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A STUDY OF CYTOKINE EXPRESSION AND FUNCTION IN  
MYELOMA CLONES

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MASTER OF SCIENCE

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## ACKNOWLEDGEMENTS

I wish to express my appreciation to Professor Ian Franklin for his role as supervisor.

I would like to thank Gordon, Heather and Mike for advice and help.

I wish to thank Diane for her help and advice on word processing.

I also wish to thank Anne for being a good friend and proof reading this thesis.

Finally to my husband Tom and daughters Rachel and Rebecca, for their love and understanding.

## SUMMARY

Multiple myeloma (MM) is a B cell malignancy characterised by the presence of a monoclonal population of terminally differentiated plasma cells in the bone marrow. It generally occurs in older people. Clinical manifestations result from monoclonal protein (immunoglobulin) production and accumulation in the serum and / or the urine, anaemia, lytic bone disease, hypercalcaemia, renal insufficiency and immune deficiency.

IL-6 is thought to be the major growth factor of MM but the part played by other cytokines in the pathogenesis of the disease is unclear. In the use of cloned U266 cells we hoped to gain an insight into the cytokines that are important in the survival / expansion of the myeloma clone. In this study we demonstrate that these myeloma clones express a variety of cytokine mRNA transcripts, IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-15 and TGF- $\beta$ .

IL-6, although considered the major proliferative factor, was observed in one clone only. In contrast TGF- $\beta$  mRNA transcripts were expressed by many clones. These clones were capable of suppressing T cell responses. However if IL-15 and TGF- $\beta$  mRNA transcripts were co-expressed, the suppression of T cell proliferation was more pronounced.

Due to these findings, it was decided to further investigate the effects of IL-6 and IL-15 on myeloma clones. Cell studies examining the effects of exogenous IL-6 or IL-15 alone or in combination with dexamethasone on cell cycle, proliferation, apoptosis and cell signalling were performed. The U266 cells were found to be sensitive to the anti-proliferative effect of

dexamethasone, while most of the myeloma clones were resistant. IL-6 and IL-15 were able to protect the U266 cells from the effects of dexamethasone at 50µg / ml but not at 100µg / ml. However we observed a significant reduction in proliferation when IL-15 was added in combination with dexamethasone to clone M3 (expresses mRNA transcripts for IL-15, TGF-β). This clone was resistant to dexamethasone alone. The role of IL-15 is complex and seems to behave differently depending on what other cytokines are present. Further studies may help elucidate the role of IL-15 in the pathogenesis of this disease.

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## ABBREVIATIONS

CNTF	ciliary neurotropic factor
CR	complete response
CRP	C-reactive protein
DMSO	dimethylsulphoxide
cDNA	complementary deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
HMCL	human myeloma cell line
IFN- $\alpha$	interferon-alpha
IL	interleukin
LI	labelling index
LIF	leukaemia inhibitory factor
OSM	oncostatin
MM	multiple myeloma
MRI	magnetic resonance imaging
MUD	matched unrelated donor
PBS	phosphate buffered saline
PBSC	peripheral blood stem cell
PCR	polymerase chain reaction

## ABBREVIATIONS

RNA	ribonucleic acid
SAC	staphylococcus aureus cowan
TBI	total body irradiation
TBS	tris buffered saline
VAD	vincristine, adriamycin <sup>®</sup> , dexamethasone
VEGF	vascular endothelial growth factor

## **DECLARATION**

I hereby declare that the work presented in this thesis is my own, except where stated in the text. The work has not been submitted in any previous application for a degree

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# CHAPTER ONE

## INTRODUCTION

## **Multiple myeloma**

### *Historical perspective*

The term 'multiple myeloma' (MM) was first coined by von Rustizky in 1873. His patient, a 47-year old man, was found at autopsy to have eight separate soft reddish tumours, which he called 'multiple myeloma', in the ribs, manubrium, thoracic vertebrae and right humerus. On microscopic examination, round vesicle-like cells were observed with a nucleus which was close to the plasma membrane. However patients with symptoms of the disease had been recognised from the mid 1800's by physicians such as Samuel Solly (1844), who observed his patient, Sarah Newbury, to have fractures of right radius and ulna, left tibia and fibula, and both femurs at post-mortem. Destruction of the bones was also recorded.

Dalrymple, a member of the Microscopical Society, described a 'red gelatiniform' substance comprising nucleated cells akin to myeloma cells in a sample he had been sent from Dr Macintyre's patient, Mr McBean, who had died of 'atrophy from albuminuria' in 1846. Both Dalrymple and Macintyre arrived at a post-mortem diagnosis of malignant bone disease.

