-conjugated isotype control) and incubated for 20mins at RT.

• Finally, the cells were again washed cells in 1ml of “PermWash” and re-suspended in 500µl of FACS buffer containing 20µl of 7-AAD solution and analysed by flow cytometry.

2.2.4 Cell cycle analysis (Propidium Iodide)

This assay is used to quantify the percentage of cells in each cell cycle phase as well as the apoptotic fraction.
• 5 x 10^5 cells were resuspended in 500µl of FACS buffer containing 0.1% sodium citrate and 0.1% Triton X-100.

• PI and RNAse were added, both at a concentration of 50µg/ml, before the cells were incubated at 37ºC for 30 min and then analysed by flow cytometry

2.2.5 [3H]-thymidine incorporation assay

This assay is also used to determine cell proliferation by measuring the incorporation of a radiolabelled nucleotide analogue [3H]-thymidine into DNA.

• MM cells (either HMCLs or CD138+ primary MM cells) were plated in triplicate in a 96 well plate at a concentration of 1 x 10^5/100ml of medium per well, before stimulation with MM growth factors and incubated at 37º for a defined period

• 1µCi of [3H]-thymidine was added to each well for the final 2-4 hours of incubation before the cell suspensions were harvested onto a filtermat and allowed to dry.

• The dry filtermat was then placed on silicon paper on a hot plate which had been heated to >90º before the solid scintillant was applied to the filtermat and allowed to melt before being set aside to cool.

• Once cool, the filtermat was then sealed in a sample bag

• DNA incorporation was then measured with a scintillation counter

The CD138/Ki67/Propidium Iodide cell cycle assay is outlined in Chapter 3
2.3 Flow Cytometry

2.3.1 Antibodies and reagents

- Rat monoclonal antibody to BCMA (Vicky-1, Abcam, UK)
- Rat monoclonal antibody to TACI (1A1, Abcam, UK)
- PE-conjugated goat polyclonal antibody to rat IgG (Abcam, UK)
- Anti NFκB, p65 active subunit (Chemicon, USA)
- Phospho-AKT (Ser473, 193H12, Cell Signalling Technology, Hitchin, UK)
- FITC-conjugated Anti-AnnexinV (Roche)
- AnnexinV buffer (150mM NaCl, 10mM Hepes, 10µM CaCl₂, pH 7.4)
- FlowCheck Fluorospheres (Beckman Coulter)

2.3.1 Detection of surface expression of BCMA and TACI on purified MM cells

- 1 x 10⁵ CD138+ cells were placed in each of 3 separate FACS tubes in 50µl of FACS buffer (tubes A, B and C).
- 1µl each of primary unconjugated anti-BCMA and anti-TACI antibody was added to 2 of the tubes (A and B) and incubated for at least 60 minutes on ice
- No primary antibody was added to the third tube which acted as a negative control (C)
- The cells were then washed in FACS buffer (1500rpm, 5 minutes) and 2µl of PE-conjugated goat anti-rat secondary antibody was added to each of the 3 tubes after which the cells were incubated on ice for 30 minutes
- The cells were washed once in FACS buffer and resuspended in 500µl of FACS buffer before analysis by flow cytometry.

The methods for the detection phospho-AKT and the activated form of p65 are discussed separately in chapter 6.
2.4 Apoptosis and Survival

2.4.1 AnnexinV/Propodium Iodide Staining

- This assay is used to quantify the proportions of cells within a suspension that are viable, apoptotic and necrotic on the principle that cells undergoing apoptosis undergo changes in the integrity of the cell membrane, which leads to externalisation of the membrane phospholipid, phosphatidylserine.

- AnnexinV is a calcium-dependent phospholipid binding-protein that binds to cells with exposed phosphatidylserine.

- Propidium iodide (PI) is taken up by the non-viable cells as their membranes are not intact whereas PI is excluded by live cells. Thus, labelling cells with both anti-AnnexinV and propidium iodide permits the identification of apoptotic cells (AnnexinV +ve) and dead cells (double AnnexinV+ve and PI+ve) (see Figure 2.1).

- FlowCheck Fluorospheres may be consecutively analysed with the cell suspension to count the absolute number of viable cells (see Figure 2.1).

Method:

- After 24-72 hours in culture 1 x 10^5 cells were carefully harvested from the wells of 24-96 well plates and placed in FACS tubes on ice before the solution was made up to in 500µL with additional PBS.

- To each tube was added: 100µL AnnexinV buffer, 0.25µL of FITC-conjugated anti-AnnexinV, 25µL of Fluorospheres, and 5µL of propidium iodide solution (2.5mg/ml).

- The cell suspension was then analysed immediately by flow cytometry.
Figure 2.1

**Figure 2.1: Representative example of AnnexinV/PI analysis.** MM1S cells were prepared as described in 2.4.1. Left Panel: R2-MM1S Cells, R3-Fluorosphere Beads. Middle Panel: R7-Live cells (AnnexinV/PI negative), R8-Apoptotic cells, R6-Necrotic cells. Right Panel: R1 – Non-viable cells (AnnexinV positive).

### 2.4.2 MTS Assay

- This assay employs a colorimetric method for determining the number of viable cells in a cell suspension.
- MTS is an abbreviated term for the tetrazolium compound \[\text{[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium]}\] that is reduced by cells to a formazan product that is soluble in culture medium.
- Conversion of MTS to the formazan product is dependent on dehydrogenase enzymes found in cells that are metabolically active.
- The quantity of formazan product is measured by absorbance at 490nm and is proportional to the number of viable cells.

**Method:**

- \(1 \times 10^5\) cells in 100µL of medium were cultured in each well of a 96-well tissue culture plate (in triplicate).
• The cell suspensions were incubated at 37°C for a defined period culture before 20µL of MTS solution was added to each well and incubated for 1-4 hrs.
• Absorbance was measured at 490nm using a Varioskan plate-reader.

2.4.3 Caspase 3 activity assay

• This method outlined below is based on the assay described by Gonzalez et al (2009).
• Caspases are a family of cysteine proteases that play an important role in the intrinsic apoptotic pathway, through cleavage of multiple protein substrates.
• Caspase-3, downstream of Caspase-8 and-9, is activated via multiple pathways.
• In this assay, the activity of Caspase-3 is determined by cleavage of a fluorogenic substrate and measured by a fluorescent spectrophotometer at a wavelength of 344nm and an emission wavelength of 436nm.

Method:

• The cells to be analysed were centrifuged at 1500rpm for 5 minutes and then resuspended in 0.7mL Assay buffer ((20mM HEPES, 2mM EDTA, 0.1% CHAPS, 5mM DTT, 8.25µM Caspase substrate)
• The cells were then sonicated for 5 minutes before incubation at 37°C for 1 hour.
• Equal volumes of lysate were then added to the wells of a 96-well plate in triplicate and caspase-3 activity was measured by spectroscopy at an excitation wavelength of 344nm and an emission wavelength of 436nm.
• Protein concentration was measured concurrently and measurements were adjusted according to protein concentration.
2.5 Western blotting analysis

2.5.1 Materials, buffers and reagents

- ECL Western blotting detection reagent (GE Healthcare)
- High performance chemiluminescence film (GE Healthcare)
- Full-range molecular weight markers (GE Healthcare)
- Methylene Bis-acrylamide (National Diagnostics, USA)
- Ammonium persulphate (APS, Sigma Aldrich, Poole, UK)
- Bromophenol blue (VWR, Lutterworth, UK)
- Hybond-C-Extra nitrocellulose membrane (Amersham, UK)
- Tween-20 (Sigma Aldrich, poole, UK)
- Dried milk (Marvel)
- Tris (Hydroxymethyl) methylamine (VWR, Lutterworth, UK)
- Double-distilled H₂O (dd H₂O)

Buffers:

- Loading Buffer (2x): 4% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris HCL
- Running buffer: (30g Tris, 188g Glycine, 10g SDS, made up to 1L with H₂O
- Transfer buffer: (29g Tris, 145g Glycine, made up to 1L with H₂O)
- Tris-buffered saline: (24g Tris 88g NaCl, pH 7.5 made up to 1L H₂O) with 0.05% Tween-20 (TBS-T)

Gels and Method:

- Resolving gel (12.5%): 150μl 10% SDS, 3ml distilled H₂O, 6.25ml Acrylamide, 5.5ml 1M Tris (pH 8.8), 50μl 25% ammonium per-sulphate (APS), 6μl TEMED
• Stacking gel: 3.5ml distilled H₂O, 835µl acrylamide, 625µl 1M TRIS (pH 6.8), 50µl 10%SDS, 12.5µl 25% APS, 10µl TEMED

• Resolving gels were prepared the evening before use and poured into gel casters (Mighty-Small Hoeffer gel caster), covered with ddH₂O, sealed and stored at 4º

• The ddH₂O was removed and the stacking gel was layered gently onto the resolving gel, before a 10 or 15 well comb was inserted.

• Cells were incubated in lysis buffer [50 mmol/l Tris–HCl, pH 7·4, 150 mmol/l NaCl, 1% Nonidet P40 (NP40), 0·5% sodium deoxycholate, 0·1% sodium dodecyl sulphate (SDS), 1 mmol/l ethylenediaminetetraacetic acid], Complete protease inhibitors (Roche, Welwyn, UK) and Phosphatase inhibitor cocktail II (Calbiochem, Nottingham, UK) on ice for 5 min.

• 10-20 µg of lysate per sample was added to an equal volume of loading buffer and was boiled for 5 minutes (along with molecular weight markers), loaded onto the gel and then run at 160V until separated.

• Proteins were transferred to nitrocellulose membranes by “wet” transfer, and protein loading was initially visualised by Ponceau-S staining.

• Membranes were then blocked for 1 hour with 5% powdered milk/TBS-T

• Membranes were incubated with primary antibodies diluted in 5%BSA/TBS-T overnight at 4ºC

• Membranes were incubated with secondary antibodies diluted in 5% powdered milk/TBS-T

• Membranes were washed for 10 minutes in TBS-T x 4 between primary and secondary antibody stages

• ImageJ software was used to quantify protein expression (http://rsbweb.nih.gov/ij/).
2.5.3 Primary antibodies

All antibodies were used at a 1/1000 dilution unless otherwise indicated.

- Cyclin D2 (M-20, Santa Cruz Biotechnology, Heidelberg, Germany)
- Cyclin D1 (DCS-6, Santa Cruz Biotechnology, Heidelberg, Germany)
- Cyclin E2 (Cell Signaling Technology, Hitchin, UK)
- CDK-4 (C-22, Santa Cruz Biotechnology, Heidelberg, Germany)
- CDK-6 (C-21, Santa Cruz Biotechnology, Heidelberg, Germany)
- Phospho-pRb (Ser$^{807/811}$, Cell Signaling Technology, Hitchin, UK)
- P27 (C-19, Santa Cruz Biotechnology, Heidelberg, Germany)
- P18 (N-20, Santa Cruz Biotechnology, Heidelberg, Germany)
- Poly ADP-Ribose Polymerase (PARP, Cell Signaling Technology, Hitchin, UK)
- MCL-1 (S-19, Santa Cruz Biotechnology, Heidelberg, Germany)
- BIM (Cell Signalling Technology, Hitchin, UK)
- Phospho-MAPK (Thr202/Tyr204, p44/p42, Cell Signaling Technology Hitchin, UK)
- MAPK (Santa Cruz Biotechnology, Heidelberg, Germany)
- Phospho-AKT (c14-6, Biosource, UK)
- AKT (C67E7, Cell Signalling Technology, Hitchin, UK)
- Anti-NFκB subunit p65 (12H11, Millipore, USA)
- IKBa (C-21, Santa Cruz Biotechnology, Heidelberg, Germany)
- Phospho-IκBα (Ser 32/36, 5A5, Cell Signalling Technology, Hitchin, UK)
- Anti-NFκB p52 (Mouse monoclonal, Millipore, USA)
- cMaf (M-153, Santa Cruz Biotechnology, Heidelberg, Germany, SC-7866, 1/200 dilution)
- B-Actin (AB-5, BD Transduction Laboratories, Oxford, UK)
• GAPDH (6C5, Abcam, UK)

2.5.4 Secondary Antibodies

• Anti-rabbit IgG horseradish peroxidise-linked antibody (1/5000, GE healthcare)
• Anti-mouse IgG horseradish peroxidise-linked antibody (1/5000, GE healthcare)

2.6 Immunohistochemistry

Immunohistochemical staining of tissue sections was performed on the Bond-maX system (Leica Biosystems, Newcastle, UK) in the Department of Histopathology, University College London, in collaboration with Dr. Teresa Marifioti, Dr. Manuel Rodriguez-Justo and Ms. Philippa Munson.

Antibodies:

• CD138 (Dako, dilution 1/50)
• Cyclin D1 (rabbit monoclonal antibody SP4, Lab Vision Products, 1/50)
• Cyclin D2 (Cell Signalling Technology, Hitchin, UK, cat # 2924, at 1/50, using the Bond maX ER2 30' antigen retrieval protocol)
• CDK6 (ABNOVA, Clone 8H4, dilution 1/50, Bond Max ER2)
• CDK4 (ABCAM, ab 7955-1, 1/50, Bond Max ENZ1)
• Phospho pRb (Cell Signaling Technology, phospho-Rb ser608, 1/50, Bond Max ER1)
• APRIL (C-terminal, secreted form, rabbit polyclonal, Millipore (AB3635) used at a dilution between 1/50-100 with a Citrate-based antigen retrieval buffer)
• BLIMP-1 (Spanish National Cancer Institute (CNIO), PRDM1 Clone ROS195G; dilution 1:4)
• APRIL (N-terminal – Stalk fragment - rabbit, AXXORA, UK, 2223-C100)
• Specimens were viewed with an Olympus BX51 microscope and images were taken with an Olympus DP12 camera.

2.7 Statistical Analysis

• Differences between sample groups or treatment conditions were analysed using the two-tailed paired or unpaired Student’s t-test

• Data are presented as mean ± S.D. or S.E.M (as appropriate) unless otherwise indicated.
Chapter 3: In-vitro culture of primary MM cells and measuring the proliferative fraction

3.1 Introduction

3.1.1 Background

Although HMCLs provide a widely available and reproducible model for the study of MM cell behaviour and responses to anti-myeloma therapies, they are unlikely to be truly representative of the MM tumours in the vast majority of MM patients. Firstly, HMCLS are almost always isolated from the peripheral blood of MM patients with plasma cell leukaemia (PCL) or myelomatous pleural effusions. In these situations, MM cells are no longer thought to be dependent on the BM microenvironment for growth and survival and instead are capable of autonomous growth with an increased proliferation rate. In contrast, in the majority of MM patients, MM is an intramedullary tumour with a low proliferation rate that relies on microenvironmental factors for growth and survival. Furthermore, IgH/TCs that are found in approximately 50% of MM patients with de novo disease are present in up to 80% of patients with extramedullary disease and nearly all HMCLs. Hence, the heterogeneity that exists amongst MM patients is not fully represented within HMCLs, as there is currently a lack of cell lines displaying hyperdiploidy and absence of an IgH/TC, which is a hallmark of MM in approximately 50% of cases. Thus, it is imperative that patient-derived material is studied to understand the biological basis for MM cell behaviour and responses to anti-myeloma treatments. Unfortunately, to date, such experiments have been hampered by the tendency of primary MM cells to grow poorly in vitro, perhaps emphasising their dependence on the bone marrow microenvironment. Therefore, one of the primary objectives of this project was to optimise a culture medium/system that would facilitate the survival of primary MM cells, to the extent that the critical early events involved in MM cell cycle progression could be studied in vitro.
3.1.2 In-vitro culture conditions

To date, very few publications have documented the survival characteristics of purified (CD138-selected) MM cells in vitro. It is widely reported and generally accepted that purified MM cells survive poorly in vitro when cultured in standard media. This has prompted some researchers to culture MM cells as part of a mononuclear cell layer (MNCs) to harness the growth and survival potential of microenvironmental factors. Whilst such an approach is valuable for flow-cytometry experiments where MM cells are easily identified, western blotting experiments and, for example, [3H]-thymidine incorporation assays require highly purified MM cell preparations, thus it is necessary to culture MM cells as a purified population which may diminish cell survival.

Recently, Zlei et al (2007) compared the effect of 5 different culture media/conditions on the survival of purified primary MM cells from 10 patients including 5 with de novo disease. The different culture conditions consisted of 3 different combinations of cytokines added to RMPI1640/10%FCS; combination A: IL-6, VEGF and IGF-I, combination B: IL-6, IGF-I, VEGF and HGF, combination C: IL-6, IGF-I, CXCL-12, Galectin-1, and IL-1α. Alternatively, MM cells were cultured with either osteoclasts or BMSCs. Overall it was found that the viability of primary MM cells reduced significantly by 3-4 fold during the first 3 days in culture, after which the number of viable cells in culture remained stable for up to 11 days. It was also found that both osteoclasts and BMSCs were superior to any of the cytokine combinations in preserving MM cell viability. In an earlier study, Abroun et al (2008) showed that the viability of primary MM cells could be maintained for 14 days in vitro by culturing the cells in the presence of IL-6, SDF-1 and Galectin-1. This combination of cytokines was found to be superior to control medium, and notably these authors found that a serum-free culture
medium (AlbuMax) was superior to RPMI1640/10%FCS. Crucially, Abroun et al cultured unselected MM cells as part of a BM mononuclear layer, thus harnessing the survival effects of co-cultured micro-environmental cells. Other investigators have shown that co-culture of primary MM cells with osteoclasts significantly improves survival in vitro. Moreaux et al (2011) showed that co-culture of the XG-1 and XG-20 myeloma cell lines with osteoclasts led to significantly improved viability of MM cells in contrast to those cultured in medium alone. Furthermore, in this study <10% of CD138-selected cells (Miltenyi system) from 2 MM patients were found to be apoptotic after 6 days culture on osteoclasts in contrast with >60% of cells maintained in culture medium alone. Indeed, Yaccoby (2005) co-cultured CD138-selected cells (Miltenyi system) from 16 MM patients with osteoclasts for between 6-20 weeks and found that the CD138+ cell viability after these time periods was remarkably >95%. In this study, it was also noted that MM cells cultured under these conditions underwent phenotypic changes, including loss of CD138 expression, acquisition of CD45 expression and acquisition of a more plasmablastic morphology.

3.1.3 Three-dimensional culture systems

The well-described tendency of primary CD138-selected (purified) primary MM cells to undergo apoptosis during in-vitro culture has prompted several investigators to evaluate so-called “3-D culture” systems (three dimensional). The objective of these culture systems is to promote the long-term survival of primary MM cells ex-vivo by providing a supportive “scaffold” of BM micro-environmental factors which have been shown to promote MM cell survival in vitro, such as fibronectin, collagen and bone marrow stromal cells (BMSCs). A culture system that could support the prolonged in-vitro survival of primary MM cells might also offer a template for the study of so-called “precursor” cells and could also provide a system for the study of new anti-MM agents.
Kirshner *et al* (2008) recently described a unique 3-D in-vitro model for the long-term culture of primary MM cells. This system had several important components: The first step in this system was to create a layer of collagen and fibronectin in the well of a standard tissue culture plate, after which a layer of matrigel was applied. Finally, MM cells were added as part of a mononuclear cell layer in a culture medium consisting predominantly of RPMI1640 and a 20% concentration of plasma pooled from MM patients. By tracking the MM cells using RQ-PCR to identify patient-specific VDJ sequences and by identifying proliferating cells by the loss of CFSE-labelling, the authors found that this system facilitated the expansion of the entire “MM clone” after prolonged culture (>25 days) along with the identification of possible CD20-expressing MM stem cells which retained CFSE-labelling. Furthermore, it was shown that melphalan treatment led to the enrichment of putative “MM precursor cells” whilst CD56-negative MM cells were resistant to bortezomib. Importantly, neither normal donor plasma nor bovine serum supported the survival of MM cells.

More recently, Italian researchers sought to improve upon the SCID-Hu murine MM model (*Calimeri et al*, 2011). The SCID-Hu model, which involves the direct injection of purified MM cells into human foetal bone chips that have been implanted into SCID mice, is limited by the heterogeneity in the gestation of the bone chips. In their model, *Calimeri et al* replaced the fetal bone chips with a three-dimensional (3D) bone-like poly-ε-caprolactone polymeric scaffold (PCLS) that was coated with BMSCs before implantation in SCID mice. Primary MM cells were then injected into this scaffold, as part of a BM mononuclear layer. This model facilitated MM cell growth and survival over a 3-4 week period as was demonstrated by increasing measurements of monoclonal light chains in mouse sera and expression of Ki67 by MM cells.
Thus, several systems have been shown to enhance the viability of primary MM cells during in vitro culture, but only when MM cells are cultured as part of an MNC layer or in direct contact with either BMSCs or osteoclasts. One of the principle objectives of this project was to correlate the proliferative behaviour of primary MM cells with D-type cyclin expression by western blotting. Therefore, a culture medium was required that would support the survival of highly purified CD138-selected cells. The observations of Kirshner and co-workers provided a starting point for the development of an optimal culture medium for these purposes.