When the urine from such patients was heated it was found to 'abound in animal matter', and its reactions with acid were reported by Macintyre (1850), a Harley Street consultant and physician to the Metropolitan Convalescent Institution and to the Western General Dispensary. Henry

Bence Jones, a physician with a reputation as a chemical pathologist, was also sent urine samples, including a specimen from Macintyre, in which Bence Jones confirmed the protein content and emphasized the part this played in the diagnosis of the disease, thereby lending his name to the urine protein in MM.

Owing to the limited understanding of physiology and pathology of the time it was not realised that all these individuals were suffering from the same condition with varying presentations. It is only with hindsight that we know today the patients described in these case reports were suffering from Multiple Myeloma (MM).

### *Epidemiology*

MM accounts for 10% of haematological malignancies, 1% of all malignancies in Caucasians but is more common in the Afro-American population (2% of all cancers) (Riedel & Pottern, 1992). The incidence is 4.7 per 100,000 in Caucasian men and 3.2 in women, but 10.2 and 6.7 in Afro-American men and women, respectively. MM is a disease in which the incidence increases with age. The median age of patients at time of diagnosis is 65 years.

### *Aetiology*

The genetic factors underlying MM remain unknown. There is a correlation with the HLA-Cw5 antigen and the G3m (g5) allotype (Leech et al, 1983:1985), which is more prevalent in the Afro-American population, and MM. Social factors apparently do not account for the racial differences in the incidence of the disease. Occupational factors however may play a role. Exposure to radiation (Riedel & Pottern, 1992), insecticides, herbicides, and other organic solvents, may explain the higher incidence of MM in farmers, and workers associated with petroleum products.

### *Clinical Presentation*

MM is a B-cell malignancy characterised by a monoclonal proliferation of plasma cells, which accumulate in the bone marrow, and the presence of the so-called M-component, a clonal immunoglobulin, in the serum and/or in the urine (Boccardo & Pileri, 1995) which is produced by these cells. The most common feature of the disease used to be osteolytic lesions with bone pain being the most frequent symptom. However owing to a greater awareness of the disease the incidence of back pain has decreased to 37% (Riccardi et al, 1991) with 20% of MM patients being asymptomatic. Pneumonia and pyelonephritis are the most frequent sequelae of recurrent bacterial infections. These infections occur due to the clonal expansion of

one type of non-functioning antibody producing plasma cell, usually IgG or IgA with occasional IgM or IgD or mixed cases, leading to the lack of production of normal antibodies. The suppression of bone marrow function by the increased tumour burden leads to anaemia. Renal insufficiency is also observed in many patients, which may be owing to various reasons, including hypercalcemia, hyperuricemia, amyloid deposits, and tubular damage due to monoclonal light chain secretion.

MM is confirmed when the bone marrow shows increased plasma cells (>10%), lytic bone lesions are detected by magnetic resonance imaging (MRI) (X-rays in the United Kingdom) and M-protein is found in the serum or urine. Patients who lack all other criteria for MM but have a monoclonal protein in the serum or urine are classified as having monoclonal gammopathy of undetermined significance (MGUS). One in four of these patients will progress to MM whilst in others the M-protein remains stable (Kyle, 1994).

### ***Prognosis***

MM has an overall survival rate ranging between one and ten years depending on the stage of the disease at diagnosis and treatment history. The tumour proliferation rate is assessed by the following factors: the bone marrow plasma cell labelling index (LI), serum interleukin-6 (IL-6) level, and the serum C-reactive protein (CRP) level. The serum level of  $\beta_2$ -microglobulin, the percentage of bone marrow plasma cells, the serum

haemoglobin level, and the extent of lytic bone lesions are all linked to tumour burden. Prediction of patient survival is linked to these clinical factors using the so-called Durie and Salmon staging system. (Durie & Salmon, 1975) There are three stages to this system (Table: 1). Patients are stratified according to biochemical and medical markers such as haemoglobin, serum calcium, the M-component level and extent of bone lesions. Renal function of the patients is also used. Patients are categorised into low, medium or high risk. This system is often used to assign patients who need chemotherapy, with improved knowledge of objective markers of poor prognosis e.g. monosomy 13, it is likely that therapy will be linked increasingly to prognosis rather than simply staging.

### ***Treatment of Multiple Myeloma***

#### *Conventional anti-neoplastic chemotherapy*

Currently, the most efficacious drugs in the treatment of MM are the alkylating agents (melphalan (L-phenylalanine mustard) and cyclophosphamide) and glucocorticoids. In the 1960's a combination of melphalan and prednisone was introduced. It is the principle treatment for MM if no neutropenia or thrombocytopenia is present. The melphalan and prednisone combination has a response rate of 50-60% in MM patients: 3% of patients lose the M-component (Alexanian et al, 1977) however in the

## **Table 1: Durie and Salmon staging system**

### **Stage I: Low tumour mass**

All of the following must be present:

- A. Haemoglobin  $>10.5$  g/dl or Haematocrit  $>32$  %
- B. Serum calcium level normal
- C. Low serum or urine myeloma protein production rates:
  - 1. peak  $< 5$  g/dl
  - 2. IgA peak  $< 5$  g/dl
  - 3. Bence Jones protein  $< 4$  g/24h
- D. No bone lesions or osteoporosis

### **Stage II: Intermediate tumour mass**

All patients who do not qualify for high or low tumour mass categories are considered to have intermediate tumour mass.

### **Stage III: High tumour mass**

One of the following abnormalities must be present:

- A. Haemoglobin  $< 8.5$  g/dl or haematocrit  $< 25$  %
- B. Serum calcium  $> 12$  mg/dl
- C. Very high serum or urine myeloma protein production rates:
  - 1. IgG peak  $> 7$  g/dl
  - 2. IgA peak  $> 5$  g/dl
  - 3. Bence Jones protein  $> 12$  g/24h
- D.  $>3$  lytic bone lesions

majority of patients the M-component decreases or stabilises (reduced in 60%, stabilises in 30% and increases in 10%).

In the 1970's further combination therapies were introduced, in the hope that additional drugs given together would be considered more effective than as monotherapies for example. Other drug combinations have been administered, for example using various permutations of the anti-metabolites (methotrexate and cytosine arabinoside) with the vinca alkaloids (vinblastine, vincristine and vindesine), nitrosoureas and anthracyclines (Adriamycin<sup>®</sup>), but no improvement in outcome has been convincingly proven.

Patients relapsing after melphalan and prednisone are usually treated with VAD (vincristine, Adriamycin<sup>®</sup>, dexamethasone) chemotherapy (Barlogie et al, 1984). VAD or VAD based regimens reduce tumour burden more rapidly and are usually used in patients with acute or chronic renal failure due to the fact that the drug removal is mostly non-renal.

### *High dose chemotherapy*

Patients who do not respond to conventional treatment can benefit from high dose chemotherapy. McElwain et al (1983) showed a dose-response relationship in MM. In refractory patients a high rate of complete response

(CR) was induced by the use of melphalan. However mortality from the intensive treatment was high (up to 30%) as the myelosuppression was so long lasting. Autologous bone marrow or peripheral blood stem cell (PBSC) transplantation was shown by Barlogie (1986) to reduce the haematological toxicity of the high dose melphalan and most young patients are now offered this form of treatment, once the disease has been stabilised following initial therapy.

#### *Autologous peripheral blood stem cell transplant*

This method of rescue therapy is recommended for patients who are less than seventy years old at presentation of the disease. Patients are first treated with VAD or similar agents for a few months which reduces the number of tumour cells. VAD has the advantage of inducing prompt responses without producing long-term damage to marrow stem cells. The patient's stem cells are mobilised and collected, chemotherapy or total body irradiation (TBI) then follows. Finally patients receive their previously collected stem cells for bone marrow reconstitution. This procedure is now considered safe with less than a 2% death rate due to toxicity. A disadvantage of this treatment is that even after intensive chemotherapy / TBI, myeloma cells remain. Tumour cells may contaminate the stem cells collected for the transplant, although no good evidence exists that this is detrimental to the outcome.

### *Allogeneic bone marrow transplantation*

For younger patients (e.g. those under fifty years of age), an alternative may be offered, using an allogeneic transplant of bone marrow from a "tissue type" matched sibling donor or matched unrelated donors (MUD). A high death rate persists because of toxicity / infection but 30% of patients who achieve a complete remission continue to be disease free six years post-transplant (Bensinger et al, 1996). New "reduced intensity" allogeneic transplants may extend the scope for this form of treatment in future.

### *Maintenance therapy*

Relapse is not delayed by the use of conventional chemotherapy during remission. Therefore other maintenance treatments have been sought. The cytokine interferon-alpha (IFN- $\alpha$ ) has been used, and in earlier studies survival and remission were shown to be increased (Mandelli et al, 1990). Later studies, however, suggested an increased duration of remission but not in overall survival. IFN- $\alpha$  has been used in combination with dexamethasone with survival duration of 48 months (Salmon et al, 1994). However further studies need to be carried out to confirm the effect was due to the combination and not just dexamethasone alone.