3.1.4 Assays to measure cell cycling or proliferation

In the literature to date, three principal techniques have been used to measure or analyse the proliferative or “growth fraction” of MM cells. The percentage of cells in S-phase in a given population can be determined by the scintigraphic measurement of the uptake of [3H]-thymidine (a nucleoside analogue) by those cells, and such a technique is a standard method of measuring growth or proliferation in vitro. Using this technique Drewinko et al (1981) found the proliferative fraction in untreated MM patients to be low at <4%. They also found that patients with relapsing disease had significantly higher rates of proliferation. In 1982 the same group of researchers showed that the rate of proliferation was linked to prognosis when they found, again using [3H]-thymidine incorporation, that patients with a proliferation rate of <1% before treatment had a better prognosis. Despite their many advantages, [3H]-thymidine incorporation assays are limited somewhat by their sensitivity, with seemingly minor alterations in sample purity having a significant bearing on measurements. An alternative technique involves the assessment of bromodeoxyuridine incorporation (BrdU, another nucleoside analogue). Lokhorst et al (1986) compared the MM growth fraction (referred to as the Labelling Index, LI) in 26 patients as measured by thymidine incorporation or
alternatively by measuring BrdU incorporation, using anti-BrdU fluorescent antibody staining of BM preparations following BrdU incorporation. The two techniques gave equivalent results, however, the BrdU assay was significantly less time-consuming (<4 hours) without a requirement for radioactive materials thus offering significant practical advantages. In fact this technique has become widely employed to determine the baseline proliferation rate in MM, commonly referred to as the plasma cell labeling index (PCLI). Studies by Durie and Greipp in 1980 and 1988 respectively demonstrated that an elevated pre-treatment proliferative index was a powerful predictor of prognosis in MM patients with de novo disease, and remains so in the era of novel anti-MM agents when measured by gene-expression profiling (Durie et al, 1980, Griepp et al, 1988, Hose et al, 2010).

A third method of determining the MM growth fraction is based on the determination of the % of MM cells expressing the Ki67 protein, which is a nuclear antigen expressed by cells in G1, S and G2M phases, but not by cells in G0. Lokhorst et al (1988) showed that the percentage of plasma cells expressing Ki67 increased significantly from MGUS (median 1.6%), to untreated MM (median 9.6%) and to relapsed MM (median 41.8%) suggesting a significant increase in the proliferative fraction with MM disease progression. Later studies from Falini et al (1988) and Girino et al (1991) employing immunocytochemical staning of MM bone marrow sections confirmed the findings of Lokhorst et al in showing a significant increase in MM cells expressing Ki67 with disease progression. More recently, Drach et al (1992) conducted a detailed analysis of Ki67 expression in MM cells in which he stained BM MNCs from MM patients with anti-CD38, anti-Ki67 and Propidium Iodide (Ki67/PI). The S/G2M fraction of the MM cells was then calculated by determining the % of CD38-positive cells that expressed
both Ki67 and PI. As the initial assay required only three-colour flow cytometry, this assay also offered the possibility of analyzing the proliferative capacity of sub-populations of MM cells (e.g. CD56, CD45 or CD20-expressing). Therefore, an assay based on the expression of CD138, Ki67 and PI was explored during initial and subsequent experiments.

3.2 Materials and Methods

3.2.1 CD138/Ki67/Propidium Iodide Cell Cycle Assay

This assay permits the detection of the S/G2M or “proliferative” fraction of MM cells by identifying MM cells that express Ki67, a nuclear protein that is expressed by cells in the G1 and S/G2M phases of the cell cycle, and show increasing DNA content. The assay can be used to determine the S/G2M fraction of MM cells, either purified, or in bone marrow MNCs. The Ki67/PI assay was first optimised using HMCLs and freshly isolated human CD34+ cells as shown in Figure 3.1. Two representative examples (One HMCL and one primary MM sample) of CD138/Ki67/PI FACS analysis are shown in Figures 3.1B and 3.1C and the gating order is outlined in the figure legend. The final gate includes the S/G2M fraction, as indicated in these figures. The method for CD138/Ki67/PI staining of CD138-selected MM cells is outlined in detail below. During the optimization phase of this assay, it was found that the duration of the “fixation” and “permeabilisation” phases were critically important. Initial experiments employed a 10-minute fixation and permeabilisation phase, but this was found to be too harsh, for primary MM cells in particular. Ultimately, a rapid (1 minute) period of fixation and permeabilisation was found to provide optimal results.
Method:

- 5 x 10^5 purified (CD138-selected) cells were resuspended in 50µl of FACS buffer (PBS/0.1%BSA) in FACS tube A and 1-2 x 10^5 CD138-selected cells were resuspended in 50µl of FACS buffer in tube B.
- 5µl of APC-conjugated anti-CD138Ab was added to tube A and 5µl of APC-conjugated isotype control was added to tube B and the cell suspensions were incubated in the dark for 30 minutes on ice.
- The cells in tubes A and B were then “fixed and permeabilised”
- Firstly, the cells in each tube were washed once in FACS buffer (300g for 5 minutes) and then resuspended cells in 500µl of ice-cold PBS.
- 500µl of ice-cold 4% paraformaldehyde was added to each FACS tube for 1 minute only on ice, before the cells were again washed in FACS buffer (300g for 5 minutes).
- The cells in each tube were then resuspended in 500µl of PBS before 500µl of 0.1% Triton-X solution was added to each tube - again for 1 minute only at room temperature.
- The cells were washed again in FACS buffer (300g for 5 minutes) before being resuspended in 50µl of FACS buffer.
- At this point the cell suspension in tube A was divided between 2 tubes (A1 and A2) and the total solution in each tube was made up to 50µl with FACS buffer.
- Then 10µl of FITC-conjugated anti-Ki67Ab was added to tube A1 and 10µl of FITC-conjugated IgG1 isotype control was added to tube A2. No further antibody-solution was added to tube B as this served as a control for CD138 expression.
- The cells were then incubated for 1 hour on ice in the dark, after which they were again washed in FACS buffer (300g for 7 minutes) and resuspended in 500µl of FACS buffer.
- 5µl of a 2.5mg/ml propidium iodide solution was added to tubes A1 and A2 and the cells were then analysed by flow cytometry.

The process of MM cell purification from BM aspirates, by both positive and negative selection methods, has previously been outlined in detail in Chapter 2. Depending on the cell numbers available, primary MM cells were routinely cultured in 96, 48 or 24 well plates at a concentration of 1 x 10⁶ cells per ml of culture medium. Culture media containing pooled serum and plasma from myeloma patients and normal donors were prepared as previously described. MM cell viability was assessed by trypan blue exclusion and annexinV/PI staining, and cell cycle progression was determined by Ki67/PI staining as described above along with the other methods described in chapter 2. The results section of this chapter will focus on a comparison of the abilities of different culture media to sustain MM cell survival in vitro, whereas the results of CD138/Ki67/PI staining for MM cells taken from MM patients at different disease stages will be discussed in chapter 4.
Figure 3.1

A

B

DNA content
Figure 3.1: Determining the S/G2M fraction with CD138/Ki67/PI staining. (A) The H929 and KMS12BM myeloma cell lines and freshly isolated human CD34+ cells were first fixed and permeabilised before staining with FITC-conjugated anti-Ki67 and PI as shown. (B) MM1S cells and (C) Purified (CD138-selected) primary MM cells were stained with APC-conjugated CD138 or APC-conjugated anti-isotype control. Following fixation and permeabilisation the cells were incubated with either FITC-conjugated anti-Ki67, or FITC-conjugated anti-isotype control and PI. Cells expressing both Ki67 and PI constitute the S/G2M fraction as was determined using the gating sequence as shown in Figure 3.1B and C. The S/G2M fraction is indicated in the top right panel in each figure.

3.3 Results

3.3.1 In-vitro culture in 20% MM plasma improves primary MM cell viability

Initial experiments were aimed at determining the culture medium that would provide optimal survival for purified MM cells. Therefore, freshly isolated MM cells were set
up in culture with RPMI-1640 containing 20% pooled MM plasma (hereafter called culture medium – CM) versus standard culture medium; RPMI-1640/10% fetal calf serum medium (10%FCS) and cell survival compared after 72 hours. These experiments employed MM cells purified from 38 patients with progressive MM (relapsed MM n=34, de novo MM n=4). CM was found to be superior in promoting MM cell survival, enabling a recovery of 60.2 ± 5.3% (mean ± SEM) of the starting number of CD138+ cells after 72 hours, compared with 40.6 ± 3.9% in 10%FCS (p<0.001, Figure 3.2A). This finding was confirmed by annexinV/PI staining in 14 further cases, where again the mean viability for cells cultured in CM was >50% after 72 hours, and significantly greater than that achieved with RPMI/10%FCS (Figure 3.2B, p<0.0001). Representative FACS plots from 4 different patients are shown in Figure 3.2C and these emphasise the superior viability achieved with the plasma containing CM compared with RPMI/10%FCS. The viability of CD138+ cells was also found to be dependent on the concentration of pooled myeloma plasma within the culture medium increasing significantly from 10% to 40% as shown in Figure 3.2D. Increasing the concentration of FCS led to a modest improvement in CD138+ cell viability, but at all concentrations examined was found to be significantly inferior to the plasma-containing medium. The superior anti-apoptotic effect of CM over RPMI/10%FCS was confirmed by the increased levels of the cleaved form of PARP protein in CD138+ cells cultured in RPMI/10%FCS (Figure 3.2E).
Figure 3.2

A

B
Figure 3.2: Survival of primary CD138+ MM cells in culture. (A) Primary CD138+ MM cells were cultured for 72 hours in RPMI with either 10% foetal calf serum (10%FCS) or 20% plasma pooled from MM patients (culture medium, CM). Viable CD138+ cells after 72 hours, expressed as a percentage of the initial viable CD138+ population, mean ± SEM, n = 38. (B) CD138+ cells (n=14) were cultured in RPMI alone (0%), RPMI/10%FCS or CM for 72 hours. Viability was determined using annexinV/PI staining and FACS analysis: viable fraction was AnnexinV-/PI-negative. (C) Four representative cases from B are shown (% indicates viable fraction). (D) CD138-selected cells from a patient with t(11;14) were cultured for 72 hours in increasing concentrations of FCS and CM with RPMI1640. Data shown are mean of triplicates ± S.D. (E) CD138-selected cells from a patient with t(11;14) were cultured in both RPMI/10%FCS and CM for 72 hours, after which PARP expression was analysed by western blotting as shown.
3.3.2 Culture in pooled myeloma plasma preserves the proliferative fraction of CD138+ cells

The next experiments were aimed at determining whether the proliferative fraction of purified CD138+ cells could be maintained during in vitro culture in either RPMI/10%FCS or plasma-containing medium. The proliferative fraction of CD138+ cells was measured by staining for Ki67/PI expression, and in keeping with the findings of the viability assays, CM was superior to RPMI/10%FCS in maintaining the proliferative fraction of primary MM cells. After 72 hours in CM, both the absolute number and % of CD138+ cells in S/G2M was significantly higher in cells cultured in plasma compared with cells cultured in RPMI/10%FCS (p<0.05, Figure 3.3A and 3.3B). Culture of CD138+ cells from nine patients in CM also resulted in a substantial increase in [3H]-thymidine incorporation after 72 hours, although the difference between CM and RPMI/10%FCS did not reach statistical significance (Figure 3.3C, p=0.07).

Figure 3.3

A
Figure 3.3: Effect of pooled myeloma plasma on CD138+ cell proliferation. (A) Absolute number of CD138+ cells in S/G2M (proliferative fraction) at the time of isolation (0 hrs) and following 72 hours culture in either 10%FCS or CM. Data are presented as mean ± SEM (n=34). (B) CD138+ cells (n=34) were cultured for 72 hours in RPMI/10%FCS or CM before Ki67/PI staining. The % S/G2M fraction was determined as described in methods. (C) [3H]-thymidine incorporation at 0 hrs and following 72 hours culture in RPMI/10%FCS or CM. Data are mean ± SEM (n=9). CPM: counts per minute.
3.3.3 Normal human plasma is equivalent to myeloma plasma in maintaining the in-vitro survival of primary MM cells

Having found that CM provided a significantly superior medium for the culture of primary MM cells when compared with standard culture medium (RPMI/10%FCS), the next experiments compared the effects of plasma and serum from normal donors and MM patients on the in vitro survival of primary MM cells. Kirshner and colleagues (2008) had observed that the addition of plasma pooled specifically from MM patients was a vital requirement for the survival of primary MM cells in 3-D culture. In their experience, culturing MM cells in either FCS or normal donor plasma failed to give equivalent results. For these experiments, plasma was collected from healthy volunteers in an identical fashion to the collection of plasma from MM patients. CD138-selected MM cells from 8 patients were cultured for 72 hours in both RPMI/20% pooled normal plasma (NP) and CM and somewhat surprisingly, no difference in MM cell viability was observed between the 2 media (Figure 3.4A). Jones et al (2003) compared the effects of both human plasma and human serum on the in vitro survival of primary CLL cells and found that culture in plasma significantly improved the viability of CLL cells when compared with culture in serum-containing medium. The effects of pooled human serum (from normal donors and myeloma patients) were then compared with those of pooled human plasma (normal and myeloma). Results indicate that that the plasma-containing media modestly improved the survival of CD138+ cells (p=NS), in comparison with the media containing both normal donor and myeloma serum, which gave equivalent results (Figure 3.4A). The differential effects of culture in either serum or plasma-containing media on the proliferative fraction of CD138+ cells were also compared in purified MM cells from 2 patients (Figure 3.4B and 3.4C). In keeping with results from the viability assays, culture in the plasma-containing media led to a greater proportion of CD138+ cells in S/G2M after 72 hours, in comparison with both serum-
containing media. In a further experiment, CD138+ cells from a patient with deletion 17p were cultured in 10% and 20% concentrations of normal and myeloma serum and plasma as well as RPMI/10%FCS. As shown in Figure 4D, [3H]-thymidine uptake after 72 hours was significantly greater in the cells cultured in both normal and myeloma plasma at 10% and 20% concentrations in comparison with all of the other conditions regardless of the concentration of serum.

Figure 3.4

A

B
Figure 3.4: Effect of normal donor serum and plasma on CD138+ cell viability. (A) CD138-selected cells (n=8) were cultured for 72 hours in RPMI, RPMI/10%FCS, RPMI/20% pooled normal donor serum (NS), RPMI/20% pooled normal donor plasma (NP), RPMI/20% pooled myeloma serum (MS) or RPMI/20% pooled myeloma plasma (MP). Viability was assessed by AnnexinV/PI staining and data are expressed as mean ± SEM. (B) and (C) CD138-selected cells from 2 patients were cultured as in A the % of CD138+ cells in S/G2M was determined by Ki67/PI expression as outlined in the Methods section of this chapter. The % of cells in S/G2M at the time of isolation is shown for comparison. (D) CD138-selected cells from a patient with 17pdel were cultured for 72 hours in RPMI, RPMI/10%FCS, RPMI/10-20% pooled normal donor serum (NS), RPMI/10-20% pooled normal donor plasma (NP), RPMI/10-20% pooled myeloma serum (MS) or RPMI/10-20% pooled myeloma plasma (MP), before proliferation was measured by [3H]-thymidine incorporation after 72 hours. Data shown are the results of triplicates ± S.D.
3.3.4 Effects of supplemental albumin and heparin on MM cell survival

Jones et al (2003) demonstrated that culturing primary CLL cells in plasma rather than serum significantly improved their in vitro survival. They went on to identify albumin as the critical component of plasma that was responsible for the highly significant survival difference between plasma and serum-containing media. Therefore, CD138+ cells from a patient with 17p deletion were cultured in RPMI with human serum albumin (HAS) added at concentrations increasing up to 5% and viability was assessed after 72 hours. As shown in Figure 3.5A, the viability of CD138+ cells was only marginally better than that achieved with RPMI alone and was significantly below that achieved after culture in CM. As the blood samples from which the plasma was prepared were collected in lithium-heparinised containers, increasing concentrations of heparin sulphate were added to RPMI/10%FCS, before the viability of CD138+ cells from a patient with t(14;16), cultured in the heparin-containing media for 72 hours, was assessed by annexin-V/PI analysis (Figure 3.5B). Interestingly, the addition of heparin to RPMI/10%FCS significantly increased the viability of CD138+ cells above that seen with RPMI/10%FCS alone in a non-dose dependent fashion. However, the viability of CD138+ cells cultured in the heparin-containing media was still substantially less than that achieved with RPMI/10% pooled myeloma plasma (Figure 3.5B). Blood samples from MM patients were also collected in non-heparinised containers (citrate-lined) and the viability of CD138+ cells cultured in RPMI and a 20% concentration of citrated plasma was less than that achieved after culture in the standard plasma containing medium (Figure 3.5C). Lastly, the ability of a serum-free culture medium to maintain the viability of purified CD138+ cells in vitro was assessed by culturing CD138+ cells from a patient with t(14;16) in X-VIVO for 72 hours and assessing viability in comparison with other culture conditions. As shown in Figure 3.6, both the percentage
and absolute number of viable cells remaining after 72 hours culture in X-VIVO was substantially less than with the other culture conditions.

Figure 3.5

A

![Graph A showing % Viability vs Albumin with 0%, 1%, 2%, 3%, 4%, 5%, 10% FCS, and 10% CM conditions.]

B

![Graph B showing % Viability vs Heparin with 10% FCS and different concentrations of Heparin (0.5μl/ml to 10μl/ml).]
Figure 3.5: Effects of supplemental heparin and albumin on MM cell survival. (A) CD138-selected cells from a patient with 17p deletion were cultured in the presence of RPMI1640 alone or with the addition of 10%FCS, increasing concentrations of human serum albumin, or 10% pooled myeloma plasma (10%CM). Viability was assessed after 72 hours culture by annexinV/PI staining. (B) CD138-selected cells from a patient with t(14;16) were cultured for 72 hours in RPMI/10%FCS ± increasing combinations of unfractionated heparin sulphate. Viability was assessed as in A. (C) CD138-selected cells were cultured for 72 hours in RPMI alone, RPMI/10%FCS or RPMI/20% pooled myeloma plasma (CM), where the plasma was collected in either lithium heparin-lined containers or citrate-lined containers. The % of apoptotic cells is indicated.
Figure 3.6: Effect of serum-free culture medium on MM cell survival. CD138-selected cells from 2 different patients (A and B) were cultured in RPMI1640, X-VIVO10, RPMI/10%FCS and RPMI/20% myeloma plasma (CM). The viable fraction (as indicated) was determined by staining with annexinV/PI. Absolute cell numbers were quantified by simultaneously analysing fluorospheres.
3.4 Discussion

Maintaining the viability of primary MM cells in vitro is notoriously difficult, and the tendency of the majority of CD138-selected cells to undergo apoptosis after 48-72 hours in vitro has hampered experiments examining the mechanisms responsible for cell cycle progression in MM cells. The results presented above indicate that a culture medium consisting of RPMI and 20% plasma pooled from myeloma patients (CM) is significantly superior to RPMI/10%FCS at maintaining MM cell viability in vitro (Figure 3.2). Furthermore, CM preserved the proliferative fraction of CD138+ cells and in some cases substantially encouraged in vitro growth (Figure 3.3D). Importantly, these results were obtained using purified MM cells from a broad variety of MM cases where all major IgH/TCs were represented. The majority of these cases had relapsed disease (n=38, relapsed MM=34, de novo MM=4). These data add weight to the findings of Kirshner et al (2008) who found that plasma pooled from MM patients was a critical factor in promoting the survival of primary MM cells in 3D culture.

There are but a few other publications in the scientific literature that compare the in vitro growth-promoting or survival effects of human plasma with those of serum. Okuno et al (1991) examined the growth pattern of myeloma cells in response to both IL-6 added to both liquid suspension and semi-solid culture media. Although not the principal finding of the study, these authors noted that adding human plasma to the culture media led to enhanced growth and survival of MM cells in comparison with added FCS, which was found to be inferior. In two later studies, firstly Wickremasinghe et al (2001) found that autologous plasma was superior to FCS in protecting CLL cells from spontaneous and chlorambucil-induced apoptosis in vitro through activation of the PI3K/AKT signalling pathway. In agreement with the findings presented in this thesis,
these authors also observed that plasma from normal donors was equally effective at inhibiting apoptosis as plasma derived from CLL patients. They further confirmed this finding by adding neutralizing antibodies targeted at IL-4, IFN-alpha and IFN-gamma to human plasma and found none of these diminished the ability of plasma to inhibit apoptosis. Thus, they postulated that the critical anti-apoptotic factor in human plasma was likely to be a component of normal human plasma.

In a follow-up study from the same group, Jones et al (2003) showed that human albumin was likely to be the plasma protein responsible for the cytoprotective effects of human plasma. In this study, the depletion of human plasma of albumin significantly abrogated its cytoprotective effects whilst the addition of albumin to cultures restored the antiapoptotic effect of plasma as well as its ability to activate the AKT signalling pathway. They also found that plasma from a patient with hereditary analbuminaemia had a significantly reduced antiapoptotic effect in comparison with normal plasma. As both serum and plasma contain similar concentrations of albumin, they subjected human serum to charcoal extraction and found that this process significantly increased the anti-apoptotic effects of human serum. This finding suggested that differences in lipid profiles between serum and plasma may account for the observed differences between the two in their respective abilities to protect CLL cells from apoptosis.

As shown in Figure 3.3A, both normal plasma and myeloma plasma were found to be equivalent in terms of protecting primary MM cells from apoptosis. These results are in keeping with those of Wickremasinghe et al but differ from those of Kirshner et al who observed that normal plasma was ineffective in supporting the longterm growth of primary MM cells in 3-D culture. It remains possible that a key MM growth factor may
be present at increased concentration in pooled plasma from MM patients, thus explaining the improved viability of primary MM cells cultured in this medium, however the results of the experiments with normal donor plasma and the data regarding CLL cells would not support this hypothesis. Wickremasinghe and Jones saw a significant difference between the abilities of human serum and human plasma to attenuate CLL cell apoptosis and although a difference between these two media in attenuating MM cell apoptosis was observed in some experiments, the effect was variable being more pronounced in some patients (Figure 3.3B and 3.3D) and somewhat more modest in others (Figure 3.3A and 3.3C). In keeping with the results obtained with plasma containing media, normal donor serum and serum pooled from myeloma patients were equivalent in their antiapoptotic effects (Figure 3.3A).

As Jones et al (2003) showed that the addition of human albumin to CLL cultures led to a significantly enhanced antipoptotic effect, human serum albumin was added to RPMI in increasing concentrations up to 5% but overall this medium had a deleterious effect on MM cell survival, apart from a 1% solution which led to a small increase in MM cell survival (Figure 3.4A). The MM cell viability achieved with this medium was significantly less than that achieved with CM in the same experiment (Figure 3.5A). MM plasma had been collected in heparinized containers (as described previously, Jones et al, 2003), therefore, in further experiments unfractionated heparin sulphate was added in increasing concentrations to RPMI/10%FCS and this led to a small but significant increase in the MM cell viability after 72 hours in culture, however the viability achieved was substantially less than that seen with CM (Figure 3.5B). Interestingly, plasma collected in citrated containers was less effective in maintaining MM cell viability than plasma collected in heparinised containers (Figure 3.5C). As
expected a serum-free medium (X-VIVO) was inferior to both serum and plasma containing media (Figure 3.6).