## *Clinical Trials*

### *Amino-bisphosphonates*

Osteolytic bone destruction is a major clinical symptom of this malignancy. Although patients respond to chemotherapy, progression of skeletal disease may occur (Belch et al, 1991). Randomised trials of first-generation bisphosphonates etidronate or clodronate or the second-generation amino-bisphosphonates pamidronate have been published (Berenson et al, 1996; McCloskey et al 1998). Whilst early small studies showed a reduction in bone pain and healing of lesions (McCloskey et al, 2001), no differences were found in larger later studies with etidronate. Clinical trials evaluating the anti-myeloma effect of new second-generation amino-bisphosphonates (inhibitors of osteoclast activity), such as pamidronate are in progress (Terpos et al, 2000). A significant reduction in bone pain, skeletal events such as the frequency of fractures and hypercalcaemia that is responsible for renal destruction was experienced by patients. Therefore second-generation amino-bisphosphonates may represent a new type of treatment for MM.

### *Thalidomide*

Studies have confirmed that a high micro vascular density has a poor prognostic outcome in MM (Munshi et al, 1998). This correlation has brought about clinical trials with thalidomide as this agent has anti-

angiogenic effects. Thalidomide has many different functions; it has been shown to have anti-proliferative and pro-apoptotic effect on myeloma cells (Hideshima et al, 2000). The density of cell surface molecules involved in the adhesion cascade has been shown to be altered by the administration of thalidomide (Geitz et al, 1996). Myeloma cell adhesion to bone marrow stromal cells stimulates the secretion of growth cytokines; the alteration of this may contribute to the anti-myeloma effect of thalidomide. The secretion, biological activity, or removal of cytokines from the bone marrow microenvironment from where they control the growth and survival of myeloma, may be affected by thalidomide (Corral et al, 1999). TNF- $\alpha$  was suppressed by improved removal of TNF- $\alpha$  mRNA (Morcira et al, 1993) and by increasing the effects of  $\alpha$ 1-glycoproteins which have anti-TNF- $\alpha$  activity (Turk et al, 1996), both of these events were attributed to thalidomide. Thalidomide is also responsible for the down regulation of IL-6 receptor mRNA and genes involved in cell metabolism and apoptosis (Shaughnessy et al, 2000).

Cytotoxic T-cells proliferate and secrete IFN- $\gamma$  and IL-2 in the presence of thalidomide, more importantly it induces the expansion of non-MHC restricted natural killer cells that were able to kill autologous myeloma cells (Davies et al, 2001).

The exact mechanism that is responsible for the anti-myeloma activity of thalidomide remains unknown.

### *Biology of Multiple Myeloma*

Monoclonal gammopathy of undetermined significance (MGUS) is a common pre-malignant monoclonal expansion of plasma cells which often comes before MM. It occurs in 1% of the adult population and progresses at a rate of 1% per year to MM. However after years of research there is no known way to identify those who will progress from MGUS to MM, or how to prevent this progression.

However in 50% of MM tumours IgH translocations occur and it has been suggested that this is a primary event but that secondary IgH or IgL translocations can occur throughout tumour progression from MGUS to intramedullary MM to plasma cell leukaemia (PCL) to cell lines (fig 1).

### *Oncogenesis of multiple myeloma*

The cytogenetics of MM has been examined in several studies (Dewald et al, 1985; Duric B, 1992). The most important chromosome abnormality in MM is a monosomy involving chromosome 13. This abnormality is observed in 50% of patients. Chromosome 13 abnormalities (13q-) are associated with an adverse outcome and advanced stages of MM but it is not clear if it is a causative lesion.

The 14q32 chromosomal region was the most recognized abnormality (Bergsagel et al, 1996; Nishida et al, 1997). Translocations with different chromosomal partners were shown in 70-75% of patients (Avet-Loiseau et al, 1998). The most frequent recurrent translocations were t (11; 14), with

Figure 1 : Primary and secondary translocations in the pathogenesis of MM

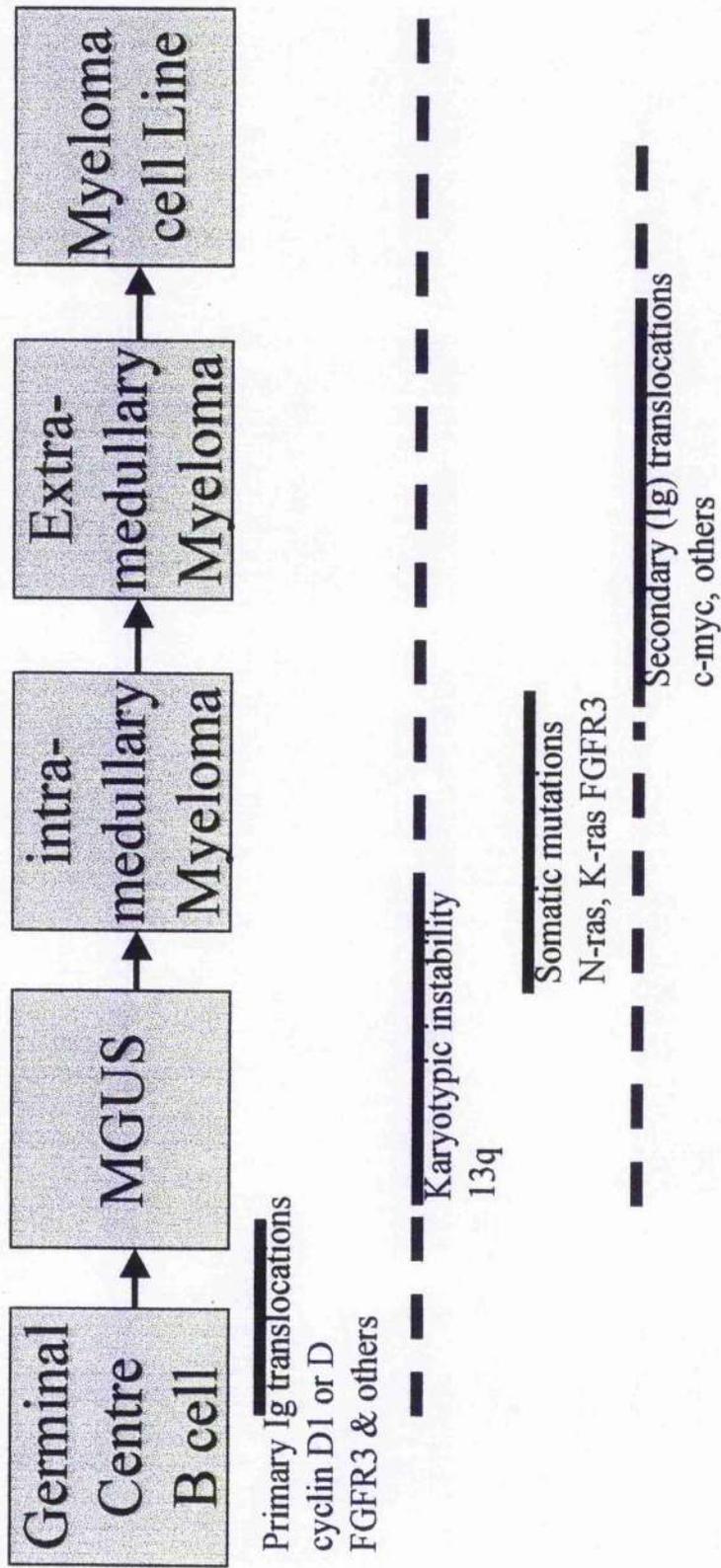


Figure 1 : Horizontal lines indicate specific oncogenic events, with solid lines showing most likely time of occurrence

the upregulation of the cyclin D1; and t (4; 14) which up regulates the receptor 3 for the fibroblast growth factor (FGF). Although these two translocations occur in 33% of instances involving 14q32, in other cases the partner is unknown. Hyperdiploidy as a second chromosomal abnormality is seen in 50-65% of patients, the chromosomes gained are mainly odd numbered (3, 5, 7, 9, 11, 15, 19) and associated with poorer prognosis (Barlogie et al, 1983)

In spite of progress in the understanding of cytogenetic changes, their part in oncogenesis of MM remains uncertain.

### *Oncogenes*

MM disease progression is associated with the p53 tumour suppressor gene and the Myc, Bcl-2 and Ras oncogenes. Malignant plasma cells from patients with MM often over express Myc, and p21 Ras or ectopically express Bcl-2 oncoproteins. The constitutive c-myc and Ras gene expression in MM may be an early event within the transformation process or a result of either an imbalanced cytokine network or activation of other upstream oncogenes within the signal transduction pathway. At diagnosis 40% of patients have mutations of N and K-ras increasing to 49% at time of relapse (Liu et al, 1996)

Point mutations of the p53 gene have been observed in 4% of intramedullary MM, and in 25-40% of patients with extramedullary MM or acute relapsed MM and in 60% of HMCL (Corradini et al, 1994).