In these experiments, a flow cytometric assay incorporating CD138, Ki67 and PI was employed to measure the S/G2M or proliferative fraction of primary MM cells. As mentioned in the introduction to this chapter, this assay was based on the work of Drach et al (1992) who analysed the proliferative fraction of MM cells by using flow cytometry to detect MM cells expressing CD38, Ki67 and PI in mononuclear layers from MM patients. Using this assay, they found that the proliferative fraction of MM cells increased with disease progression in contrast to the plateau phase when the percentage of S/G2M cells was lower. This observation lent weight to the findings of an earlier study, which found that the percentage of Ki67+ MM cells increased with disease progression, as did the percentage of BrdU+ MM cells (Girino et al, 1991). The CD138APC/Ki67PI was first optimized on HMCLs and freshly isolated human CD34+ cells, both of which have relatively high percentages of cells in S/G2M phase (Figure 3.1A and 3.1B) before the S/G2M fraction of purified primary MM cells was determined as shown (Figure 3.1C).

The main objective of these experiments was to optimise a culture medium that would maintain the survival of and proliferative fraction of purified MM cells so that the effects of specific cytokines on MM cell cycle behavior could be studied. As western blotting and thymidine uptake experiments were important parts of the experimental plan, it was critical that purified MM cells were studied. These initial experiments clearly showed that a plasma-containing culture medium was superior to one containing FCS, and therefore this medium (CM) was adopted as the standard culture medium for
future experiments involving primary MM cells. In addition, an assay incorporating
CD138APC/Ki67Ftc and PI was found to provide a reproducible system for the
determination of the percentage of primary MM cells in S/G2M phases and this was
used to study the proliferative behaviour of primary MM cells during in vitro culture in
future experiments.
Chapter 4: Cell cycle analysis of primary myeloma cells and effects of APRIL & BAFF

4.1 Introduction

Having optimized a culture medium (CM) that maintained the survival and preserved the proliferative fraction of primary MM during 72 hours in culture, the next series of experiments were aimed at determining if MM cells could undergo cell cycle progression in response to APRIL and/or BAFF and other MM growth factors. To date, only a handful of studies have sought to determine whether malignant plasma cells undergo cell cycle progression in response to micro-environmental factors. In particular, few studies have addressed the role of D-type cyclins in mediating cell cycle progression in MM cells. Furthermore, most of the available data regarding cell cycle behaviour of MM cells has been gleaned from experiments predominantly using HMCLs which are unlikely to be fully representative of intramedullary MM which accounts for the vast majority of MM patients. Ludwig et al (1983) reported that interferon stimulation of primary MM cells in vitro led to a significant increase in the incorporation of tritiated thymidine in 4 out of 21 samples, indicating that it was possible to increase the % of MM cells in S-phase following cytokine stimulation in vitro. Furthermore, two intriguing studies from Jelinek et al (1997) and Arora et al (1998) examined the responsiveness of different HMCLs to interferon-alpha. In the first study, they found that of 5 IL-6-dependent HMCLS (ANBL-6, ANBM-6, DP-6, and KP-6, KAS-6/1) just two (KAS-6/1 and KP-6) were responsive to interferon-alpha whilst all five cell lines were responsive to IL-6, perhaps providing an early clue that the clinical heterogeneity that is known to exist amongst myeloma patients might be underscored by differential cytokine responsiveness. A second study sought to determine the mechanism underlying the ability of interferon-alpha to promote proliferation of the KAS-6/1 cell line but not the ANBL-6 cell line, as the initial study
had shown that this was not mediated through activation of the JAK/STAT pathway. This subsequent study showed that interferon-alpha stimulation of KAS-6/1 cells led to an increased percentage of S/G2M cells, as well as increased expression of cyclin D2 protein and phosphorylated retinoblastoma protein. Furthermore, interferon-alpha stimulation of ANBL-6 cells led to growth arrest via induction of p19 (INK4d) protein without any significant change in the expression of p15, p16, p18 or p21. In more recent work from our laboratory, Glassford et al (2007) examined the effects of IGF-I stimulation on cell cycle progression in MM cells. Firstly, they found that D-type cyclins, CDK4, CDK6, p27 and phosphorylated-pRb were expressed, not only by HMCLs, but also in a large panel of primary MM cells. They also found that IGF-I stimulation of primary MM cells expressing cyclin D2 protein, led to increased thymidine incorporation as well as increased expression of cyclin D2, CDK4, CDK6 and phosphorylated-pRb proteins indicating cell cycle progression. In a related study, Ely et al (2005) examined the expression of cell cycle proteins in MM patients by immunohistochemical staining of patient BM biopsies as well as by western blotting. Importantly, their work questions the role of cyclin D1 protein in promoting cell cycle progression (in those cells where it is expressed), as they found there was no correlation between the expression of cyclin D1 and Ki67 proteins. Furthermore, they found that the expression of cyclin D1 protein did not significantly alter with disease progression.

On the same theme, Glassford et al (2007) found that cyclin D1 protein expression in KMS12BM cells and primary MM cells carrying t(11;14) was not upregulated in response to IGF-I stimulation. Neither was [3H]-thymidine incorporation increased in the same cells in response to IGF-I stimulation. Although, these findings question the role of cyclin D1 in cell cycle progression in MM cells, Glassford et al also showed that siRNA-mediated knockdown of cyclin D1 led to cell cycle arrest in KMS12BM cells.
confirming a key role for cyclin D1 in maintaining cell cycle progression in MM cells with t(11;14) (Glassford 2007). These findings suggested that perhaps MM cells with t(11;14) may not rely upon mitogenic stimulation by growth factors such as IGF-I for cell cycle progression. Somewhat surprisingly, Ely et al found that CDK6 expression was restricted to MM cells where cyclin D2, but not cyclin D1 was expressed, suggesting exclusive cyclin D-CDK pairings in MM cells (Ely 2005). This work contrasted somewhat with the work of Glassford et al (2007), where CDK6 expression was found in primary MM cells expressing cyclin D1 as well as HMCLs.

Although it has been shown that APRIL and BAFF play a significant role in mediating drug resistance in MM cells, little is know of their ability to promote cell cycle progression in MM cells. Although Moreaux et al (2009) have shown that APRIL stimulation significantly increases thymidine incorporation in XG-1 and LP-1 HMCLs in comparison with control after 96 hours in culture, this has been in the context of drug treatments of the MM cells, therefore it is difficult to determine if these responses were related primarily to a survival or a cell cycle-promoting effect. Furthermore, the effect of APRIL and BAFF on D-type cyclin and cell cycle regulatory protein expression has not been studied to date. Therefore, the next series of experiments was firstly aimed at determining the baseline proliferative fraction of primary MM cells. The second objective was to stimulate primary MM cells with APRIL and other MM growth factors and document the cell cycle response along with changes in cell cycle protein expression. Further objectives included determining the expression of the APRIL-receptors BCMA and TACI by primary MM cells as well investigating whether the expression of D-type cyclin protein expression (along with other cell cycle regulatory proteins) could be determined by immunohistochemical staining of MM BM biopsies.
4.2 Materials and Methods

The assays and culture conditions used in the experiments outlined below have already been detailed extensively in chapters 2 and 3. Briefly, the proliferative fraction of CD138-selected primary MM cells was calculated using the Ki67/PI FACs assay, both at the time of isolation and also following 72 hours culture in CM ± APRIL or BAFF (both 200ng/ml) as outlined in chapter 3. The culture medium (CM, RPMI/20% pooled myeloma patient plasma) was prepared in the same manner as outlined in chapters 2 and 3. The methods and materials employed in the FACS analysis of BCMA and TACI expression, Western blotting experiments and immunohistochemistry are all detailed in chapter 2. As before, depending on the cell numbers available, primary MM cells were routinely cultured in 96, 48 or 24 well plates at a concentration of 1 x 10^6 cells per ml of culture medium. The disease characteristics of the MM patients from whom the samples used in these experiments were obtained are summarized in Table 4.1.

4.3 Results

4.3.1 The proliferative fraction in freshly purified MM cells increases with disease progression

MM cells were freshly purified either by CD138-positive selection or Rosette-Sep negative selection with >90% purity. The proliferative (S/G2M) fraction was then determined using the CD138/Ki67/PI FACs assay as described previously. The S/G2M fraction was determined by gating on the CD138+, Ki67+ population that showed increasing PI staining (see gates in Figure 4.1A). In total, 143 samples were examined and the percentage of CD138+ cells in S/G2M varied significantly with disease stage. As expected, the S/G2M fraction of CD138+ cells during plateau phase following autologous transplantation or in smouldering MM was low (1.1 ± 0.29% and 1.1 ± 0.39% respectively, mean ± SEM). The S/G2M fraction was also low in newly
diagnosed samples, but then was found to increase significantly with disease progression (Figure 4.1B). For example, in 31 patients with newly diagnosed MM, the % of CD138+ cells in S/G2M was 1.7 ± 0.3%, compared with 3.4 ± 0.3% for 91 patients with relapsed disease (p<0.001, Figure 4.1B). Unsurprisingly, the highest proliferation rate was seen in CD138+ cells from patients with plasma cell leukaemia or myelomatous pleural effusions (6.8 ± 1.8%, p<0.05 compared with relapsed disease group). The purified MM cells in these cases were obtained from several sources. Of these 9 cases, CD138+ cells were obtained from BM in 5 cases, peripheral blood in 3 cases and pleural fluid in 1 final case. Purified MM cells were used in all cases except for the post-autograft cases where mononuclear cells were used because of the low numbers of CD138+ cells frequently found in these samples.
Figure 4.1: The S/G2M (proliferative) fraction of freshly purified CD138+ cells varies in accordance with disease stage. (A) Freshly purified primary MM cells were first stained with APC-conjugated anti-CD138, then fixed and permeabilised, before intracellular staining with FITC-conjugated anti-Ki67 and DNA-staining with propidium iodide (PI). Gating was on the CD138+ cells and the proliferative fraction in S/G2M was gated as shown in Figure 4.1A, which illustrates representative patients at different MM disease stages. (B) Data are mean ± standard error (SEM) for each group: SMM – n=4, De Novo MM – n=31, Plateau – n=8, Relapse – n=91, PCL/PE – n=9.
4.3.2 APRIL promotes cell cycle progression predominantly in cyclin D2-expressing MM cells with IgH/TCs

Having established an assay to determine the S/G2M fraction of primary MM cells, the overall aim of the next experiments was to determine the effect of APRIL and BAFF on the cell cycle behaviour of primary MM cells. When primary MM cells were cultured in RPMI-1640 alone, neither the addition of APRIL nor BAFF had a significant effect on the %CD138+ cells in S/G2M (data not shown). Therefore, APRIL and BAFF were added to CM to try and harness the survival effects already observed with this culture medium in the absence of added growth factors. In the presence of CM, APRIL was seen to increase the fraction of CD138+ cells in S/G2M (Figure 4.2A). Initial experiments employed a time course up to 72-96 hours and these indicated that maximal cell cycle progression in response to APRIL occurred at 48-72 hours (Figure 4.2A). As before, the S/G2M fraction was calculated as in Figure 4.1A by gating on the CD138+ cells. Figure 4.2A shows the responses to APRIL stimulation in 3 cases with cyclin D2 expression: Cases #1 and #13 carrying the t(14;16) translocation, with case #12 expressing cyclin D2 in the absence of an IgH/TC (See Table 4.1 for case details)

Thereafter, I examined the effect of APRIL and BAFF on CD138+ cells after incubation for 72 hours. Twenty-six cases were examined, and these showed a marked variation in responsiveness to growth factors (see Table 4.1 for details of cases). Figure 4.2B shows the FACS profiles from a typically responsive case, where after 72 hours in culture the % of CD138+ cells in S/G2M increase from 1.5% in CM to 10.3% with APRIL. This patient also showed a marked response to IL-6 with somewhat lesser responses to BAFF and IGF-I. In contrast, the case shown in Figure 4.2C shows minimal responsiveness to either APRIL, or to any of the other growth factors (BAFF, IL-6 or
IGF-I), despite a significant proportion of CD138+ cells being in S/G2M phase at time of isolation (0 hours), and also after 72 hours in CM alone. Importantly, in Figure 4.2B, the MM cells were purified from a patient with de novo MM whose cells carried t(14;16), in contrast to the case detailed in Figure 4.2C where the CD138+ cells expressed cyclin D1 (aggressive 2nd relapse) and harboured t(11;14).

**Figure 4.2**

A

![Graphs showing cell distribution](image)

B

**Patient #1 – Cyclin D2+ t(14;16)**

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</tr>
<tr>
<td>IGF-I</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
</tbody>
</table>

DNA Content
Figure 4.2: APRIL promotes cell cycle progression in primary MM cells. (A) Purified CD138+ MM cells were cultured in 20% MM patient plasma (CM) ± APRIL (200ng/ml). Cell cycle responses were calculated as for Figure 4.1. Results are expressed as the percentage of CD138+ cells in S/G2M. Three representative cases showing time course of APRIL-induced cell cycle progression of CD138+ MM cells in the presence of CM alone or CM + APRIL (control=CM alone, APRIL=CM+APRIL). (B and C). Representative examples of cell cycle responses to cytokines in primary MM cells, incubated in CM alone (control) or with APRIL, BAFF (both 200ng/ml), IGF-I (500ng/ml) or IL-6 (100ng/ml). FACS plots show the S/G2M fraction of CD138+ cells following 72 hours culture in CM ± growth factors, in comparison with freshly isolated cells (0 hours). (B) Cyclin D2-expressing case with t(14;16) (#1, Table 4.1) (C) Cyclin D1-expressing case with t(11;14) (#20, Table 4.1).
Next, the cell cycle responses to APRIL and BAFF were compared according to D-type cyclin class. The expression of cyclins D1 or D2 was determined for each case by immunohistochemical staining of BM biopsies and/or western blotting. Twelve cases were found to express cyclin D2 and 14 to express cyclin D1. Table 4.1 shows the disease characteristics, D-type cyclin, and IgH/TC status for these patients. When the effects of APRIL and BAFF stimulation were examined according to D-type cyclin expression, MM cells expressing cyclin D2 showed a significant increase in %CD138+ cells in S/G2M following 72 hours culture in CM+APRIL compared to CM alone, (8.3 ± 2.1% with CM versus 13.4 ± 2.2% with APRIL p<0.01, mean ± SEM, Figure 4.3A). A lesser effect was seen with BAFF (p<0.05). In contrast, the mean S/G2M fraction for 14 cyclin D1-positive cases following culture in CM was 3.1% ± 0.5 with no significant increase observed with APRIL or BAFF (3.4% ± 0.6 and 2.7% ± 0.7 respectively, Figure 3A). Cases expressing cyclin D2 were then further sub-divided into those with and without IgH/TCs. While 8 cases with IgH/TCs (t(4;16) n=5, t(4;14) n=3) showed a marked response to APRIL, cases without IgH/TCs had lesser responses (Figure 4.3B).

Figure 4.3
A
Figure 4.3: APRIL promotes cell cycle progression in primary MM cells expressing cyclin D2 but not cyclin D1. (A) Effect of APRIL (200ng/ml) or BAFF (200ng/ml) on the cell cycle progression of CD138+ MM cells cultured in CM. Results are presented as mean ± SEM % of CD138+ cells in S/G2M. Fourteen patients expressed cyclin D1 (#16-29, Table 1) and 12 patients expressed cyclin D2 (#1-12, Table 1). The S/G2M fraction at the time of isolation (0 Hrs) is shown for comparison. (B) The cell cycle response to APRIL in cyclin D2 expressing CD138+ cells harbouring IgH/TCs (n=8) compared to cells without IgH translocations (n=7), #1-15, Table 4.1.

Further confirmation that APRIL induces DNA synthesis in MM cells expressing cyclin D2 was obtained by using [3H]-thymidine and bromodeoxyuridine (BrdU) incorporation assays. As shown in Figures 4.4A and 4.4B, APRIL stimulation of CD138+ cells from a patient with t(14;16) led to a significant increase in [3H]-thymidine incorporation after 72 hours culture in comparison with control, as well as a substantial increase in S/G2M cells as determined by bromodeoxyuridine incorporation (Figure 4.4B).
Figure 4.4: APRIL-induced proliferation in primary MM cells bearing t(4;14) and t(14;16). (A) CD138+ cells from patient #3 (expressing cyclin D2 with t(14;16)) were cultured in RPMI with (CM+) or without plasma (CM-) alone or with APRIL or BAFF (as indicated) for 72 hours and pulsed with [3H]-thymidine for the last 2 hours. Data is presented as mean ± SD of triplicates. (B) CD138+ cells were pulsed with BrdU for 2 hours then stained as described in chapter 2. FACS plots (gated on CD138+ cells) showing BrdU uptake at the time of cell isolation (0 hrs) and after 72 hours culture in CM alone (control) or with APRIL or BAFF (200 ng/ml) from one representative case harbouring t(14;16), and expressing cyclin D2 (patient #3, Table 4.1). The S/G2M fraction, with % cells, is indicated in each FACS plot.
These experiments were carried out in CM because this medium allowed superior recovery of viable cells after 72 hours in comparison with standard culture media. However, similar results were obtained when CD138+ cells from 3 cases were cultured in RPMI/10% FCS ± APRIL 200ng/ml (Figure 4.5). In keeping with the results of experiments in CM, only the CD138+ cells from the case with t(14;16) were APRIL-responsive, in contrast to the CD138+ cells expressing cyclin D1 and D2 in the absence of an IgH/TC (Figure 4.5).

**Figure 4.5**

**Figure 4.5**: Effect of RPMI/10%FCS ± APRIL on cell cycle progression. CD138+ cells from 3 patients were cultured in RPMI/10%FCS ± APRIL 200ng/ml and CM ± APRIL 200ng/ml for 72 hours. The effect on cell cycle was analysed by quantifying the number of CD138+ cells in S/G2M in each of the culture conditions. Patient details: (A) Relapsed disease - Cyclin D2+ without an IgH/TC (B) Relapsed disease - Cyclin D1+ with RPMI/10%FCS (C) De Novo Disease – Cyclin D2+ with t(14;16)
Further experiments were aimed at showing that the effect on cell cycle progression was specific to APRIL. By measuring [3H]-thymidine incorporation after 72 hours incubation, cell cycle responses to APRIL were found to be dose-dependent and maximal at 100ng/mL (Figure 4.6 left panel). Furthermore, the proliferative effect of APRIL on CD138+ cells from a patient with t(4;14) was inhibited the addition of TACI-Fc (Figure 4.6, right panel). Identical results were seen in experiments with the OPM2 cell line (data not shown). As these experiments had shown that APRIL had a greater effect on cell cycle than BAFF, further experiments were focused on APRIL.

**Figure 4.6**

**Figure 4.6: Dose Response to APRIL and effect of TACI-Fc.** Left panel: Dose response to APRIL in 1 representative patient. CD138+ purified cells (Patient #7) were cultured with increasing concentrations of APRIL for 72 hours, and pulsed with [3H]-thymidine for the last 2hrs. Mean ± SD of triplicates. Right panel: TACI-Fc blocks APRIL-induced proliferation. TACI-Fc (10µg/ml) was added to CD138+ MM cells prior to culture for 72 hours, and DNA synthesis assessed as above. Mean ± SD of triplicates (Patient #7).
4.3.3 Expression of BCMA and TACI by MM cells

APRIL binds BCMA, TACI and HSPGs all of which have been shown to be present on the surface of MM cells. The next experiments examined the expression of BCMA and TACI on MM cells and their possible role in APRIL-mediated cell cycle progression and whether the pattern of expression was related to D-type cyclin expression. BCMA and TACI expression were determined by flow cytometry. Antibody staining was first optimised using the K562 cell line that had been transduced separately with both BCMA and TACI (kindly provided by Dr. Martin Pule, UCL Cancer Institute). A large panel of HMCLs was first stained with both anti-TACI and anti-BCMA as described in chapter 2. Representative histograms are shown in Figure 4.7A with the cell lines grouped according to IgH/TC. As shown, expression of BMCA and TACI was found to be variable but was also found across all molecular groups. In particular BCMA expression was found in 8/9 HMCLs analysed, with just the KMM1 cell line showing no expression, whereas TACI expression was found in 3/7 cell lines examined. The expression of BCMA and TACI by primary MM cells was then studied in CD138+ cells from 28 patients. Although levels varied, most patients showed low to moderate expression of both of these receptors. Figure 4.7B shows BCMA and TACI expression by primary MM cells that are grouped according to IgH/TC. In keeping with the findings in HMCLs, expression of both receptors was found in MM cells expressing either one of t(11;14), t(4;14) or t(14;16). Overall, MM cells showed a greater intensity of staining for BCMA compared with TACI, median MFIr 1.9 (range 0.8 – 13.0) vs.1.3 (range 0.8 – 5.3, Figure 4.8). Importantly, no significant difference in median BCMA or TACI expression was observed between cyclin D1 and cyclin D2 positive MM cells (Figure 4.8).
Figure 4.7

A

\[
\begin{align*}
&\{t(4;14)\} \\
&\{\text{OPM2}\} \\
&\{\text{JJN3}\} \\
&\{t(14;16)\} \\
&\{\text{RPMI8226}\} \\
&\{\text{MM1S}\} \\
&\{\text{KMS27PE}\} \\
&\{t(11;14)\} \\
&\{\text{KMS12BM}\}
\end{align*}
\]
Figure 4.7: BCMA and TACI expression by flow cytometry. (A) HMCLs and (B) Freshly purified CD138+ cells were first incubated with anti-BCMA or anti-TACI followed by staining with PE-conjugated goat anti-rat IgG A. Representative histograms showing BCMA and TACI expression in MM cells with varying IgH/TCs.

Figure 4.8

Figure 4.8: Expression of BCMA and TACI according to D-type cyclin status. Levels of BCMA and TACI expression according to D-type cyclin class (MFIr = Median Fluorescence Index ratio). Median values are indicated (n=14 in each group).

4.3.4 APRIL differentially activates cell cycle proteins in cyclin D2- but not cyclin D1-expressing primary MM cells

The expression and modulation of cell cycle regulatory proteins in primary MM cells was next studied, both at the time of isolation and then in response to APRIL stimulation. Firstly, the expression of cyclin D1, cyclin D2, CDK4, CDK6 and phosphorylated pRb was determined by immunohistochemical staining of MM patient BM trephine biopsies. These results were further confirmed by western blotting. By immunohistochemistry, cyclin D2 expression was observed in fifteen cases; five harboured t(14;16), three t(4;14), and seven were negative by FISH for IgH/TC. Of
these last seven, four had 17p deletion. Figure 4.9 shows the expression of cyclin D2 in
BM MM cells from cases #1 and #6 known to have t(14;16). These MM cells were also
found to express CDK4 and CDK6, with prominent expression of phosphorylated pRb
(Figure 4.9), suggesting significant levels of cell cycle progresssion in vivo. In patients
with t(11;14), BM plasma cells strongly expressed cyclin D1, and also CDK4 and
CDK6. Expression of phospho-pRb was variable, but some cases showed significant
expression (#20 and #30). CDK6 expression was found by western blotting and
immunohistochemistry in four cyclin D1+ cases, three of which carried t(11;14).

**Figure 4.9**

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**Figure 4.9: Expression of cell cycle regulatory proteins in myeloma cells.**
Expression of cyclin D and other cell cycle proteins by immunohistochemistry on bone
marrow trephine biopsies. Representative sections from 2 cases with t(14;16) (Case #1
and #6) and 2 cases with t(11;14) (#20 and #30 showing the expression of the
appropriate D-type cyclin (x600 for both), phosphorylated pRb (x 600), CDK4 and
CDK6 (both x 600).
Next, the modulation of cell cycle proteins in response to APRIL was examined by western blotting. All experiments used BM-derived CD138-selected MM cells. In vitro culture for 72 hours with APRIL was found to increase the expression of cyclin D2 as well as CDK4, CDK6 and phospho-pRb in MM cells from patient #13 (Figure 4.10A), in keeping with the cell cycle responses seen in Figure 4.2A (upper left panel). Similarly, in MM cells from patients #1 and #7 with t(14;16) and t(4;14) respectively, in vitro culture with APRIL again upregulated cyclin D2, CDK4 and -6, and phospho-pRb (Figure 4.10B). Furthermore, APRIL stimulation led to upregulation of cyclin E2 in MM cells expressing t(14;16) (patient #13), however the expression of p27 was unaltered (Figure 4.11A). Similar findings were observed in the MM1S cell line which carries the t(14;16) translocation (Figure 4.11B). In contrast, APRIL had minimal effect on cell cycle proteins in cyclin D1 expressing MM (#30 and #31, Figure 4.10C), despite the fact that MM cells from patients with the most aggressive cyclin D1-expressssing disease were used for these experiments. Patient #30 had an aggressive relapse with circulating plasma cells, whilst patient #31 also had an aggressive relapse, just months after autologous transplantation.
Figure 4.10

A

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B

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Figure 4.10: Effect of APRIL on cell cycle protein expression in primary MM cells.

(A) Bone marrow-derived CD138+ cells from a case with t(14;16) (patient #13) were cultured in CM (control) or CM with APRIL 200ng/ml for up to 72 hours, and analysed by western blotting for expression of cell cycle proteins. (B) Expression of cell cycle proteins in BM CD138+ cells from cases #1 and #7 harbouring t(14;16) and t(4;14) respectively, at time of purification, and after 72 hours culture with or without APRIL. (C) Effect of APRIL on the expression of cyclin D1 and other cell cycle proteins in bone marrow-derived CD138+ MM cells with t(11;14) (patients #30 and #31). Protein expression in B and C was quantified using ImageJ software and results are expressed as a ratio to control.
Figure 4.11: Effect of APRIL on Cyclin E expression and the MM1S cell line. (A) CD138+ cells from a case with t(14;16) (patient #13) were cultured as in Figure 4.10 and the expression of cyclin E2 and p27 was examined after 72 hours in vitro stimulation with APRIL. (B) MM1S cells were cultured for 48 hours in the presence of RPMI/10%FCS ± APRIL 200ng/ml, before cell cycle proteins were analysed by western blotting as shown.
Table 4.1 Disease characteristics of patients whose cells were used in cell cycle experiments

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<th>Case</th>
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Legend: Asterisk denotes patients who had circulating plasma cells, Nil=no IgH/TC, LC=light chain, BM= bone marrow, PB = peripheral blood.
4.4 Discussion

4.4.1 APRIL Promotes Cell Cycle Progression in Primary MM cells

Although, it is well established that APRIL is required for the generation and survival of BM plasma cells, and the closely related BAFF is necessary for B-cell activation and survival, as part of normal humoral responses, little is known of the role these cytokines play in promoting the growth of primary MM cells, despite the observation by two independent groups of BCMA and TACI expression in primary MM cells. In the experiments outlined above, APRIL was found to induce cell-cycle entry in primary BM-derived MM cells. However, surprisingly this effect was most apparent in those MM cases that expressed cyclin D2, and particularly those expressing cyclin D2 in the context of an IgH/TC. In contrast, cyclin D1 expressing cells were significantly less responsive. APRIL-stimulation of primary MM cells induced G1/S progression in a time- and dose-dependent fashion and crucially was also accompanied by the up-regulation of cyclin D2, CDK4, CDK6, and phosphorylated-pRb. In contrast, in primary MM cells expressing cyclin D1 with t(11;14), cell-cycle regulatory proteins were unaffected by APRIL, in keeping with the lack of cell-cycle responses seen in the Ki67 FACS analysis.

4.4.2 Expression of BCMA and TACI by MM cells

Both BCMA and TACI expression were found on the surface of a large panel of HMCLs and broad range of primary MM cells (n=28), where the most common IgH/TCs were well represented. These findings reinforce the results of 2 other groups who also found BCMA and TACI to be expressed by MM cells. In particular, BCMA expression was found in the majority of primary MM cells, in keeping with the findings of Moreaux et al (2009) who found BCMA expression and TACI expression in MM
cells from 19 and 15 out of 22 MM patients respectively. Amongst the HMCLs, BCMA expression was found in 8/9 cell lines examined with only KMM1 which carries the t(6;14) translocation showing no expression. TACI expression was found in 3/7 HMCLs examined, which again accords with the data of others who have found TACI expression less commonly than BCMA on HMCLs. Notably, the OPM2 cell line was found not to express TACI in keeping with the results of Moreaux et al (2009). These results also raise two important points: Firstly, as the median expression of both BCMA or TACI was found not to differ significantly between cyclin D1 and cyclin D2 expressing MM cells, these findings suggest that the differential expression of APRIL-receptors is not responsible for the observed difference between cyclin D1 and cyclin D2 expressing MM cells in cell cycle responses to APRIL. Secondly, it is not clear what the key APRIL-receptor interaction is promoting MM cell cycle progression. The interaction between APRIL and TACI has recently been shown to mediate proliferation of follicular NHL cells by activating the PI3-kinase/AKT pathway, which led to the upregulation of cyclin D1. In this study, Gupta et al (2009) showed that siRNA-mediated TACI-knockdown abrogated the growth-promoting effect of APRIL, an effect that was not seen by the addition of heparin to cultures or the treatment of follicular lymphoma cells with BCMA-siRNA. However, Moreaux et al (2009) have observed APRIL-mediated growth in the TACI-negative XG-1 cell line and APRIL-stimulation of OPM2 cells (also TACI-negative) led to the upregulation of phosphorylated-AKT (phospho-AKT, see chapter 6). Thus, APRIL appears capable of stimulating MM cells in the absence of TACI-expression (at least below the limits of detection using antibodies that are commercially available for flow cytometry).

Clearly, APRIL also binds HSPGs and it is possible that many of the APRIL-stimulated effects seen in these experiments may be wholly or partly mediated through APRIL-
HSPG interaction. In fact, MM cells that were repeatedly responsive to APRIL both in cell cycle and drug resistance experiments showed no TACI expression (patient #13, Table 4.1). Further evidence for such an interaction comes from the work of Moreaux et al (2009) who have shown that heparin treatment of MM cells inhibited the growth-promoting effect of APRIL in the XG-1 cell line and APRIL-mediated dexamethasone-resistance in LP1 and L363 cells. The same authors have shown that APRIL, TACI and CD138 interact on the surface of MM cells suggesting a complex interaction may exist between APRIL and its receptors’ that remains to fully elucidated.

4.4.3 Expression of Cell Cycle Regulatory Proteins

Firstly, the expression of both cyclin D1 and cyclin D2 proteins was demonstrated by immunohistochemical staining of MM BM biopsies. Unsurprisingly the strongest expression of cyclin D1 protein was observed in cases where the t(11;14) translocation was found. Importantly, cyclin D2 expression was only observed in cases where there was a complete lack of cyclin D1 expression. Although the original description of the TC groups includes a group comprised of a small percentage of MM patients where increased expression of both cyclins D1 and D2 was found, co-expression of significant amounts of cyclin D1 or D2 proteins was not observed during these experiments, although the number of cases analysed is almost certainly too few to exclude co-expression of cyclins D1 and D2 at the protein level in a minority of cases. The demonstration of cyclin D2 protein expression in MM cells by immunohistochemical staining is an important as this has not been previously recorded to date in the scientific literature. A publication subsequent to this work has since confirmed that this technique is feasible in MM BM biopsies (Neri et al, 2011).
Subsequently, the expression of cell-cycle regulatory proteins was further confirmed by western blotting of cell lysates from purified CD138+ cells from MM patients, emphasising the relevance of the data from the BM biopsy immunohistochemistry, as well as providing further evidence of cell cycle progression in-vivo. In agreement with the findings of the findings of the immunohistochemistry experiments and the findings of Glassford et al (2009), expression of both CDK4 and CDK6 was found in both cyclin D1 and cyclin D2-expressing cases. These findings also support the majority of the data of Ely et al (2005) who documented the expression of cell cycle regulatory proteins by both BM immunohistochemistry and western blotting.

4.4.4 Differential Response to APRIL-stimulation According to D-type Cyclin Expression

The most interesting question raised by these findings is the reason for the difference between cyclin D1- and cyclin D2-expressing MM cells in their relative responsiveness to APRIL stimulation. It would seem that the lack of response in cyclin D1 cells is not necessarily the result of a lower baseline proliferative rate because the % of S/G2M cells was similar in D1 and D2 samples at the time of purification. In addition, only cases that were clinically progressing (de novo or relapsing) were selected to study regulation of cell-cycle progression. Part of the answer may lie in the differential regulation of the genes encoding cyclin D1 and cyclin D2 in MM cells. Cyclin D2 is upregulated in normal B-lineage cells in response to growth factors and mediates cell-cycle entry. Therefore, it may not be surprising that the expression of cyclin D2 is increased in malignant plasma cells in response to growth factor stimulation. In MM cells, IgH/TCs, such as t(4;14) and t(14;16), lead to elevated cyclin D2 mRNA expression indirectly via FGFR3/MMSET or MAF transcription factors, but the level of cyclin D2 protein expression may be dependent on exogenous stimuli, which may also
modulate expression via the natural cyclin D2 gene promoter. Thus, in these cells, increased expression of cyclin D2 occurs as a consequence of mitogen-activated signalling pathways, which may also modulate levels of the CDKs and CKIs resulting in cell-cycle entry. In these experiments, APRIL-stimulation led to the upregulation not only of cyclin D2, but also of CDK4 and CDK6, in responsive MM cells. The up-regulation of cyclin D2 and CDK4 in response to APRIL is somewhat similar to the responses to BAFF seen in resting murine splenic B cells. Although in this study, BAFF alone did not promote S-phase entry, suggesting that additional pathways may be activated in primary MM cells (Huang et al., 2004). The difference in APRIL-induced proliferation between cyclin D2+ MM cells bearing IgH/TCs and those without IgH/TCs, may reflect the relative independence of the latter from external mitogen-activated pathways. In contrast to cyclin D2, cyclin D1 expression in primary MM cells occurs as a direct result of the IgH/TC t(11;14) or via unexplained mechanisms in cells with hyperdiploidy, rather than as a downstream response to mitogen-activated signaling. Cyclin D1 expressed via t(11;14) is not under the control of its natural mitogen-responsive promoter. Furthermore, because cyclin D1 is not normally expressed in B-lineage cells, it is possible that signalling pathways that would normally modulate this protein are not active in MM cells.

4.5 Summary

Several important findings have resulted from the experiments described within this chapter. It has been shown that APRIL promotes cell cycle progression in primary MM cells expressing cyclin D2 (particularly in the presence of an IgH/TC) whilst having a lesser impact on cells expressing cyclin D1. This effect occurred independently of BCMA or TACI expression and was accompanied by the upregulation of cyclin D2 protein and other cell cycle regulatory proteins, whilst cyclin D1 protein was
unaffected. The next chapter will focus on determining whether APRIL promotes drug resistance in primary MM cells, and if so, whether such an effect also segregates with respect to D-type cyclin class. In addition, the pattern and extent of APRIL expression in the MM BM microenvironment will also be explored.
Chapter 5: Effect of APRIL on survival and drug resistance in primary MM cells

5.1 Introduction

Having observed that APRIL was able to promote cell cycle progression in primary MM cells expressing cyclin D2, whilst having less impact on cells expressing cyclin D1, the experimental plan then moved on to focus on the effect of APRIL on survival and drug resistance in primary MM cells. If the observed differential cell cycle response to APRIL between cyclin D1 and D2 expressing MM cells was (as hypothesised) related to differences in the regulation of the signalling pathways controlling cell cycle progression in MM cells, then it might be expected that APRIL would elicit equivalent pro-survival or anti-apoptotic effects in primary MM cells irrespective of D-type cyclin or translocation class.

APRIL was first shown to have provided a survival stimulus to plasma cells in murine studies that also showed BCMA-deficient mice to have significantly reduced mature plasma cell numbers (O’Connor et al, 2004). In addition, a further study that employed murine tissue showed that APRIL provides a critical survival stimulus for plasma cells in mucosa-associated lymphoid tissue (MALT) by increasing the expression of MCL-1, BCLXL and BCL-2 anti-apoptotic proteins (Huard et al, 2008). More recently, macrophage-derived APRIL was shown to be a critical factor for early plasmablast survival in the murine BM. In this study, APRIL-knockout mice failed to support plasmablast survival and APRIL was shown to mediate its survival effect through an interaction between HSPGs and plasma cells that led to increased Bcl-XL expression (Balnoue et al, 2008). However, there is a lack of data relating to the effect of APRIL (and BAFF) on the survival of MM cells with just a handful of publications to date. Moreaux et al (2004) studied the effects of APRIL and BAFF on survival and drug
resistance in HMCLs and primary MM cells. They first demonstrated that APRIL could rescue several IL-6 dependent HMCLs (XG-13, XG14 and XG-20) from the pro-apoptotic stimulus of IL-6 withdrawal and further demonstrated that APRIL attenuated dexamethasone-induced apoptosis in the RPMI8226 and L363 myeloma cell lines. They confirmed the protective effect of APRIL against dexamethasone-induced apoptosis in primary MM cells from 8 patients and also showed that the addition TACI-Fc to mononuclear cell cultures from MM patients led to the reduced survival of MM cells, suggesting significant APRIL-secretion in the MM BM microenvironment. They further showed that APRIL could up-regulate the anti-apoptotic proteins BCL-2 and MCL-1 in the XG-13, XG-14 and RPMI8226 HMCLs without an effect on BCL\textsubscript{X}L expression.

The same researchers confirmed some of their earlier findings when they showed in a subsequent paper that APRIL and TACI interact with syndecan-1 on the surface of MM cells to promote MM cell survival. In this study they again demonstrated that APRIL could protect HMCLs (L363, LP1 and XG1) from dexamethasone-induced apoptosis (Moreaux et al., 2009). Furthermore, the addition of TACI-Fc to co-cultures of primary MM cells and osteoclasts overcame the pro-survival effect of osteoclasts on MM cells, suggesting that anti-apoptotic effect of osteoclasts on MM cells, is at least partially attributable to APRIL secretion (Abe et al., 2008). Although it is also possible that this effect may have been related to BAFF-inhibition, it has more recently been shown that osteoclasts express abundant APRIL mRNA but relatively little BAFF mRNA, making it more likely that APRIL-inhibition mediated the anti-apoptotic effect (Moreaux et al., 2011). In more recently published in vitro experiments, Moreaux et al (2011) further confirmed osteoclasts as an APRIL-rich source, by demonstrating that the addition of TACI-Fc to osteoclast co-cultures with the XG-19 and XG-20 MM cell lines attenuated the antiapoptotic effect of osteoclasts on MM cells. In separate studies, other
researchers employed an in-vivo model of myeloma, where patient MM cells are implanted into human foetal bone in a SCID mouse (so called SCID-Hu model) to study the effects of blocking APRIL and BAFF interactions with MM cells (Yaccoby et al, 2008). Using TACI-Fc, (which binds both APRIL and BAFF) and BAFFR-Ig, which binds only BAFF, it was shown that only TACI-Fc significantly reduced the growth and survival of MM cells in contrast to BAFFR-Ig which had a significantly lesser effect. These results suggest that inhibiting BAFF in isolation may provide an inadequate anti-myeloma effect and that blocking APRIL-MM cell interactions may be critical in inhibiting MM cell growth and survival. An alternative explanation is that both APRIL and BAFF inhibition is required to produce an anti-myeloma effect in-vivo. In keeping with previous studies, these authors also found that the pro-survival effect of osteoclast co-culture on primary MM cells was inhibited by the addition of TACI-Fc to the cultures (Abe et al, 2008, Moreaux et al, 2011). Another important finding of this study was the demonstration that patients with “high” expression of TACI, as measured by mRNA expression, were more sensitive to TACI-Fc treatment than those with “low” expression of TACI.

5.2 Materials and Methods

As detailed in Chapter 2, primary CD138+ MM cells were freshly isolated from MM BM samples and cultured in RPMI ± 20% pooled MM plasma (culture medium (CM)) ± APRIL 200ng/ml or BAFF for 48-72hrs. Depending on the cell numbers available, primary MM cells were routinely cultured in 96, 48 or 24 well plates at a concentration of 1 x 10^6 cells per ml of culture medium. CD138+ cell viability was measured by AnnexinV/PI staining. For the drug-induced apoptosis assays, cells were treated with dexamethasone 0.01-1µM (Dex), lenalidomide 50mM, bortezomib 20nM and melphalan 50nM, alone or in combination with APRIL and/or BAFF (200ng/ml).
Caspase 3 levels were assessed by spectroscopic analysis after adding a caspase-3 substrate to cultured cells as described in chapter 2. Apoptosis was also evaluated using Western blotting to detect PARP protein. The method and materials used to carry out immunohistochemistry have been described in chapter 2.

5.3 Results

5.3.1 Effect of APRIL on spontaneous apoptosis of MM cells in vitro

The first experiments were designed to determine if APRIL could attenuate the tendency of primary MM cells to undergo spontaneous apoptosis in vitro. Freshly purified CD138+ cells from 15 patients (cyclin D1+ n=9, cyclin D2+ n=6, see Table 5.1 for details) were cultured in CM ± APRIL 200ng/ml as for cell cycle experiments. Following culture for 72 hours, cells were harvested and stained with FITC-conjugated anti-Annexin V and PI and then analysed by flow-cytometry. Viable cells were those found to be Annexin V-negative and PI negative. The mean viability of 51.9 ± 4.7% (mean ± SEM) at 72 hours was similar to the viability achieved after 72 hours during cell cycle experiments (see chapter 3). As shown in Figure 5.1A, the addition of APRIL to the cell cultures had no consistent significant impact on attenuating apoptosis, in MM cells expressing either cyclin D1 and cyclin D2. In subsequent experiments where cells were harvested at different time-points (24, 48, 96 and 120 hours), no consistent APRIL-induced inhibition of spontaneous apoptosis was observed. Figure 5.1B shows representative FACS plots from a patient with a t(14;16) translocation whose cells were very responsive to APRIL in cell cycle experiments (patient #13, Table 4.1). As shown, the percentage of viable cells at 24, 48, 72 and 96 hours was not substantially increased by the addition of APRIL to the cell cultures. Additional experiments with purified CD138+ cells from patients with relapsed MM and employing serum-free culture media alone or with bovine serum (RPMI±10%/FCS, n=7, Figure 5.1C) also failed to
demonstrate an APRIL-mediated anti-apoptotic effect. Further experiments in the H929, OPM2, KMS12BM, U266, MM1S, JJN3 and RPMI8226 HMCLs yielded similar results (data not shown). These results were somewhat surprising given that APRIL is widely reported to be a critical factor for plasma cell survival. On the other hand, there are no published reports of APRIL inhibiting spontaneous apoptosis of MM cells in vitro, and it is interesting to note that a recent study demonstrating that APRIL-induced cell cycle progression in follicular lymphoma cells was not accompanied by a significant anti-apoptotic effect (Gupta et al, 2009).

Figure 5.1

A

![Graph showing the effect of APRIL on cyclin D2 and D1 levels](image)

B

![Flow cytometry plots showing apoptosis induction by APRIL](image)
Figure 5.1: Effect of APRIL on apoptosis in primary myeloma cells. (A) CD138+ cells (n=15; 6 cyclin D2+ and 9 cyclin D1+) were cultured in CM ± APRIL (200ng/ml) for 72 hrs and stained with FITC-conjugated anti AnnexinV and PI. Viability (annexin-V and PI-negative fraction) in the presence or absence of APRIL is shown according cyclin D class. Data are presented as mean ± SEM. (B) CD138+ cells (patient #13, Table 1) were cultured in CM ± APRIL for up to 96 hours. The viable fraction, (AnnexinV/PI-negative), is indicated by the arrows. (C) CD138+ cells (n=7) were cultured in RPMI, RPMI/10%FCS or CM ± APRIL (200ng/ml) for 72 hrs. Cells were harvested and the viable fraction was determined as in A.