### *Immunophenotype of plasma cells*

The CD38 antigen is used to identify plasma cells (Harada et al, 1996). Although this marker is widely distributed in the haemopoietic system, plasma cells express this antigen at a much higher intensity than other cells allowing it to become in effect a specific marker for plasma cells (Leo et al, 1992). Antibodies PCA-1, PCA-2 (Anderson et al, 1983), 62B1, 8A (Tazzari et al, 1987), R1-3 (Gronchoroff et al, 1986) have been produced that can identify antigens on plasma cells but they are not specific as they also recognise antigens on other haemopoietic cells. Benign and malignant plasma cells express syndecan-1 (a heparan sulphate-bearing proteoglycan) which was recently recognised by the antibody B-B4 / CD138 (Wijdenes et al, 1996). The CD138 is not expressed on any of the other cells within the haemopoietic compartment. Therefore CD38 and CD138 are the markers used for recognising plasma cells.

Plasma cells show a phenotypically heterogeneous profile. Reactivity was shown for CD38, CD9, CD10, HLA-DR, CD20 and for the myeloid markers CD13 and CD33 (Terstappen et al, 1990). This heterogeneity may represent the differentiation from the immature plasmoblast (sIg<sup>+</sup>, CD10<sup>+</sup>, CD19<sup>+</sup>, CD20<sup>+</sup>, HLA-DR<sup>-</sup>, CD38<sup>+++</sup>) to the mature plasma cell (sIg<sup>-</sup>, CD10<sup>-</sup>, CD19<sup>-</sup>, CD20<sup>-</sup>, HLA-DR<sup>-</sup>, CD38<sup>+++</sup>) (Tominaga et al, 1989).

Phenotypic characteristics of myeloma plasma cells varied from normal plasma cells on their expression pattern of CD19 and CD56 (Rawstron et al, 1997). Bone marrow myeloma cells expressed the phenotype CD19<sup>-</sup> CD56<sup>+</sup>

whereas normal plasma cells from different tissues expressed the phenotype CD19<sup>+</sup> CD56<sup>-</sup> (Harada et al, 1993). Recently, weak CD56 expression was observed on a minor subpopulation of normal plasma cells (Ocqueteau et al, 1998). CD56 is an adhesion molecule, an isoform of N-CAM which was involved in homophillic and heterophillic interactions.

It was also observed that, although the normal plasma cells had the phenotype CD19<sup>+</sup>, there was a small population that remain negative for this marker. Therefore the phenotype that best distinguishes the malignant plasma cells usually is lack of expression of CD19 but strong expression of CD56<sup>++</sup>.

#### *Adhesion molecules*

Some molecules are required for adhesion to the intercellular and bone marrow matrix, namely CD44 (H-CAM), CD49, CD56, galactosyl residues, rhamn (hyaluron), syndecan-1, and fibronectin. Others are required for intercellular communication, such as CD11a, CD54 (I-CAM-1), and CD58 (LFA-3).

Normal plasma cells have been shown to express VLA-4 (CD49d/CD29), VLA5 (CD49e/CD29),  $\beta$ -2 integrins, CD44 and I-CAM-1 (Leo et al, 1992, Harada et al, 1993, Barker et al, 1992, Van Riet I, Van Camp B. 1993). N-CAM expression has been shown to be low (Pellat Deceunynck et al, 1994,1995) or not found (Barker et al, 1992, Leo et al, 1992). On normal plasma cells LFA-3 was not expressed (Barker et al, 1992) while LFA-1