5.3.2 Effect of APRIL in attenuating dexamethasone-induced apoptosis in MM cells
As previous reports had shown that APRIL could protect MM cells from drug-induced apoptosis, the next experiments sought to determine if APRIL could attenuate dexamethasone-induced apoptosis in primary MM cells and to correlate the results with D-type cyclin group and translocation status. Primary MM cells were cultured in CM ± APRIL 200ng/ml. Dexamethasone 0.01-1µM was added to the cell cultures within five minutes of the cells being stimulated with APRIL. Apoptosis was determined as before by staining with FITC-conjugated anti-AnnexinV and PI followed by FACS analysis.
APRIL was found to inhibit dexamethasone-induced apoptosis maximally after 48-72 hours cell culture as shown in Figure 5.2.

**Figure 2**

![Figure 2](image.png)

**Figure 5.2: Effect of APRIL on dexamethasone-induced apoptosis in CD138+ cells.**

Two representative examples of MM cells expressing cyclin D2: CD138+ cells from 2 patients were treated with dexamethasone 1µM ± APRIL 200ng/ml for 72 hours. The percentage of viable cells is shown, demonstrating the protective effect of APRIL in these cells in the setting of dexamethasone treatment. In the upper panel, the cells carry the t(14;16) translocation with the cells depicted in the lower panel bearing t(4;14).

In total sixteen cases were examined, 9 expressing cyclin D1 and 7 expressing cyclin D2. As shown in Figure 2, APRIL substantially attenuated dexamethasone-mediated apoptosis in MM cells expressing cyclin D2. After 48-72 hours in culture, the mean viability of cyclin D2-expressing cells treated with dexamethasone was 54.2% (± 4.8%,...
mean ± SEM) in contrast to the APRIL-treated cells cyclin D2+ cells where the mean viability was 71.1% (± 7.3%, mean ± SEM) after dexamethasone-addition (Figure 5.3A). When all 16 cases were evaluated, it was evident that the protective effect of APRIL was confined to Cyclin D2+ MM cells, including 1 case with t(14;16) and 3 with t(4;14) (See Table 5.1) although this effect did not reach statistical significance (p=0.08). There was no consistent evidence of APRIL-mediated protection from apoptosis seen in Cyclin D1+MM cells all of which carried t(11;14) (Figure 5.3A).

Where adequate CD138+ cells were available, BAFF 200ng/ml was used as a positive control and APRIL-mediated protection against dexamethasone-induced apoptosis was not seen in the absence of BAFF-mediated protection. In addition, the effect of APRIL in the setting of MM cell apoptosis induced by lenalidomide was also examined in 12 cases, of which 5 expressed cyclin D2 (1 with t(14;16), 2 with t(4;14), 2 with no IgH/TC) and 7 expressed cyclin D1 (all cases with t(11;14)). As shown in Figure 5.3B, the mean survival of both cyclin D1 and cyclin D2-expressing cells was not increased by the addition of APRIL in the context of lenalidomide treatment.

**Figure 5.3**

A
Figure 5.3: APRIL protects cyclin D2-expressing CD138+ cells from Dexamethasone-mediated apoptosis. (A) Primary CD138+ cells (n=16, cyclin D1+ n=9, cyclin D2+ n=7) were cultured as in Figure 2. Apoptosis was quantified by Annexin V/PI analysis. Data are presented as viability as a % of control. Apoptosis with dexamethasone (Dex) alone compared to Dex + APRIL. Cyclin D2+ MM cells derive greater protection from APRIL compared to Cyclin D1+ MM cells although this effect does not reach statistical significance (p=0.08). (B) Primary CD138+ cells (n=7, cyclin D1+ and n=5, cyclin D2+), were cultured as above except the cells were treated with lenalidomide 50µM (Len) instead of dexamethasone.

Treatment of primary MM cells with both bortezomib and melphalan in vitro led to a significant reduction in cell viability after 48-72 hours in culture (typically <5-10%), making it very difficult to determine if APRIL mediated protection against apoptosis following treatment with either of these 2 agents (See Figures 5.4A and B). Consistent with the cell cycle experiments detailed in Chapter 4, the experiments examining whether APRIL mediated drug resistance were performed using RPMI/20% pooled myeloma plasma (CM) as the standard control medium. Figure 5.4B shows that the effect of APRIL in mediating protection against dexamethasone-induced apoptosis in cyclin D2 expressing cells was also evident when these experiments were carried out in
RPMI/10%FCS. Again in keeping with the findings described in Chapter 3, viability was substantially improved when the cells were cultured in CM (Figure 5.4A and B).

**Figure 5.4**

A

![Graph A](image)

B

![Graph B](image)
Figure 5.4: APRIL attenuates dexamethasone-mediated apoptosis in both human plasma and bovine serum-containing media. (A) CD138+ cells from a patient carrying a t(14;16) translocation were treated with dexamethasone 1μM (DEX), lenalidomide 50μM (LEN), melphalan 50nM (MEL) and bortezomib 20nM (VEL) ± APRIL 200ng/ for 72 hours after which the % of viable cells was determined by Annexin V/PI analysis. Results for MM cells cultured in RPMI/20% pooled myeloma plasma are shown in A with the results for MM cells cultured in RPMI/10%FCS shown in B, where BAFF was employed as a positive control (B).

5.3.3 Mechanism of APRIL-mediated protection against dexamethasone in MM cells
To confirm that the protective effect of APRIL was mediated by a reduction in the activation of apoptotic pathways, caspase-3 activity was measured in primary CD138+ cells in the setting of dexamethasone-induced apoptosis. In this context, caspase-3 activity was significantly reduced by APRIL stimulation of primary MM cells expressing t(14;16), whilst a lesser effect was observed in cells bearing t(11;14) (Figure 5.5A). In keeping with these findings, APRIL-stimulation of primary MM cells expressing t(14;16) reduced PARP cleavage in the face of dexamethasone treatment (Figure 5.5B).

Figure 5.5
A
Figure 5.5: APRIL attenuates dexamethasone-mediated Caspase 3 activity and PARP cleavage in CD138$^+$ cells. (A) CD138$^+$ cells from 2 patients were cultured as in Fig 2. After 48 hours, cells were harvested and caspase-3 activity was determined. (B) CD138$^+$ cells expressing cyclin D2 with t(14;16) were cultured as in Figure 2. PARP-cleavage was then assessed by Western blotting.

5.3.4 Expression of APRIL in the MM bone marrow microenvironment by immunohistochemistry

To further understand the biological relevance of APRIL in MM, the next experiments sought to characterise the extent of APRIL expression within the MM bone marrow microenvironment. A further objective was to determine whether MM cells produce APRIL in autocrine loops or whether the secretion of APRIL occurs in a paracrine fashion in the MM BM microenvironment. BM biopsies from MM cases representing different molecular groups (see Table 4.1) underwent immunohistochemical staining to detect APRIL expression. This work was carried out in collaboration with Dr. Teresa Marafioti, Dr. Manuel Rodriguez-Justo and Ms. Philippa Munson in the Department of Histopathology at University College London (PM performed the immunohistochemical staining and optimized the antibody dilutions and antigen retrieval methods that were employed). The immunohistochemical staining protocol was first optimized using tonsillar sections that showed strong cytoplasmic APRIL expression in subepithelial plasma cells (Figure 5.6). Using an antibody that recognizes the secreted form of
APRIL, strong uniform expression was detected on the surface and in the cytoplasm of MM cells from 2/3 cyclin D2+ cases and 3/3 cyclin D1+ cases (Figure 5.7A), while dual staining for BLIMP-1 confirmed the positive cells to be plasma cells. APRIL-producing cells in the MM BM microenvironment were identified using an antibody that recognizes the N-terminal proximal extracellular domain (APRIL-Stalk) with dual staining for CD138. With this approach, the predominant APRIL-producing cells in the BM microenvironment were found not to be MM cells, which did not stain with the anti-Stalk antibody, but were cells of myeloid origin (Figure 5.7B).

**Figure 5.6**

![Image](image_url)

**Figure 5.6: Optimisation of APRIL immunohistochemistry.** Immunohistochemical staining of MM bone marrow biopsies with anti-APRIL antibody (C-terminal secreted form) was first optimised on tonsillar sections as shown. (A) x 100, (B) x 200, Higher power images (C) x 400 and (D) x 600 show intense membrane and cytoplasmic APRIL staining in plasma cells lining the subepithelium.
Figure 5.7

A

Cyclin D2+ - t(14;16) - #1

(i) Cyclin D2+ - t(14;16) - #1
(ii) Cyclin D2+ - t(14;16) - #1
(iii) Cyclin D2+ - t(14;16) - #1

Cyclin D1+ - t(11;14) - #29

(iv) Cyclin D1+ - t(11;14) - #29
(v) Cyclin D1+ - t(11;14) - #29
(vi) Cyclin D1+ - t(11;14) - #29

APRIL x 100  APRIL x 600  APRIL + BLIMP-1 x 600

B

Cyclin D2+ - t(4;14)

(i) Cyclin D2+ - t(4;14)
(ii) Cyclin D2+ - t(4;14)

(iii) Cyclin D2+ - t(4;14)
(iv) Cyclin D2+ - t(4;14)

APRIL x 100  APRIL x 600  APRIL + CD138 - x 100  APRIL + CD138 - x 600
Figure 5.7: APRIL expression in the MM bone marrow microenvironment. (A) Detection of secreted APRIL by immunohistochemistry using anti-APRIL (C-terminal, secreted form) in representative BM sections from 2 MM cases (#1 and #29, Table 4.1). Both of these BM biopsies were heavily infiltrated with CD138-positive cells and dual staining with anti-BLIMP-1 antibody identified plasma cells (panels (iii) and (vi)). (B) Detection of APRIL-producing cells by immunohistochemistry in BM sections from a patient with t(4;14). Sections are stained with anti-APRIL (N-terminal, Stalk) only in (i) and (ii) and with both anti-CD138 (blue) and anti-APRIL (brown) in (iii) and (iv).

5.4 Discussion

The experiments outlined in this chapter examined whether APRIL behaved as an anti-apoptotic factor for primary MM cells and then switched focus to determine the extent and pattern of APRIL expression within the MM BM microenvironment. Firstly, it was found that APRIL protected primary MM cells from dexamethasone-induced apoptosis, thus confirming the previous findings of other researchers (Moreaux et al, 2004). These findings were supported by the demonstration of reduced PARP cleavage in MM cells with t(14;16) following treatment with APRIL and dexamethasone, in comparison with those cells treated with dexamethasone alone. Caspase-3 activity was also significantly reduced by APRIL treatment. However, the most interesting finding of these experiments is that APRIL protected primary MM cells expressing cyclin D2 from dexamethasone-induced apoptosis (n=7). Although this effect approached, but did not reach statistical significance, follow-up work involving additional primary samples confirmed a statistically significant effect (Percy et al, 2011). In contrast, there was no significant effect on primary MM cells expressing cyclin D1 (n=9). This finding was somewhat surprising as it was postulated that the differential responses to APRIL, in terms of cell cycle progression, between cyclin D1+ and cyclin D+ cells, were perhaps related to the dysregulation of signalling pathways controlling cell cycle progression.
One possible explanation for this observation may relate to differential expression of the cMaf protein between MM cells of different molecular subgroups. In 2004 overexpression of cMaf mRNA was found in almost 50% of a large panel of HMCLs (Hurt et al., 2004). This was a surprising finding as IgH/TCs leading to overexpression of cMaf are found in just 5% of primary MM cells and approximately 15% of HMCLs. Furthermore, these authors found that cMaf expression in HMCLs was most prevalent amongst those cells bearing the t(4;14) and t(14;16) translocations, and largely absent from those HMCLs with t(11;14). They also found cMaf expression in approximately 50% of primary samples, but the molecular characteristics of these cases were not detailed. A further important finding of this study was the demonstration that the overexpression of cMaf in the KMS12BM cell line led to increased expression of cyclin D2, the β-7-integrin adhesion molecule and C-C chemokine receptor-1, suggesting increased expression of cMaf may be associated with dependence on specific micro-environmental stimuli. Importantly, it was subsequently shown that APRIL-stimulation increased expression of cyclin D2, cMaf and β-7-integrin in HMCLs that expressed TACI (Moreaux et al., 2007). Overall, although largely hypothetical, these results together with the findings of the experiments detailed in this Chapter, suggest that MM cells with cMaf expression are dependent on specific microenvironmental stimuli for growth and survival. As discussed in chapter 1, MM cases with t(4;14) and t(14;16) (cMaf expressing) have a worse prognosis, emphasising the importance of further study in this area. Furthermore, this segregation by genetic subtype suggests different mechanisms of drug resistance in molecular subgroups, supporting the concept of designing subgroup specific anti-MM therapy.
I focused on dexamethasone-induced apoptosis of MM cells as this agent produced a relatively predictable 50-75% reduction in MM cell viability after 48-72 hours in the myeloma-plasma containing culture medium and the anti-apoptotic effects of APRIL in this setting were consistently seen. Although the apoptotic effects of other anti-myeloma agents were explored there were several limitations. Firstly, bortezomib, and to a lesser extent melphalan induced rapid cell death in both HMCL and primary MM cells, making it difficult to establish a time-point at which to measure an anti-apoptotic effect. BAFF was previously shown to protect MM1S cells from lenalidomide-induced apoptosis (Tai et al, 2006). Despite this, no consistent evidence that APRIL was able to protect MM cells from lenalidomide-induced apoptosis was observed (Figure 5.3B).

I was unable to consistently demonstrate that APRIL could protect primary MM cells from spontaneous apoptosis or HMCLs from apoptosis induced by serum withdrawal. These results were somewhat surprising given that APRIL is widely reported to be a critical factor for plasma cell survival. On the other hand, the only published work to date showing that APRIL protects MM cells from spontaneous apoptosis comes from Moreaux et al (2004) who showed that APRIL protected IL-6 dependent HMCLs from apoptosis precipitated by IL-6 withdrawal. However, there are no published data regarding autonomously growing HMCLs or primary MM cells and it is possible that different culture methods or longer time-points may be necessary to demonstrate such an anti-apoptotic effect in primary MM cells. Although APRIL expression has been demonstrated in the microenvironment of several malignancies including follicular NHL and Hodgkin Lymphoma, no publication to date has demonstrated APRIL expression by immunohistochemistry in bone marrow trephine biopsies, so the original finding of this work demonstrating strong APRIL expression in the MM BM
underscores its physiological role in this disease. Moreaux and co-workers (2004), working with HMCLs suggested that MM cells produce APRIL (and/or BAFF) in an autocrine fashion, as they found that the addition of TACI-Fc to the RPMI8226 and L363 cell line cultures significantly reduced cell growth. They further found that that addition of APRIL and BAFF to the cell cultures overcame the inhibitory effect of TACI-Fc and restored cell growth (Moreaux et al, 2004). However, since then increasing evidence suggests that APRIL production within the microenvironment of many tumours is predominantly by cells of myeloid origin.

The identification of “APRIL-secreting” and “APRIL-binding” cells within tumour tissues has been made easier by the availability of two APRIL-specific antibodies (see Figure 5.8 for schematic representation, Burjanadze et al, 2009). One antibody recognizes the N-terminal APRIL fragment, also referred to as “APRIL-Stalk”, which remains in situ after cellular processing of APRIL. Therefore, this antibody identifies APRIL Producing cells. The second antibody recognizes the TNF-homology domain (THD) and recognizes only the secreted form of APRIL. The two antibodies have been found to produce markedly different patterns of staining (Burjanadeze et al, 2009). The employment of the anti-Stalk antibody has identified neutrophils as an important cellular source of APRIL in several settings. In one study, APRIL expression was clearly identified in sections from patients with diffuse large B-cell lymphoma and in this setting the APRIL-producing cells were shown to be neutrophils (Schwaller et al, 2007a). In a separate publication, the same researchers also showed that neutrophils within Hodgkin Lymphoma sections provided a paracrine source of APRIL (Schwaller et al, 2007b). Although it was found that serum levels of APRIL (but not BAFF) were elevated in patients with CLL, APRIL expression was not identified in CLL lymph node
sections (Planalles et al, 2007, Schwhaller et al, 2007a). Furthermore, within lymphoma tissues, macrophages are the most recently described APRIL-secreting cells, as it has been demonstrated that Gastric MALT lymphoma tissues were heavily infiltrated with APRIL-expressing macrophages (Munari et al, 2011) whilst it was also shown in this study that Helicobacter Pylori infection stimulated the release of APRIL by macrophages.

In the non-tumour setting, it has recently been shown that APRIL is mainly produced by neutrophils closely associated with plasma cells within niches in MALT tissue and that the APRIL-plasma cell interaction led to the upregulation of anti-apoptotic proteins (Huard et al, 2008). Despite the findings detailed above, the expression of APRIL in the MM BM microenvironment has not been demonstrated by immunohistochemistry to date. Although Moreaux and colleagues have shown that osteoclasts and myeloid cell within the MM BM microenvironment express abundant APRIL mRNA, it has not been demonstrated whether this expression is sufficient to lead to the expression of APRIL protein (Moreaux et al, 2009b). Gupta et al (2009) showed by western blotting that APRIL protein was expressed in the HS-5 stromal cell line, however data from gene expressing profiling experiments suggest that stromal cells from MM patients express significantly less APRIL than do osteoclasts and myeloid cells. In this thesis, employing antibodies that identified APRIL-producing cells and membrane-bound APRIL, strong APRIL expression was found in 5/6 MM cases by immunohistochemical staining of BM trephine biopsies. Whilst it was found that secreted APRIL was present in CD138+ MM cells in BM sections from both cyclin D2- and cyclin D1-expressing cases, these cells were not the producers of APRIL. Instead, the cellular sources of APRIL in MM BM sections were shown to be neutrophils and/or other myeloid cells, as has been
demonstrated in non-Hodgkin lymphoma, and in Hodgkin lymphoma tumours as detailed previously. A recent study using normal human BM has confirmed these findings, by showing that granulocytes, monocytes and myelocytes but not promyelocytes or lymphocytes were the major cellular sources of APRIL in the BM (Matthes et al, 2011).

5.5 Summary
The data presented within this section includes some important findings that require further study. Importantly, in a large series of MM cases (n=16), it was shown that APRIL protects primary MM cells from dexamethasone-induced apoptosis, thus confirming the findings of Moreaux et al (2004) who made similar findings in a smaller patient sample (n=8). However, a preliminary finding was that the protection from dexamethasone-induced apoptosis was largely limited to MM cells expressing cyclin D2 and bearing the t(4;14) and t(14;16) IgH/TCs. The reasons for this finding are unclear, but further work may include the determining the relative expression and roles of BCMA or TACI in this setting or the role of APRIL-HSPG interactions in protecting MM cells from drug-induced apoptosis. A major finding of this work was the demonstration of APRIL expression in the MM BM microenvironment, and particularly the demonstration that the cellular sources of APRIL in this setting are myeloid cells. These findings provide supporting evidence to published data showing that myeloid cells provide a paracrine source of APRIL to support the growth and drug resistance of MM cells. Further work may seek to unravel what the key MM cell-APRIL receptor interactions are between MM cells and myeloid cells, as well as dissecting the signalling pathways that are activated by these interactions.
Figure 5.8

**Figure 5.8: Cellular interactions of APRIL in the myeloma bone marrow microenvironment.** Schematic representation of APRIL-producing cells and APRIL-binding cells through APRIL-HSPG (heparin sulphate proteoglycan) interactions. Antibodies specific for APRIL-Stalk and Secreted APRIL permit the identification of APRIL-producing cells and APRIL-binding cells (Adapted from Burjanadze et al, Histol Histopathol 2009).

**Table 3.1: Characteristics of patient samples used in dexamethasone-resistance experiments**

<table>
<thead>
<tr>
<th>Cyclin D1 (n=9)</th>
<th>IgH/TC</th>
<th>No IgH/TC</th>
<th>De Novo MM</th>
<th>Relapsed MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D2 (n=7)</td>
<td>1 t(14;16)</td>
<td>3 t(4;14)</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
Chapter 6. Cytokine-mediated signalling in primary MM cells and regulation of cell cycle progression

6.1 Introduction

6.1.1 Background

In the work presented up to this point, it has been found that APRIL promotes cell cycle progression in primary MM cells as well as protecting primary MM cells from dexamethasone-induced apoptosis. Importantly, these effects have been largely restricted to MM cells expressing cyclin D2, particularly in the presence of an IgH/TC such as t(4;14) or t(14;16). In chapter 5, the results of immunohistochemical staining of MM BM biopsies show that there is abundant APRIL bound to the surface of MM cells regardless of D-type cyclin expression, so neither the absence of adequate amounts of secreted APRIL nor an inability to bind APRIL provides an explanation as to why cyclin D2-expressing MM cells were found to be more APRIL-responsive. In keeping with the results of the immunohistochemical studies, the median expression of BCMA and TACI were found to be equivalent between cyclin D1 and D2-expressing MM cells.

An important question that has not been addressed thus far is whether APRIL can activate proximal signalling pathways in MM cells irrespective of D-type cyclin expression.

One possible explanation for the apparent inability of APRIL to stimulate cell cycle progression in cyclin D1-expressing primary MM cells is that cyclin D1 is not the major functional D-type cyclin expressed by B-lymphocytes. Furthermore, in MM cells bearing t(11;14), although there is abundant cyclin D1 present, the cyclin D1 gene is not under the control of its usual promoter. In contrast, cyclin D2 is the major D-type cyclin expressed by B-lymphocytes, and the cyclin D2 gene remains under the control of its natural cytokine-responsive promoter, even in the context of the IgH/TCs, t(14;16) and
t(4;14) which induce cyclin D2 expression through an indirect mechanism. One hypothesis suggests that APRIL (and/or other growth factors) can activate proximal signalling pathways in MM cells expressing cyclin D1, but the dysregulated cell cycle mechanisms within these cells restrict progression through the G1/S phases of the cell cycle. Another hypothesis is that cyclin D2-expressing MM cells are more critically dependent on a particular signalling pathway that provides the necessary “scaffolding” of cell cycle regulatory proteins required to promote cell cycle progression. Although multiple signalling pathways have been shown to be important in the growth and survival of MM cells, few studies to date have examined whether any of these signalling pathways are differentially activated in MM cells bearing differing molecular lesions. However, one group of researchers who performed a detailed examination of the expression of AKT subunits in HMCLs and primary MM cells, found that the MM1S and OPM2 cell lines showed evidence of AKT activation by expression of phospho-AKT at baseline, in contrast to the U266 and AMO1 cell lines that did not display baseline phospho-AKT expression (Zollinger et al, 2008). Furthermore siRNA-mediated inhibition of AKT1 and AKT2 led to cell death in the MM1S cell line unlike the AMO1 cells that were not affected. They then examined the effect of pharmacological inhibition of AKT1 and AKT2 in primary MM cells from 30 patients that were divided into 2 groups (high and low) depending on their relative expression of phospho-AKT as determined by immunohistochemistry and flow cytometry. Patient cells displaying relatively strong expression of phospho-AKT were found to be more sensitive to the effects of AKT1/2 inhibition in comparison with those cells without phospho-AKT expression, suggesting that MM cells might be divided into 2 functional groups depending on the relative expression of phospho-AKT. Overall these data provide preliminary evidence that subgroups of MM cells may differ in their
dependence on different signalling pathways for growth and survival.

6.1.2 Signalling pathways activated by APRIL/BAFF in multiple myeloma cells

An early insight into the intracellular signalling pathways activated by APRIL was provided in a study where the TACI receptor was described for the first time. During these investigations, it was also found that overexpression of TACI in Jurkat cells led to activation of the NFκB pathway (Von Bulow et al, 1997). In 2000 it was further demonstrated that BCMA was bound by BAFF and that overexpression of BCMA in human 293T cells led to the activation of the NFκB pathway (Shu et al, 2000). In previous studies involving MM cells, APRIL has been shown to activate the PI3K/AKT, MAPK and canonical NFκB pathways in the XG-13, XG-14 and RPMI8226, myeloma cell lines (Moreaux et al, 2004). Specifically, APRIL stimulation of these cell lines increased the expression of the p50 NFκB subunit as measured by ELISA assay and in the RPMI8226 cell line, pharmacological inhibition of the PI3K/AKT and NFκB pathways blocked the growth-promoting effects of APRIL as measured by thymidine incorporation. Activation of the canonical NFκB by pathway by APRIL has also been demonstrated in CLL cells whereas APRIL stimulation of follicular lymphoma cells was also accompanied by activation of the PI3K/AKT pathway a process that was found to be dependent on the interaction between APRIL and TACI (Nishio et al 2005, Gupta et al, 2009). Based on these findings, the experimental plan focused on demonstrating the ability of APRIL to activate the PI3K/AKT, MAPK and NFκB pathways in primary MM cells, comparing cyclin D1- with cyclin D2-expressing MM cells.

A second line of inquiry was based on the hypothesis that MM cells bearing different IgH/TCs differed in their dependence on particular signalling pathways, this being
linked to differential effects on the assembly and activation of cell cycle proteins. The MAPK/ERK pathway has been shown to be important for the assembly and activation of CDKs and this pathway was chosen for further study (Cheng et al, 1998). Hence, differences in engagement of this pathway by mitogen-activated signalling may account for the differential regulation of cell cycle proteins in molecularly distinct MM cells. The differential effect of mitogens on cell cycle regulation depending on cyclin D-type is not restricted to APRIL but has also been described for IGF-I by our group (Glassford et al, 2007, 2012). IGF-I is the major MM growth factor and the vast majority of HMCLs are responsive to IGF-I, but segregate with respect to IgH/TC class (Sprynski et al, 2009, Glassford et al, 2007, 2012). Therefore, experiments to investigate the effect of MAPK blockade on mitogen-induced cell cycle responses employed HMCLs stimulated with IGF-I.

A final part of the work described in this chapter explored the expression of the cMaf transcription factor by MM cells. The cyclin D2 gene is a cMaf target and it has previously been shown that induced overexpression of cMaf in the KMS12BM MM cells led to increased expression of cyclin D2 (Hurt et al, 2004). In addition, a more recent study found that dexamethasone treatment of MM cell lines led to a decrease in the expression of both cyclin D2 and cMaf (Mao et al, 2007). Although approximately 50% of primary MM cells examined showed expression of cMaf RNA, much less is known about the expression of cMaf protein in MM patient samples (Hurt et al, 2004). To date, two separate immunohistochemical studies have have tried to address this question with one analysis finding cMaf expression in CD138+ cells in 30% of 73 MM patient BM trephine biopsies, whilst also noting that cMaf expression did not have prognostic significance (Chang et al, 2007). In contrast, a more recent study found cMaf expression amongst just 4% of MM patient samples again using the same antibody to
perform immunohistochemical staining of MM BM trephine biopsies (Natkunam et al, 2007). Thus, the experimental plan was to investigate whether cMaf protein was expressed in primary MM cells and whether this protein was functional. This line of inquiry was a prelude to investigating whether the expression and function of cMaf differed between distinct MM subgroups, and if so, whether this could account for the differences in cell cycle responses.

6.2 Materials and Methods

The materials for the two assays outlined below are documented in chapter 2. Both of these assays were first optimized using the MM1S cell line with IGF-I and TNFα employed as positive controls (see Figure 6.1).

6.2.1 Detection of phospho-AKT expression by flow cytometry

- 1 x 10^6 cells were fixed in 200μL of 2% paraformaldehyde for 10mins on ice and washed once in FACS buffer (PBS/0.1%BSA, 1500 rpm for 5 minutes).

- The cells were resuspended in 90% ice-cold methanol solution for 30 minutes at room temperature.

- The cells were washed once in FACS buffer and divided into 2 equal cell volumes (A and B), which were each resuspended in 50μL of FACS buffer.

- 1μL of anti-phospho-Akt antibody was added to tube A and incubated at room temperature for 60 minutes (no antibody was added to the control sample, B).

- The cells were again washed once in FACS buffer and resuspended in 50μL of FACS buffer containing APC-conjugated anti-rabbit antibody at a 1:500
• The cells were incubated for 30 minutes at room temperature, before being washed once in FACS buffer and re-suspended in 500µL of FACS buffer and analysed by flow cytometry.

6.2.2 Detection of the activated form of p65 by flow cytometry

• 1 x 10^6 cells were resuspended in 500µl PBS to which 500µl of ice-cold 4% paraformaldehyde and the cell suspension was incubated for 5 minutes on ice.

• The cells were washed once again in FACS buffer (1500 rpm for 5 minutes) and re-suspended in 500µl of PBS to which 500µl of 0.1% Triton-X solution was added and then incubated for 5 minutes at room temperature.

• The cells were washed once in FACS buffer and divided into 2 equal cell volumes (A and B), which were each re-suspended in 50µL of FACS buffer.

• 1µL of anti-p65 antibody was added to tube A and incubated at room temperature for 60 minutes (no antibody was added to the control sample, B).

• The cells were again washed once in FACS buffer and resuspended in 50µL of FACS buffer containing APC-conjugated anti-rabbit antibody at a 1:500 dilution.

• The cells were incubated for 30 minutes at room temperature, before being washed once in FACS buffer and re-suspended in 500µL of FACS buffer and analysed by flow cytometry.
Figure 6.1: Effect of APRIL on p65 and phospho-AKT expression in MM1S cells. MM1S cells were treated as described in 6.2.1 and 6.2.2 and also treated with IGF-I 100ng/ml and TNFα 20ng/ml as positive control samples for phospho-AKT and p65 experiments respectively. Expression of phospho-AKT and p65 are shown in the upper and lower panels respectively.

6.2.3 Signalling Experiments

For signalling experiments, MM cells were centrifuged at 1500rpm for 7 minutes, after which they were resuspended in RPMI1640 (at 37 degrees) without added serum or albumin. The cells were again centrifuged at 1500rpm for 7 minutes and this step was repeated to ensure that any serum or plasma had been washed from the cells. The cells were then resuspended in RPMI1640 before stimulation with APRIL 800ng/ml with
IGF-I 100ng/ml or TNFα 20ng/ml employed as positive controls. The cells were then incubated at 37 degrees for specific periods of time, after which the cells were harvested and centrifuged (1500rpm for 7 minutes) at 4 degrees in PBS. The supernatant was then aspirated and the resulting cell pellets were lysed, before protein expression was determined by western blotting as described in chapter 2. For MAPK-inhibition experiments, control MM cells were stimulated with IGF-I 100ng/ml in RPMI1640 without added serum or BSA. Alternatively, MM cells were pre-treated with the specific MAPK-inhibitor U0126 at a concentration of 10µM for 60 minutes and then stimulated with IGF-I 100ng/ml as described previously. Cell lysates for western blotting were made as outlined above.

6.3 Results

6.3.1 APRIL activates the PI3K/AKT signalling pathway in MM cells

Initial signalling experiments employed HMCLs. In keeping with earlier publications, APRIL stimulation of the RPMI8226, MM1S and OPM2 cell lines resulted in increased expression of pAKT after 30mins as demonstrated by Western blotting (Figure 6.2A). Respectively, these cell lines harbour t(14;16) (RPMI8226 and MM1S) and t(4;14) (OPM2), however, overall the increased expression of pAKT is weak (in comparison with IGF-I stimulation) and should be interpreted in the absence of a blot showing total AKT expression. In addition, APRIL stimulation led to increased expression of pAKT in cyclin D2+ve primary MM cells only, whilst pAKT expression in the cyclin D1+ve primary MM cells was unchanged (Figure 6.2B). No significant increase in pAKT was observed upon stimulation of the KMS12BM or U266 cell lines both of which carry t(11;14) (data not shown). Employed as a positive control, IGF-I induced strong
expression of pAKT in all MM cells examined, both primary MM cells and HMCLs, in keeping with published data (Sprynski et al, 2009)

Figure 6.2

A

![Image of Western blots showing pAKT and β-actin expression in RPMI8226, MM1S, and OPM2 cells under control, APRIL, and IGF-I conditions.]

B

![Image of flow cytometry histograms showing pAKT expression in MM1S and OPM2 cells and patient samples under control and APRIL conditions.]

Figure 6.2: Effect of APRIL on PI3K/AKT signalling in MM cells. (A) MM1S, OPM2 and primary MM cells (B) were incubated in medium with or without APRIL (800ng/ml) for 10 minutes after which cells were fixed and permeabilised before staining initially with anti-phospho-AKT antibody followed by APC-conjugated goat anti-rabbit IgG. Patient #29 expresses cyclin D1 without an IgH/TC and Patient #13 carries t(14;16) and expresses cyclin D2. Grey shaded histogram: control cells, black unshaded histogram: APRIL-stimulated cells.
6.3.2 APRIL activates the MAPK and Canonical NFκB Signalling Pathways

Next, the MAPK and Canonical NFκB pathways were examined. After stimulation with APRIL for 30 minutes, CD138+ cells from 2 patients (cyclin D1 and D2, n=1 each) showed increased expression of the p65 subunit whereas IGF-I stimulation did not lead to increased p65 expression (Figure 6.3A). These findings were confirmed using a flow-cytometric assay employing an antibody specific for an epitope corresponding to the nuclear location (NLS) signal of the p65 NFκB subunit which is revealed following IκB phosphorylation which results in activation of the p65 and p50 subunits. APRIL stimulation of MM1S and KMS27 cells resulted in increased p65 expression (Figure 6.3B). Similarly, primary CD138+ MM cells expressing either cyclin D1 or D2 showed increased p65 expression in response to APRIL. APRIL stimulation induced pMAPK expression in CD138+ cells expressing cyclin D2 (Figure 6.3A). However, increased pMAPK expression was not observed in CD138+ cells expressing cyclin D1 (Figure 6.3A).

Figure 6.3

A
Figure 6.3: Effect of APRIL on MAPK and NFκB signalling in MM cells. (A) CD138+ cells from 2 patients (cyclin D1 and cyclin D2, one case each) were cultured with APRIL 800ng/ml for 60 mins and analysed for expression of p65 and pMAPK by western blotting. (B) CD138+ cells from 2 patients (cyclin D1 and cyclin D2, one case each) and MM1S and KMS27 cells, were cultured with APRIL 800ng/ml for 60 mins and analysed for expression of the activated form of p65 by flow cytometry. Patient #21 expresses cyclin D1 with t(11;14) and Patient #11 carries t(14;16) and expresses cyclin D2.

6.3.3 Effect of IGF-I on signalling pathways in HMCLs bearing different IgH/TCs

Next, the effects of IGF-I on proximal signalling pathways in HMCLs were examined with a view to determining if these effects were dependent upon or differed according to IgH/TC or D-type cyclin expression. IGF-I was chosen as the growth factor for these experiments as it has recently been shown to be the MM growth factor with growth-promoting effects on the greatest number of HMCLs, when compared with HGF, IL-6, and APRIL (Sprynski et al, 2009). Four cell lines representing the main IgH/TC classes were selected for these experiments: H929, MM1S (both expressing cyclin D2 and bearing t(4;14) and t(14;16) respectively) and U266 and KMS12BM, both of which
show strong expression of cyclin D1 and carry t(11;14). IGF-I stimulation induced upregulation of pMAPK and pAKT in the KMS12BM, U266 and H929 cell lines whilst these pathways were found to be constitutively activated in the MM1S cell line (Figure 6.4A). As the MAPK pathway has been shown to be important for cell cycle regulatory protein assembly and stability, this pathway was focused on for further analysis. The effects of IGF-I on pMAPK expression were examined over a longer time course. As shown in Figures 6.4A and 6.4B, IGF-I stimulation caused a rapid induction of pMAPK in the KMS12BM, U266 and H929 cell lines. However, in the KMS12BM and U266 cell lines, both of which carry t(11;14) and express cyclin D1, induction of pMAPK was less sustained and had declined by 4 hours, whereas in the cyclin D2-expressing cell line H929, bearing t(4;14), expression of pMAPK appeared to be sustained up to 24 hours (Figure 6.4B), but not the MM1S cell line where constitutive activation of MAPK was again observed.

Figure 6.4
Figure 6.4: Effect of IGF-I on signalling pathways in MM cells. (A) MM1S, H929, U266 and KMS12BM cells were stimulated with IGF-I 100ng/ml in serum-free media and the expression of pAKT, AKT, pMAPK and MAPK was determined by western blotting at 10, 30 and 60 minutes as shown above. Cell lysates were prepared as described in chapter 2. (B) The identical MM cell lines were stimulated as in (A) and the expression of AKT and MAPK proteins were determined as before at the timepoints indicated.

6.3.4 Effect of MAPK-inhibition on the expression of cell cycle regulatory proteins in MM cells

Although IGF-I stimulation caused a rapid induction of pMAPK in all 4 MM cell lines examined, these initial experiments suggested that IGF-I induced a more sustained (up to 24 hours) activation of the MAPK pathway in the 2 MM cell lines that carry IgH/TCs leading to cyclin D2 upregulation. The next series of experiments examined the effect of pharmacologic inhibition of the MAPK pathway on cell cycle protein expression in 2
cell lines, H929 with t(4;14) and expressing cyclin D2 and KMS12BM bearing t(11;14) and strong cyclin D1 expression. For these experiments U0126, a direct inhibitor of MEK1/2, was selected to block MAPK activation. Initial experiments demonstrated that U0126 completely inhibited pMAPK induction, even in the presence of IGF-I stimulation as shown in Figure 6.5.

**Figure 6.5**

<table>
<thead>
<tr>
<th>IGF-I</th>
<th>U0126</th>
<th>30</th>
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**Figure 6.5: The MAPK-inhibitor U0126 blocks MAPK-activation in MM cells stimulated with IGF-I.** H929 and KMS12BM cells were first pre-treated with U0126 10µM for 30 minutes and then stimulated with IGF-I 100ng/ml. Alternatively, cells were cultured in serum-free medium in the absence of U0126, before stimulation with IGF-I as shown. Then pMAPK and MAPK expression were determined by western blotting as previously described.

The next experiments examined the effect of blocking IGF-I induced MAPK activation on the expression of cell cycle regulatory proteins in KMS12BM and H929 cells. As shown in Figure 6.6, the expression of cell cycle regulatory proteins was determined in H929 and KMS12BM cells at several time points up to 24 hours, in the setting of IGF-I stimulation and with or without pre-treatment with U0126. In H929 cells, as in previous experiments, U0126 pretreatment efficiently blocked pMAPK induction in the presence
of IGF-I stimulation. Secondly, in keeping with a previous report (Glassford et al, 2007), IGF-I stimulation led to an induction of cyclin D2 and CDK4 in H929 cells that occurred maximally at 8 hours in H929 cells although the expression of phospho-pRb was somewhat asynchronous (Figure 6.6A). Neither the expression of p21 or p18 was significantly altered by IGF-I stimulation. Although, U0126 completely blocked pMAPK induction, there was no significant change in the expression of any of the cell cycle regulatory proteins in these initial experiments. In particular, IGF-I stimulation led to increased cyclin D2 expression after 8 hours despite U0126 pre-treatment. In keeping with previous experiments, IGF-I stimulation of KMS12BM cells led to a less sustained upregulation of pMAPK (Figure 6.6C) and did not lead to upregulation of either cyclin D1 or phospho-pRb (taking differences in protein loading into account, Figure 6.6C). In addition, and in keeping with the observations in the H929 cells, U0126 pretreatment of KMS12BM cells did not significantly alter expression of either cyclin D1, CDKs 4/6, phospho-pRb or p21 (p18 expression was not detected in this cell line).
Figure 6.6

A

H929 – minus U0126

B

H929 – plus U0126
C

KMS12BM – minus U0126

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td>pMAPK</td>
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<td>MAPK</td>
<td>10</td>
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<tr>
<td>Cyclin D1</td>
<td>30</td>
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<tr>
<td>CDK4</td>
<td>60</td>
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<td>CDK6</td>
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<td>p21</td>
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<td>ACTIN</td>
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D

KMS12BM – plus U0126

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<th>Protein</th>
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<tr>
<td>pMAPK</td>
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<td>Cyclin D1</td>
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<td>ACTIN</td>
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Time: 0 10 30 60 4 8 24
Figure 6.6: Effect of MAPK-inhibition on the expression of cell cycle regulatory proteins in MM cells. H929 and KMS12BM cells were stimulated with IGF-I 100ng/ml without (A and C) or with U0126 pre-treatment (B and D) as described above. Cell lysates were then prepared as described previously before the expression of cyclins D1/D2, CDK4/6, pMAPK, MAPK, p21, p18, phospho-pRb and actin were determined by western blotting as previously described.

6.3.5 Expression of cMaf protein by MM cell lines and primary MM cells

Lastly, cMaf expression in MM cells was determined by western blotting. As anticipated cMaf expression was apparent in all MM cell lines bearing t(14;16) apart from MM1S cells (Figure 6A, upper panel). In addition, MM cell lines with t(4;14) also showed cMaf expression although at lower levels than found in those cells with t(14;16). In contrast, the two MM cell lines with t(11;14) failed to demonstrate any cMaf expression (Figure 6.7A, lower panel). CD138+ cells from a patient with t(14;16) were selected to determine if cMaf protein expression was evident in primary MM cells. As shown in Figure 6.7B, although baseline expression of cMaf protein was not evident in these cells, culture for 72 hours in medium containing MM plasma resulted in a striking upregulation of cMaf protein. The addition of APRIL or BAFF to the plasma-containing medium did not result in further cMaf upregulation.
6.4 Discussion

The results of the experiments detailed above provide several important insights into the mechanism of cytokine-mediated signalling in MM cells, but also pose further questions. Importantly, in these experiments, it was shown that APRIL activates the PI3K/AKT, NFκB and MAPK signalling pathways not only in HMCLs but also in primary MM cells, although the effect on PI3K/AKT pathway was modest in comparison with effects of IGF-I. These results help to reinforce and confirm the report by Moreaux et al (2004) who had documented similar findings in IL-6 dependent
HMCLs. Importantly, APRIL activated signalling pathways in MM cells expressing both cyclins D1 and D2 (Figures 6.2 and 6.3), suggesting that the observed inability of APRIL to promote significant cell cycle progression in MM cells with cyclin D1 expression is not due to the inability of APRIL to bind to these cells and activate such pathways.

These results also help to confirm the work of Sprynski et al (2009) who demonstrated that IGF-I is probably the major growth factor for MM cells (at least in vitro). In keeping with this work, IGF-I induced robust upregulation of both pAKT and pMAPK in all 4 MM cell lines examined, as well as primary MM cells irrespective of IgH/TC status (Figure 6.4). Activation of the PI3K/AKT pathway has been shown to mediate MM cell growth and drug resistance and recent studies reinforce the rationale of targeting this pathway in MM. In vitro, perifosine, an AKT inhibitor, has been shown to induce MM cell apoptosis via down regulation of the Survivin protein (Hideshima et al, 2007). More recently, a phase II study of Perifosine in combination with bortezomib in relapsed/refractory MM demonstrated an overall response rate of 41% (Richardson et al, 2011) reinforcing the PI3K/AKT pathway as a major target in MM. Although, Zollinger et al (2008) were able to divide MM cases into 2 functional groups (present and absent) based on the expression of pAKT by MM cells and the subsequent relative sensitivity to pAKT inhibition, it is not known if this differential sensitivity was in any way related to IgH/TC status. However, It is interesting to note that RAS mutations were found more frequently in MM cases expressing cyclin D1, in comparison with cases bearing t(4;14) where these mutations occurred at a much lower frequency (Chng et al, 2008). As it has been shown that oncogenic RAS may activate the PI3K/AKT pathway, the possibility exists that cyclin D1-expressing MM cells may show increased sensitivity to PI3K/AKT-blockade (Hu et al, 2003). However, it has recently been
demonstrated that silencing of oncogenic RAS in MM cell lines did not affect the AKT signalling pathway suggesting that the two pathways are not directly linked (Steinbrunn et al, 2011).