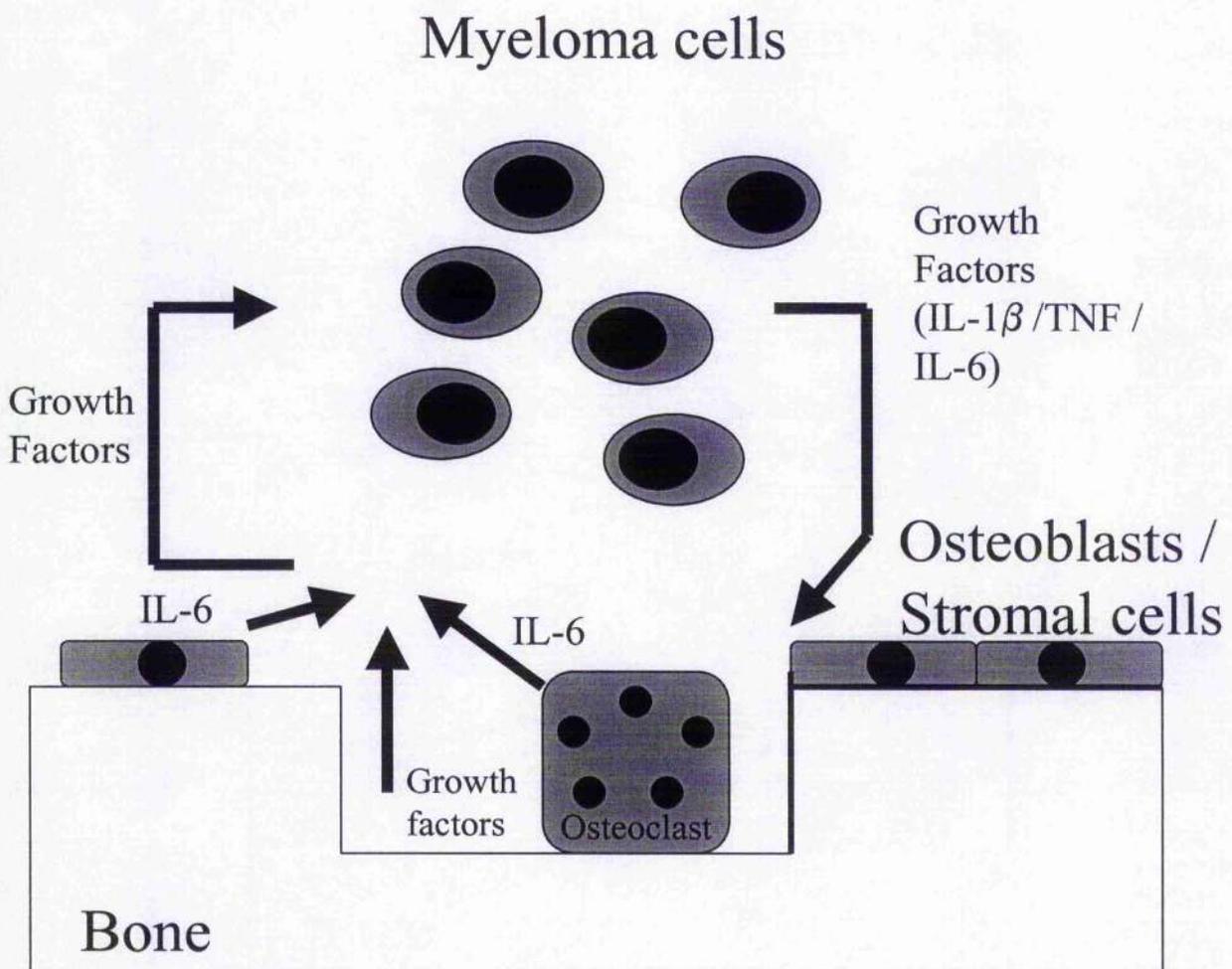
was undetectable (Ashmann et al, 1992, Barker et al 1992) or expressed at low levels (Pellat-Deccunynck et al 1995).

Myeloma cells express syndecan-1 (Wijdenes et al, 1996, Livingston et al, 1996), CD44 (Hamilton et al, 1991, Picker et al, 1988, Uchiyama et al, 1992) and CD54 (Hamilton et al 1991, Harada et al, 1993). CD44 and CD54 remain up regulated during the acute phase of MM and were expressed in extramedullary proliferation and on human myeloma cell lines. Whereas CD56 (N-CAM) which was strongly expressed on MM cells (Van Camp et al, 1990, Van Riet et al, 1991, Barker et al, 1992) was down regulated during the acute phase of the disease as well as LFA-1 (Ashmann et al, 1992). MM cells also expressed LFA-3 (Barker et al, 1992). Reduced cell-cell interaction may encourage dissemination of the disease. Loss of adhesion molecules may allow cells to escape immune detection.