Another recent study identified DEPTOR protein as a key protein that activates the PI3K/AKT pathway (Peterson et al, 2009) and also demonstrated that increased levels of DEPTOR mRNA were found in 28% of primary MM samples analysed. This finding stands in contrast with most other cancers where DEPTOR levels are relatively low. Intriguingly, DEPTOR mRNA levels were highest amongst the cases with t(6;14), t(11;14) and also those cases with maf translocations. Thus, targeting DEPTOR may provide another mechanism for blocking PI3K/AKT activity in MM cells, and may ultimately most benefit those cases bearing maf translocations. Ultimately, the results of ongoing clinical trials involving AKT-inhibitors may shed further light on any differential sensitivity amongst MM cells bearing different molecular lesions to this therapeutic approach. It also needs to be borne in mind that targeting AKT is isolation may not be adequate to optimally kill MM cells as a recent study has suggested that inhibition of the PI3K/AKT pathway may conversely lead to mTOR-mediated AKT activation thus abrogating any therapeutic advantage (McMillin et al, 2009). These authors went on to show that dual inhibition of PI3K and mTOR had a significant anti-MM effect using the compound NVP-BEZ235, suggesting that dual blockade is required to achieve a significant anti-MM effect. In fact, recent work from our laboratory has confirmed the anti-MM effect of this compound, but crucially, also demonstrates that MM cells with cyclin D2-activating IgH/TCs are sensitive to PI3K-inhibition unlike MM cells bearing t(11;14) which were found to be relatively insensitive (Glassford et al, 2012).
APRIL-stimulation of MM cells was also found to activate the canonical NFκB signalling pathway in keeping with previous findings in both CLL cells and MM cells. BAFF has also been shown to be critically important for activation of the NFκB pathway in MM cells but, unlike APRIL, activates the non-canonical (alternative) pathway by binding to BAFF-R. The majority of MM cells display increased expression of NFκB target genes (so-called NFκB index), which may result from interaction with ligands such as APRIL and BAFF within the BM microenvironment, and suggests an important role for this pathway in MM cell survival. Two recent studies, that were published simultaneously, further emphasise the importance of this pathway in MM cell biology (Keats et al, 2007, Annunziata et al, 2007). In these studies, mutations in “positive” regulators (i.e. TACI, CD40) that result in activation of the NFκB pathway, as well as loss of function mutations in “negative” regulators such as TRAF3 were found in approximately 20% of de novo MM cases and 45% of MM cell lines. Furthermore, Annunziata et al (2007) showed that pharmacological inhibition of the canonical NFκB pathway by targeting IKKβ led to a significant reduction in cell viability in a large panel of MM cell lines (although this approach targets the canonical pathway only). Recently the entire MM genome was sequenced in 38 patients and point mutations and structural rearrangements affecting 11 genes in the NFκB pathway were observed further underlining the importance of this pathway (Chapman et al, 2011). In addition, Keats et al (2007) found that MM patients harbouring inactivating mutations in TRAF3 were significantly more responsive to bortezomib treatment than dexamethasone, suggesting that cells with constitutive NFκB activation may be more sensitive to bortezomib treatment than those without mutations in TRAF3. In the experiments described here, APRIL activated the canonical NFκB pathway in MM cells expressing both cyclin D1 and cyclin D2 suggesting that the observed difference in cell
cycle progression in response to APRIL stimulation between cyclin D1 and cyclin D2-expressing MM cells is not related to differential activation of the canonical NFκB pathway. In addition, Annunziata et al (2007) did not observe differential sensitivity according to IgH/TC amongst the MM cell lines exposed to IKKβ-inhibition. To date, APRIL has not been shown to activate the non-canonical pathway, and preliminary experiments examining p52 and p100 protein expression following APRIL stimulation were inconclusive (data not shown).

As neither receptor expression nor the activation of proximal signalling pathways was found to be significantly different between MM cells bearing different IgH/TCs (and hence different D-type cyclins) it was postulated that perhaps the “kinetics” or assembly of cell cycle regulatory proteins in response to mitogen-stimulation might differ with respect to IgH/TC in MM cells. In 1995 it was found that activation of RAS led to the accumulation of cyclin D1 protein and that MAPK–activation was required for the induction of the Cyclin D1 gene (Albanese et al, 1995). Subsequently, MAPK/ERK pathway activation was shown to be crucial for the assembly of the cyclin D1 protein into active complexes with CDK4 in NIH 3T3 cells thus promoting G1-S progression (Cheng et al, 1998). It is not clear how MAPK-activation leads to increased transcription of the cyclin D1 gene, however, as the cyclin D1 gene has an AP1-binding site (activator protein-1), increased expression of AP1 proteins in response to MAPK-activation has been proposed as a possible mechanism (Meloche et al, 2007). MAPK-activation has also been shown to enhance c-Myc protein stability and thus may promote Cyclin D2 expression, a known target of c-Myc (Bouchard et al, 1999). Therefore, as the MAPK-pathway has been shown to play a crucial role in cell-cycle protein assembly and stability, it was hypothesized that blockade of the MAPK-pathway in MM cells might reveal differential effects on the expression of key cell cycle...
regulatory proteins in MM cells bearing different IgH/TCs. However, in these preliminary experiments involving two cell lines bearing t(4;14) and t(11;14) respectively, differential effects on cell cycle proteins were not observed despite efficient MAPK-inhibition. Interestingly, the kinetics of MAPK phosphorylation appeared to differ between cyclin D1 and D2-expressing MM cells, although this remains to be confirmed in a larger panel of cell lines. Ultimately, a more detailed analysis involving MM cell lines bearing a broad range of molecular lesions will be required to more fully examine this hypothesis.

Increasingly, the transcription factor cMaf is being regarded as a crucial target in MM cells. In keeping with the findings of an earlier study (Mao et al., 2007) cMaf protein expression was found in a majority of MM cell lines carrying t(14;16) and t(4;14), whilst no expression was found in two cell lines carrying the t(11;14) translocation (KMS12BM and U266). Similarly, Hurt et al showed that these two cell lines expressed relatively little cMaf mRNA in comparison to those MM cell lines with t(14;16) and t(4;14) (Hurt 2004). No study to date has demonstrated cMaf protein expression by Western blotting in primary MM cells nor examined whether cMaf expression is increased in response to mitogenic stimuli. Importantly, the experiments detailed above found that CD138+ cells from a patient with t(14;16) showed strong upregulation of cMaf protein following culture in 20% MM plasma. Intriguingly, a recent publication has shown that MM cells expressing cMaf are dependent on MAPK signalling and crucially that cMaf-expressing MM cells show increased sensitivity to the MAPK-inhibitor U0126, although it is not yet known if these results can be replicated in in-vivo models (Anunziata et al., 2011).
6.5 Summary

In the experiments outlined above, it was found that the PI3K, MAPK and NFκB pathways in both HMCLs and primary MM cells were activated in response to APRIL. Importantly, these effects were seen in both cyclin D1 and D2-expressing MM cells. Furthermore, IGF-I stimulation of HMCLs bearing either cyclin D1-and cyclin D2-activating IgH/TCs, led to upregulation of both pAKT and pMAPK irrespective of IgH/TC status. Lastly, the cMaf transcription factor was found to be expressed in MM cells and induced in response to the stimulus of MM-plasma containing culture medium. Ultimately, further studies will be required to elucidate which pathways activated in response to APRIL directly impinge on the cell-cycle machinery, and how this may differ between genetic subgroups.
Chapter 7. Conclusions and future directions

7.1 Introduction

Despite the considerable advances made in the treatment of MM patients in the last 10-15 years, MM remain incurable for the vast majority of patients with a median overall survival of 5 years (Kumar et al., Blood 2008). As outlined in chapter 1, MM displays remarkable clinical heterogeneity and although the introduction of the newer agents (bortezomib, lenalidomide and thalidomide) has led to improved survival for MM patients during the last decade, there remains a significant minority of patients (approximately 15-20%) who continue to fare badly with all currently available treatments including the newer agents. IgH/TCs are found in approximately 40-50% of all newly diagnosed MM patients and it has been repeatedly shown that patients harbouring the t(4;14) and t(14;16) lesions have inferior outcomes despite intensive treatments and the incorporation of novel agents into induction regimens (Avet-Loiseau et al., 2010, Morgan et al., 2012). Thus, patients with these IgH/TCs are amongst those with “high-risk” MM and these patients urgently require more effective anti-MM therapy. Therefore, identifying the soluble or cellular components of the MM BM microenvironment that promote cell cycle progression and survival in these poor-risk MM subgroups may identify an appropriate target for intervention or point to the most important intracellular signalling pathways. FISH-analysis of CD138-selected MM cells from MM bone marrow aspirates permits the rapid identification of these patients at diagnosis facilitating their entry into clinical trials of risk-adapted or targeted therapies.

This thesis sought to explore the interaction between primary MM cells and soluble BM microenvironmental growth factors and to correlate the proliferative and survival responses of MM cells in response to such growth factors with underlying IgH/TC and
D-type cyclin class. A culture system that promoted the survival of primary MM cells in vitro was first optimized (chapter 3) before the effect of the MM growth factor, APRIL, on the cell cycle behaviour of a large panel of primary MM cells with diverse IgH/TCs was explored in chapter 4. The effect of APRIL in promoting drug resistance in MM cells of different IgH/TC status was investigated in chapter 5 before the effect of MM growth factors on intracellular signalling pathways on MM cell lines of differing IgH/TC and D-type cyclin expression was analysed. This chapter will provide a summary of the main findings of chapters 3-6 after which directions for future studies will be discussed.

The lack of a culture medium that supports the growth and survival of purified primary MM cells has hampered attempts to study the biological behavior of primary MM cells in vitro, perhaps emphasising the dependence of these cells on the MM BM microenvironment. In chapter 3, a culture medium consisting of RPMI1640 and a 20% solution of plasma pooled from MM patients (CM) was shown to be superior to the standard culture medium of RPMI1640/10%FCS in maintaining CD138-selected primary MM cell viability (Figure 3.2A, p<0.001) and at a range of equal concentrations of pooled MM plasma and FCS. Importantly, normal human plasma was found to be as effective as pooled MM plasma suggesting that improved in vitro survival of MM cells in CM was not related to the increased concentration of a specific MM growth factor in MM plasma (Figure 3.4A). Culturing purified primary MM cells in CM was also found to be superior to RPMI/10%FCS in preserving the proliferative fraction of primary MM cells, as measured by FACS analysis of the S/G2M fraction by Ki67/PI staining (p<0.05). Importantly, this medium supported the survival of CD138-selected primary MM cells, thus facilitating the western blotting and thymidine incorporation experiments that were critical to examining cell cycle progression in vitro.
The work detailed in chapter 4 focused initially on identifying and measuring the S/G2M (proliferative fraction) of primary MM cells according to disease stage. In keeping with published data, the S/G2M fraction was seen to increase with disease progression. Subsequently, the work then concentrated on the effect of the TNF-superfamily member APRIL on MM cells. Firstly, APRIL was found to promote cell cycle progression in MM cells, but crucially, this effect was largely restricted to cyclin D2-expressing MM cells bearing the t(4;14) and the t(14;16) translocations (Figure 4.3B, p<0.01). This finding raised the possibility that the APRIL-receptors BCMA and TACI might be differentially expressed on MM cells with different IgH/TCs, however when the expression of BCMA and TACI in 28 MM cases was examined by flow cytometry, expression was found not to differ with respect to D-type cyclin class (Figure 4.7B). The effect of APRIL on MM cells bearing t(4;14) and t(14;16) was emphasised by the demonstration of cyclin D2, CDK4 and -6 and phosphorylated pRb upregulation in response to APRIL-stimulation of MM cells bearing these translocations, whilst cyclin D1 protein expression was unaffected in cells carrying t(11;14). The expression of cell cycle regulatory proteins by primary MM cell in vivo (cyclins D1 and D2, CDK4/6 and phosphorylated pRb) was further confirmed by immunohistochemical staining of trephine biopsies from MM patients.

As previous studies had demonstrated a role for APRIL in promoting drug resistance in MM cells, whether this effect was dependent on IgH/TC or D-type cyclin expression was then examined as detailed in chapter 5. Firstly, APRIL did not rescue purified primary MM cells from spontaneous apoptosis in vitro (Figure 5.1), however, APRIL consistently protected CD138-selected primary MM cells from dexamethasone-induced apoptosis (Figure 5.2). Somewhat surprisingly, APRIL-mediated protection against dexamethasone-induced apoptosis was also most pronounced in MM cells expressing
cyclin D2 and was accompanied by reduced expression of cleaved PARP protein (Figures 5.2 and 5.4). APRIL was also shown to be abundantly expressed in the MM BM microenvironment by immunohistochemical staining of MM BM trephine biopsies, with myeloid cells identified as the cellular source of APRIL unlike MM cells which expressed surface-bound APRIL only.

At this point, the work turned to focus on the effect of APRIL on intracellular signalling pathways in MM cells (chapter 6). By immunohistochemical staining, APRIL was shown to be expressed on the surface of MM cells expressing both cyclin D1 and cyclin D2, whilst the expression of TACI and BCMA was found to be equivalent between cyclin D1 and D2-expressing cases, thus neither the availability or the reduced ability to bind APRIL appeared to explain the differential response to APRIL stimulation observed between MM cells bearing different IgH/TCs. Thus the effect of APRIL on proximal signalling pathways in MM cells was examined. In keeping with published work, APRIL-stimulation of MM cells led to the induction of the PI3K/AKT, MAPK and NFκB pathways in primary MM cells irrespective of D-type cyclin class (Figures 6.2 and 6.3). The observed difference in proliferative behaviour in response to APRIL-stimulation between cells expressing either cyclin D1 or cyclin D2 suggests that critical differences exist in the cell cycle protein “machinery” (i.e. assembly and stability of cell cycle proteins) between MM cells from different IgH/TC groups. This hypothesis will ultimately require in-depth mechanistic studies.

7.2 Future Directions

This work has demonstrated that APRIL plays an important role in promoting cell cycle progression in primary MM cells. In addition, this work confirms the previous findings of Moreaux et al (2004) in showing that APRIL protects MM cells from
dexamethasone-induced apoptosis and supports the findings of Gupta et al (2009) who showed that APRIL promoted cell cycle progression in B-cell lymphoma cells via activation of the PI3K/AKT signalling pathway. From a clinical perspective, patients with newly diagnosed MM now have very high rates of response to induction therapy (>70-80%) that commonly includes a novel agent as part of a three-drug regimen. Responding patients, with good performance status, then go on to receive high-dose melphalan and reinfusion of cryopreserved stem cells as part of an autologous stem cell transplant (ASCT). Complete response (CR) rates of 40-50% are not uncommonly seen after ASCT with modern induction therapy. Despite these impressive response rates, virtually all patients relapse after a median duration of 24-30 months and ultimately die of their disease when it becomes refractory to anti-MM therapy. Similarly, older MM patients can achieve CR rates of up to 30% with modern bortezomib-containing induction regimens (Mateos et al, 2010). Therefore, identifying the bone marrow microenvironmental factors that may contribute to the ability of MM cells to proliferate after a period of quiescent disease (plateau) of over 2 years (in the case of those receiving ASCT) would appear to be critical to preventing disease-relapse.

7.2.1 APRIL as part of a bone marrow “Multiple Myeloma Niche”

In the work presented in this thesis, using dual immuno-staining of MM cells with anti-Blimp-1 and anti-APRIL, it has been shown that APRIL is bound to the surface of MM cells in the bone marrows of MM patients with floridly relapsing disease. However, it is not known what contribution APRIL (or any of the other known MM growth factors) makes to the ability of MM cells to proliferate following a period of disease plateau. We know that the percentage of CD138+ MM cells in the bone marrow of MM patients in complete remission following ASCT is typically very low (<5%), however very little is known about the localization of these cells. Although the dependence of MM cells on
multiple soluble and cellular factors for growth and survival in the MM microenvironment has been well documented, less is known about the spatial relationship of such factors during plateau phase and whether these factors are important in the development of relapsing disease. In this context, a “niche” has been described as “a specialized regulatory microenvironment, consisting of components which control the fate specification of stem and progenitor cells, as well as maintaining their development by supplying the requisite factors” (Basak et al, 2009) and such a BM niche has been described that supports the development of normal human haematopoietic stem cells consisting of osteoblasts, osteoclasts, endothelial cells, stromal cells, adipocytes and extracellular matrix proteins (HSC niche, Lymperi et al, 2010).

It is generally accepted that the MM cells responsible for disease reactivation, or “myeloma progenitors”, are dependent on specialized niches in the BM for growth and survival; a so-called “MM niche”. For example, many of the individual BM components of the HSC niche, such as osteoclasts and BM stromal cells, have been shown to promote the growth and survival of MM cells (Podar et al, 2009). In addition, support for the existence of BM niches to support the growth of haematopoietic malignancies stems from the observation of relapsed leukaemia following allogeneic transplantation, where the relapse of the leukaemia is derived from donor cells that have managed to acquire the molecular and immunophenotypic features of the patient’s original malignant cells (Flynn et al, 2007). However, a “MM niche” has not been properly characterised to date and only a few attempts have been made to describe such a niche (Basak et al, 2009). Many important questions regarding the components of a MM niche remain unanswered. Many BM microenvironmental factors have been shown to promote MM cell growth and survival, but which ones are the most critical in
provoking clinical relapse? It is possible that the MM niche may be dynamic and the key components may change with changing disease status. It is also possible that MM cells may only spend brief periods in contact or association with one or several niche components but the order of these interactions is unknown. It may also be the case that different factors may be responsible for the attraction of MM cells and whilst other factors may maintain their survival within the niche. MM cells interact with other cellular factors and ligands with a variety of cell surface receptors, but which interactions are the most crucial in a niche context is also poorly understood.

Overall, accumulating evidence suggests that APRIL would play a role in such a niche. APRIL was shown to be an important autocrine growth factor for megakaryopoiesis (Bonci et al, 2004) and megakaryocytes were further confirmed as an important cellular source of APRIL when Winter et al (2010) recently showed that 30% of BM plasma cells co-localised with APRIL-producing megakaryocytes that supported plasma cell survival in murine studies. In the same study, mice without expression of the thromboietin receptor c-mpl were found to have reduced plasma cell survival. MM cells co-cultured with osteoclasts had improved in vitro survival in comparison to MM cells culture in medium alone, an effect that was abrogated by the addition of TACI-Fc (Abe et al, 2008, Moreaux et al, 2011). The work presented in this thesis and studies in B-cell lymphoma sections and mucosa-associated lymphoid tissue (MALT) have shown that lymphoma cells, malignant plasma cells and normal plasma cells exist in close proximity to APRIL-secreting myeloid cells (Schwaller et al, 2007a, Huard et al, 2008). The importance of APRIL in maintaining plasma cell survival is underscored by the observation that APRIL is crucial for survival at the plasmablast stage (Belnoue et al, 2008). APRIL binds the BCMA and TACI receptors, but also binds to HSPGs, an interaction which may be highly important in the context of a plasma cell niche.
(Hendriks et al, 2005, Huard et al, 2008). A schematic representation of the likely key APRIL-BM niche components is shown in Figure 7.1. Ultimately, a greater understanding of the APRIL interactions in this context may permit more accurately targeted therapy for MM.

Figure 7.1

**Figure 7.1: Role of APRIL in the “Multiple Myeloma Niche”.** APRIL, secreted by megakaryocytes (MEG), myeloid cells and osteoclasts (OC), binds to MM cells bearing BCMA, TACI and HSPGs to promote MM cells survival. The niche is supported by CXCL12-bearing bone marrow stromal cells which bind CXCR4-expressing MM cells (adapted from “New friends for bone marrow plasma cells”, Brinks R, Nat Immunol. 2011 Feb;12(2):115-7).
7.2.2 APRIL-mediated drug resistance in multiple myeloma

A key issue in the treatment of patients with MM is the development of resistance to anti-MM therapies. In the work presented in this thesis, it has been shown that APRIL protects primary MM cells from dexamethasone-induced apoptosis, an effect that was most apparent in MM cells expressing cyclin D2. This observation adds to the findings of other groups who have shown that APRIL plays an important role in promoting resistance to chemotherapy. As discussed in chapters 1 and 5, APRIL was previously shown to protect IL-6 dependent HMCLs from dexamethasone-induced apoptosis, an effect that was overcome by the addition of TACI-Fc (Moreaux et al, 2004, 2009). In these studies APRIL stimulation of HMCLs led to increased expression of BCL-2 and Mcl-1 anti-apoptotic proteins suggesting a mechanism by which APRIL mediates drug resistance. Similarly, BAFF was shown to protect the MM1S and KMS28PE HMCLs from lenalidomide and dexamethasone-induced apoptosis (Tai et al, 2006). Although novel anti-MM treatments have been introduced, the response rates to these agents is significantly greater when they are used in combination with dexamethasone, confirming that dexamethasone remains part of the backbone of anti-MM therapy.

Studies in other tumour types have also demonstrated an important role for APRIL in drug resistance and tumour cell survival. In a recent publication that showed APRIL to be expressed by acute myeloid leukaemia (AML) cells, the addition of BCMA-Fc and TACI-Fc to the cell cultures led to a significant increase in AML cell death suggesting that APRIL may be produced in an autocrine fashion by AML cells (Bonci et al, 2008). Furthermore, APRIL-blockade led to a significant increase in cell death when AML cells were treated with either etoposide or camptothecin suggesting a role for APRIL in mediating drug resistance in these cells. To confirm this, these authors transduced CD34-expressing haematopoietic progenitor cells with a retroviral vector bearing the
APRIL gene and found that AML cells over-expressing APRIL displayed significant resistance to chemotherapeutic agents.

Despite the evidence demonstrating a role for APRIL-mediated drug resistance in different tumour types, the molecular basis and signalling pathways involved remain to be fully elucidated. APRIL activates the PI3K/AKT, MAPK and NFκB signalling pathways in MM cells, the NFκB pathway in CLL cells and the PI3K/AKT pathway in B-cell lymphoma cells suggesting that multiple pathways may be involved (Moreaux et al, 2004, Endo et al, 2007, Gupta et al, 2009). However, more recent studies provide a glimpse of other mechanisms that may be involved. APRIL-overexpression in CD34-expressing haematopoietic cells lead to chemotherapy resistance via upregulation of Bcl-2 protein, whilst APRIL inhibition resulted in Bcl-2 downregulation suggesting a key role for this protein in APRIL-mediated drug resistance (Bonci et al, 2008). These effects are similar to those seen in Hodgkin lymphoma cells, where both APRIL and BAFF were shown to increase the expression of Bcl-2 protein in Hodgkin-Reed-Sternberg (HRS) cells, whilst also promoting protection against doxorubicin-induced cell death (Chiu et al, 2007). Recent studies have focused attention on the Pim family of kinases, which have been shown to be overexpressed in haematopoietic malignancies and to promote cancer cell growth and survival (Brault et al, 2010). In a recent study, Pim-2 was upregulated in MM cells in response to stimulation with BAFF, APRIL and IL-6 and was associated with enhanced MM cell viability, which was reversed upon Pim-2 knockdown (Asano et al, 2011). Thus, increased expression of Pim-2 presents another mechanism by which APRIL may promote drug-resistance. It is also possible that APRIL may indirectly promote cell adhesion mediated drug-resistance (CAM-DR). In TACI-expressing HMCLs, APRIL was shown to increase expression of c-Maf, cyclin D2 and importantly, β-7-integrin mRNA levels (Moreaux et al, 2007).
Subsequently, β-7-integrin expression was found in 92% of primary MM samples with increased expression associated with a poorer prognosis and shRNA-mediated knockdown of β-7-integrin was observed to reduce MM cell adhesion to fibronectin and E-cadherin, thus sensitizing MM cells to bortezomib and melphalan treatment (Neri et al., 2011).

APRIL binds to three different receptors (BCMA, TACI and HSPGs), and although the interaction between APRIL and each of these receptors has been shown to be important in promoting tumour cell and/or normal B-cell growth and survival, the interactions that promote drug resistance are not yet fully understood. As already discussed in chapter 1, siRNA-mediated knockdown of TACI abrogated APRIL-mediated B-cell lymphoma cell growth, whilst BCMA-knockdown had no effect. Similarly in this study, pretreatment of the lymphoma cells with heparin did not affect the ability of APRIL to promote cell growth (Gupta et al., 2009). In contrast, it was subsequently shown that heparin treatment was able to overcome APRIL-mediated protection against dexamethasone-mediated apoptosis in the LP1 and L363 HMCLs both of which express TACI and APRIL-mediated growth of the TACI-negative XG-1 HMCL (Moreaux et al., 2009). Therefore, the interaction between APRIL and HSPGs may be crucial in APRIL-mediated drug resistance and is likely to be the focus of future studies as it is possible that some of the survival benefit observed in patients with metastatic cancer treated with low molecular weight heparin (LMWH) may have derived from the interruption of HSPG-mediated signalling (Klerk et al., 2005). One further possibility is that another as yet unknown APRIL receptor may mediate many of these effects.

7.2.3 Targeting APRIL in multiple myeloma
The accumulating evidence linking APRIL with tumour progression and demonstrating
APRIL as a key cytokine in the pathogenesis of autoimmune disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), has focused efforts on blocking APRIL-binding as a therapeutic strategy during the last decade (Moreaux et al., 2009, Townsend et al., 2010). The APRIL-blocking agent that has advanced furthest in development to date is “Atacicept”, a soluble, recombinant fully human chimeric molecule consisting of IgG1 fused to the extracellular ligand-binding domain of TACI (Figure 7.2). As BAFF also binds TACI, the effects of BAFF are also inhibited.

Atacicept was safely administered to 23 healthy volunteers in a Phase I study and was accompanied by a dose-dependent reduction in serum IgM levels without serious adverse events and was then shown to reduce MM cell growth in the SCID-hu murine model (Munafo et al., 2007, Yaccoby et al., 2008). Since then, three early phase studies of atacicept treatment in patients with lymphoproliferative disorders have been reported.

In the first study, 15 patients with relapsed/refractory B-cell NHL were treated with weekly subcutaneous injections of atacicept (Ansell et al., 2008). In keeping with the findings of the study in healthy volunteers, atacicept treatment led to significant reductions in serum immunoglobulin levels and importantly led to stable disease in 2 patients, who later experienced disease progression during extended atacicept treatment.

In the second, more recent study, 12 MM patients and 4 patients with Waldenstrom’s macroglobulinaemia were treated with five once-weekly subcutaneous injections of atacicept (Rossi et al., 2009). Encouragingly, 5 MM patients had stable disease after the initial phase of treatment and intriguingly, atacicept treatment led to a reduction in syndecan-1 concentrations. The most recent study examined safety and biological activity of increasing doses of atacicept in 21 patients with fludarabine-refractory CLL (Kofler et al., 2011). Interestingly, of the patients treated at the highest dose-level, 1 patient achieved a partial response and 8 patients achieved stable disease. Taken
together, these studies show that atacicept treatment is well tolerated and shows evidence of biological activity in lymphoproliferative disease suggesting larger studies of atacicept in combination with other anti-MM agents may be justified. However, the possibility of infection relating to the decline in immunoglobulin levels is highlighted by the results of a study in patients with lupus nephritis who received combination therapy with atacicept, prednisolone and mycophenylate mofetil. An unexpectedly high rate of infection in these patients led to the early cessation of the trial (Townsend et al, 2010).

As atacicept also binds BAFF, which is crucial for normal B-cell development, other researchers have focused on developing agents that are APRIL-specific. A recent study describes the development of 2 anti-APRIL monoclonal antibodies (Guadagnoli et al, 2011). These antibodies were shown to strongly inhibit APRIL-binding to lymphoma cell lines and 2 MM cell lines (RPMI8226 and L363). Furthermore, these antibodies inhibited CLL cell growth in vitro and significantly reduced B-cell proliferation in APRIL-transgenic mice. MM is a heterogeneous disease and studies with greater patient numbers treated with APRIL-blocking agents may identify subgroups of MM patients that may derive the most benefit from such a treatment approach. Moreaux et al identified 2 groups of MM patients, with both “high” and “low” TACI expression respectively (Moreaux 2005). In this study gene expression analysis showed that those patients with increased TACI expression had a signature consistent with dependence on the BM microenvironment in contrast to TACI-low patients who displayed signature representative of BM-independence. In a subsequent study, it was found that only the growth of TACI-high MM cells was inhibited by atacicept whilst TACI-low MM cells were relatively unaffected (Yaccoby et al, 2008). In the work presented in this thesis, cyclin D2 expressing MM cells bearing either t(4;14) or t(14;16) were APRIL-
responsive, both in terms of cell cycle progression and drug resistance, suggesting that these patients may perhaps derive most benefit from APRIL-inhibition. Ultimately, this will need to be demonstrated in pre-clinical models and clinical trials.

**Figure 7.2**

![Diagram of TACI-Fc](image)

**Figure 7.2: Schematic representation of TACI-Fc.** Atacicept is comprised of a soluble, recombinant fully human chimeric molecule consisting of IgG1 fused to the extracellular ligand-binding domain of TACI (Adapted from “Atacicept: targeting B cells in multiple sclerosis” by Hartung HP et al, Therapeutic Advances in Neurological Disorders. 2010 Jul;3(4):205-16)
7.2.4 D-type cyclins as targets in multiple myeloma

It is now well documented that D-type cyclins are overexpressed in >95% of MM cases, however, as discussed in chapter 1 this important finding was originally described on the basis of large gene expression profiling experiments. Less is known about the expression of D-type cyclin protein in primary MM cells, however some of the work presented in this thesis adds to and confirms previous work from our laboratory and others in showing D-type cyclin protein expression in primary MM cells by both Western blotting and immunohistochemical staining of MM BM trephine biopsies (Glassford *et al*, 2007, Ely *et al*, 2005). In particular, the demonstration of the feasibility of detecting cyclin D2 expression in primary MM cells by immunohistochemistry has since been confirmed by another group (Neri *et al*, 2011). Furthermore, in keeping with earlier findings, D-type cyclins were shown to be functional, as cyclin D2 protein was upregulated in response to APRIL stimulation in primary MM, a feature that was accompanied by increased expression of Ki67 and BrdU uptake (Glassford *et al*, 2007).

Along with the earlier gene expression profiling studies, these findings highlight D-type cyclins, and other cell cycle regulatory proteins, as potential therapeutic targets in MM. Although D-type cyclins are at the core of the cell cycle machinery, one problem with this strategy is the possibility that malignant cells would be able to undergo cell cycle progression even in the absence of D-type cyclin expression. Alternative mechanisms for cell cycle progression have been suggested on the basis of knockout studies in mice lacking expression of cyclin D1, D2 and D3 (Kozar *et al*, 2004). In these mice, fibroblasts lacking D-type cyclin expression were found to show near normal patterns of proliferation, in contrast to hematopoietic stem cells that were found to be dependent upon D-type cyclin expression for proliferation. Other studies have documented the
phenotype of CDK-knockout mice, and shown that mice lacking CDK4 and CDK6 had
defective haematopoiesis (Malumbres et al., 2004, Berthet et al., 2006). However, MM
cells demonstrate cell cycle dysregulation by overexpressing one single D-type cyclin,
which is in contrast to cells in normal tissue where the three D-type cyclins are co-
expressed and have been shown to be interchangeable (Lahti et al., 1997). This
characteristic of MM cells may render them more susceptible to the targeting of one
particular overexpressed D-type cyclin.

Tiedemann et al (2008) investigated the susceptibility of MM cells to individual D-type
cyclin knockdown, via shRNA-mediated silencing of cyclin D1 and D2 expression, and
found that knockdown of each of these 2 cyclins in turn led to cell cycle arrest and
cytotoxicity after prolonged in vitro culture. By screening a chemical library for agents
that could inhibit transactivation of the cyclin D2 gene, they identified kinetin riboside
(a synthetic cytokinin) as an agent that suppressed cyclin D1 and D2 expression and led
to early G1-phase arrest in MM cell lines as well as inducing apoptosis in primary MM
cells and inhibiting tumour growth in MM xenograft models. The observation, in this
study, that knockdown of cyclin D1 led to cell cycle arrest is in keeping with previous
work from our laboratory (Glassford et al., 2007), although another study found that
overexpression of cyclin D1 in MM cells was insufficient to promote cell cycle
progression suggesting that inhibition of cyclin D1 in isolation may be insufficient to
have a meaningful clinical impact in MM (Ely et al., 2006). Furthermore, a recent study
emphasises the complexity of targeting individual D-type cyclin proteins, even when
that D-type cyclin is overexpressed, as is the case in mantle cell lymphoma (MCL),
which has it’s hallmark a t(11;14) translocation and overexpression of cyclin D1. Klier
et al (2008) performed shRNA-mediated knockdown of cyclin D1 in MCL lines and
observed a reduction in the percentage of cells in S-phase and increased expression of
However, they also observed a consistent compensatory upregulation of cyclin D2 protein and disappointingly, combined inhibition of both cyclins D1 and D2 did not further enhance the modest effects seen with cyclin D1 knockdown alone (Klier et al, 2008). In addition, similar knockdown experiments in the U266 MM cell line led to a decrease in S-phase percentage cells but had no effect on cell survival, an effect that was possibly explained by a compensatory increase in cyclin D2 protein (Tchakarska et al, 2009). Together the results of these studies would appear to suggest that targeting cyclin D1 in isolation in MM (and MCL) is unlikely to result in meaningful clinical benefit.

This has prompted other groups to focus on the CDK4 and -6 proteins as they form complexes with all three of the D-type cyclins. One group has shown that in vitro treatment of primary MM cells with PD0332991, a highly selective, orally active inhibitor of CDK4 and -6, led to early G1 arrest and also inhibited tumour growth in a MM xenograft model (Baughn et al, 2006). In a follow-up study, this agent was investigated as a combination treatment with bortezomib in the 5T33MM murine model, with the combined treatment showing enhanced activity over single-agent treatment (Menu et al, 2008). More recently, another CDK-inhibitor (P276-00) that targets CDK9 and CDK1 in addition to CDK4 was shown to induce cell cycle arrest and cytotoxicity in MM cell lines and an RPMI8226 xenograft model (Manohar et al, 2011). Overall, the specific targeting of both CDKs and D-type cyclins in MM is at an early stage, and the results of early phase studies of CDK4/6-inhibitors are awaited with much interest.
7.2.5 Clinical and biological heterogeneity in myeloma

MM patients show remarkable variation in their clinical features and their responses to treatment and we now understand that this heterogeneity is related to different disease subtypes that are underpinned by distinct genetic changes, implying that the efficacy of anti-MM therapies may vary depending upon the genetic subtype. As discussed above and throughout this thesis, dysregulation of a D-type cyclin occurs in >95% of MM cases. Upwards of 90% of MM patients express either cyclin D1 or cyclin D2 and these cases are further subdivided according to the presence or the absence of an IgH/TC, providing the basis for the TC classification (Table 7.1). In addition to previous work from our laboratory, the work presented in this thesis suggests that cyclins D1 and -D2 are regulated differently, providing a functional validation of the TC classification. Primary MM cells expressing cyclin D2 and bearing t(4;14) and t(14;16) were shown to undergo cell cycle progression in response to APRIL, whereas other cell types were largely unresponsive. In addition, APRIL was also observed to attenuate dexamethasone-induced apoptosis in cyclin D2-expressing primary MM cells, but had minimal effect in cyclin D1-positive cells. These findings suggest that distinct subtypes of MM, with different mechanisms of cyclin D dysregulation, differ in the cellular mechanisms that regulate proliferation and drug resistance.

Our knowledge of the mechanisms underlying the heterogeneity within MM continues to expand and grow in complexity, with the universal overexpression of D-type cyclins at the heart of the major molecular classifications of MM (Table 7.1).
Table 7.1: Comparison of different molecular classifications of multiple myeloma

<table>
<thead>
<tr>
<th>Group</th>
<th>TC</th>
<th>Gene</th>
<th>%</th>
<th>UAMS</th>
<th>Hovon</th>
<th>Key Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cyclin D translocation</strong></td>
<td>11q13</td>
<td>D1</td>
<td>15%</td>
<td>CD-1</td>
<td>CD-1</td>
<td>Different clinical outcome for CD-1 vs CD-2</td>
</tr>
<tr>
<td></td>
<td>6p21</td>
<td>D3</td>
<td>2%</td>
<td>and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12p13</td>
<td>D2</td>
<td>&lt;1%</td>
<td>CD-2</td>
<td>CD-2</td>
<td></td>
</tr>
<tr>
<td><strong>MMSET translocation</strong></td>
<td>4p16</td>
<td>MMSET</td>
<td>15%</td>
<td>MS</td>
<td>MS</td>
<td>FGFR3 expressed in 75%</td>
</tr>
<tr>
<td><strong>MAF translocation</strong></td>
<td>16q23</td>
<td>c-maf</td>
<td>5%</td>
<td>MF</td>
<td>MF</td>
<td>Overexpression of beta-7-integrin</td>
</tr>
<tr>
<td></td>
<td>20q12</td>
<td>maB</td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8q24</td>
<td>maA</td>
<td>&lt;1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hyperdiploid</strong></td>
<td>D1</td>
<td>D1</td>
<td>33%</td>
<td>HY</td>
<td>HY CD-1 NFkB CTA PRL3 PR CTA</td>
<td>NFkB target gene expression may be ligand-dependent or result from activating mutations</td>
</tr>
<tr>
<td></td>
<td>D1+D2</td>
<td>D1 + D2</td>
<td>7%</td>
<td>PR</td>
<td></td>
<td>D1 + D2 presumed to be progression from D1, PR contains 5-10% of each TC group, but &gt;40% and None</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>None</td>
<td>No D</td>
<td>2%</td>
<td>PR</td>
<td>PR CTA</td>
<td>Bi-allelic RB deletion</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>D2</td>
<td>18%</td>
<td>LB</td>
<td>LB CTA PRL3</td>
<td>PRL3 lack poor risk features and are enriched for good risk patients</td>
</tr>
</tbody>
</table>

Key: In UAMS classification: PR=proliferation, HY=hyperdiploidy, LB=low bone, MS=MMSET/FGFR3, CD-1, CD-2, MF=MAF. In Hovon classification: additional groups are CTA=cancer testis antigen, NFkB, PRL3=PTP4A3, a protein tyrosine phosphatase. Other groups are as in UAMS classification. (Adapted from "Many multiple myelomas: making more of the molecular mayhem" by Chesi M et al. American Society of Haematology Education Program. 2011:344-53)

This point is emphasised by the recent publication of third classification system based on gene expression profiling (Broyl et al, 2010, Table 7.1), which showed significant similarities with the previously published UAMS classification (Zhan et al, 2005). This
classification system identified 10 molecular subgroups that corresponded to the
different IgH/TCs and D-type cyclin expression whilst also identifying an additional 3
subgroups to the UAMS classification based on the expression of cancer testis antigens
(CTA), NFκB target genes and the phosphatase PTP4A3/PRL3 (PRL3). Importantly,
Broyl et al identified subgroups with different clinical behaviour. For example, the CD-1
group was found to have a rapid response to therapy and a high rate of complete
remission (CR), but the overall duration of CR was relatively short. In contrast, the CD-2
group responded slowly to therapy with a low overall CR rate, however once patients
in this group achieved CR, they remained in CR for a relatively long period (Chesi et al,
2011). Thus, classification systems based on IgH/TC and D-type cyclin class, continue
to provide clinically relevant insights into MM disease behaviour. The TC classification
(and related classification systems) offers MM researchers and clinicians opportunities
to tailor treatment to specific TC groups on several fronts. Firstly, the genetic mutations
that drive the overexpression of a particular D-type cyclin continue to be targets for
therapeutic intervention. For example, the t(4;14) translocation results in overexpression
of FGFR3 which is oncogenic (Zingone et al, 2010) and a recent study showed that
shRNA-mediated knockdown of FGFR3 in bladder carcinoma cells led to cell cycle
arrest and reduced tumour growth in xenograft models, effects that were also mediated
via a specific anti-FGFR3 monoclonal antibody (Qing et al, 2009).

Alternatively, MM cells bearing different IgH/TCs appear to be dependent on different
intracellular signalling pathways for growth and survival. Recent work from our
laboratory has shown that MM cells bearing t(4;14) are sensitive to effects of dual
blockade of PI3K and mTOR pathways, unlike t(11;14) bearing cells which were found
to be insensitive (Stengel et al, 2012). Further emphasising the importance of this
PI3K/AKT inhibition in MM, DEPTOR (mTOR interacting protein) expression was
found to be greatest in the 11q13, 6p21 and maf TC groups suggesting that these groups may benefit from therapy targeted to this pathway (Peterson et al, 2009). Lastly, other groups have made recommendations for “risk-adapted therapy” of MM. One such model (mSMART, Mayo Stratification of Myeloma and Risk-Adapted Therapy), is based on an initial risk-stratification according to IgH/TC and also includes other important prognostic markers such as FISH-identified 17p deletion, cytogenetic evidence of 13q deletion and a high baseline proliferation fraction into consideration along with other variables (Kumar et al, 2009, Figure 7.3). Although not universally adopted, such a model represents one of the first attempts to tailor specific therapies to MM patients with specific genetic lesions. Finally, although the molecular landscape of MM becomes more complex with identification of new mutations, such as BRAF mutations in 4% of MM patients (Chapman et al, 2011), IgH translocation and D-type cyclin overexpression remain the starting point for current models of the molecular pathogenesis of MM, and are likely to remain the focus of targeted therapy for MM patients in the near future (Figure 7.4).

**Figure 7.3**
Figure 7.3: Risk-adapted anti-myeloma therapy. Adapted from “Management of Newly Diagnosed Symptomatic Multiple Myeloma: Updated Mayo Stratification of Myeloma and Risk-Adapted Therapy (mSMART) Consensus Guidelines (Kumar et al, Mayo Clin Proc. 2009 Dec;84(12):1095-11).

Figure 7.4

Figure 7.4: Model for the molecular pathogenesis of myeloma. Cyclin D dysregulation, occurring in a post-germinal centre B-cell(s) is thought to be the primary tumour-initiating event in MM, with 2 largely exclusive pathways – Non-hyperdiploid (Non-HD, enriched for IgH/TCs) and hyperdiploid (HD, IgH/TCs rare). Disease progression from MGUS to intramedullary MM and then to extramedullary MM is accompanied by the acquisition of cytogenetic abnormalities and mutations as shown (Adapted from “Many multiple myelomas; making more of the molecular mayhem”, Chesi M et al, American Society of Hematology Education Program, 2011; 2011:344-53)
7.3 Conclusions

In conclusion, this thesis has documented the role of APRIL in promoting cell cycle progression and drug resistance in primary MM cells and therefore provides further evidence of its role as a significant MM growth factor. Furthermore, MM cells expressing cyclin D2 in the presence of an IgH/TC were shown to be particularly APRIL-responsive suggesting that MM cells bearing different IgH/TCs interact differently with signals from the BM microenvironment in order to grow and survive. These findings represent an important contribution to our understanding of the mechanisms of proliferation and drug resistance in MM.
Chapter 8. Publications relating to this thesis

8.1 Publications in peer-reviewed journals


3. Optimal induction of myeloma cell death requires dual blockade of phosphoinositide 3-kinase and mTOR signalling and is determined by translocation subtype. Stengel C, Cheung CW, Quinn J, Yong K, Khwaja A. Leukemia. 2012 Mar 14


References