#### *Bone marrow microenvironment*

The clonal tumour cells have plasma cell morphology, and are present in, and spread through, the haematopoietic bone marrow. Extramedullary disease is present in advanced cases, with many myeloma plasma cells accumulated in the peripheral circulation. It is acknowledged that the bone marrow microenvironment may contribute to tumour cell survival and proliferation (Meredith et al, 1993). Cytokines and growth factors existing in the microenvironment may control cell growth, survival and migration of the tumour cell (fig 2). Myeloma cells can produce factors such as IL-1 $\beta$ , Tumour necrosis factor (TNF $\alpha$ ) and lymphotoxin which promotes the

Figure 2 : Representation of the possible interaction between myeloma cells and bone. Myeloma cells produce factors that are able to promote bone re-absorption. The environment generated by resorbing bone, including the products released by the bone, osteoclasts and osteoblasts can promote the proliferation and survival of myeloma cells



synthesis of IL-6 by isolated osteoclasts (Garrett et al, 1995; Giovanni et al, 1996). Bone marrow stromal cells secrete IL-6 which is a paracrine growth factor for myeloma. Activation of the JAK2 / STAT3 by IL-6, results in the over expression of Bcl-X<sup>L</sup> and inhibition of CD95 induced apoptosis (Cartlett-Falcone et al, 1999). Tumour survival can also be controlled by cell-cell or cell-ECM (extracellular matrix) adhesion (Clark & Brugge, 1995). CD95 induced apoptosis was blocked by myeloma cell adhesion to fibronectin (Shain et al, 2002). Therefore these host-tumour cell interactions may provide the means by which the myeloma clone can survive and proliferate.

#### *Cytokines and growth factors*

Interleukin-6 (IL-6) is a pleiotropic cytokine that was originally identified as B-cell stimulatory factor 2 (BSF-2) (Kishimoto 1989). Owing to its multifunctional roles IL-6 was also formerly described as interferon- $\beta_2$  (IFN- $\beta_2$ ), hybridoma / plasmacytoma growth factor (HPGF), and hepatocyte-stimulating factor (HSF). IL-6 is produced by many cell types, including T cells, B cells, bone marrow stromal cells, endothelial cells monocytes and tumour cells. Myelomas and plasma cell leukaemias express IL-6 mRNA (Freeman et al, 1989). Light and electron microscopy were used to detect cytoplasmic IL-6 in myeloma cells of the bone marrow (Ohtake et al, 1990). Patients with advanced MM were given neutralising anti-IL-6 murine monoclonal antibodies (Klein et al, 1991), which in some cases reduced the survival and proliferation of malignant plasma cells,

suggesting that this cytokine has a central role in the pathogenesis of MM. IL-6 supports survival of MM plasma cells by the prevention of programmed cell death (Lichtenstein et al, 1995) and / or inducing tumour expansion and resistance to dexamethasone (Hardin et al, 1994).

This cytokine was originally described as an autocrine growth factor for myeloma cells (Kawano et al, 1988; Barut et al, 1992), but evidence now exists supporting the theory of a paracrine secretion of IL-6 from the tumour environment of the bone marrow (Klein et al, 1989; Klein 1995). Bone marrow stromal cells, osteoblasts and osteoclasts secrete IL-6 (Caligaris-Cappio et al, 1992) that in turn is thought to be controlled by IL-1 $\beta$  that was released by the tumour cells (Klein 1995).

The IL-6 receptor (IL-6R) has two subunits: the  $\alpha$  chain (gp80 or IL-6R $\alpha$ ) and the  $\beta$  chain (gp130 or IL-6R $\beta$ ). Receptor activation necessitates the binding of IL-6 to the IL-6R $\alpha$  followed by phosphorylation of the IL-6R $\beta$  (transducer chain). IL-6 is part of the family of six cytokines that use the gp130 as the transducer chain: oncostatin M (OSM), leukaemia inhibitory factor (LIF), IL-11, ciliary neurotropic factor (CNTF) and cardorphin 1. Some MM cell lines react to LIF, OSM or CNTF when an appropriate receptor is expressed (Zhang et al, 1994). The soluble form of the IL-6R $\alpha$  was found to amplify the sensitivity of myeloma cell lines to IL-6 and may therefore lead to expansion of myeloma cells (Gaillard et al, 1993). While a poor prognosis is envisaged if high levels of IL-6 are found in the serum no such correlation has been found with soluble IL-6R.

Interleukin-10 (IL-10) is a pleiotropic cytokine expressed by many cell types including monocytes, B cells and macrophages. This cytokine inhibits pro-inflammatory cytokines (Huhn et al, 1996) and enhances the proliferation of human B-cell precursors and mature B cells activated by anti-IgM mAbs, *Staphylococcus aureus* Cowan (SAC)(Rousset et al, 1992). IL-10 is a growth factor for malignant plasmablastic cell lines, and IL-10 dependent MM cell lines have been developed (Lu et al, 1995). MM patients have raised levels of IL10 in their serum which correlates with the M-protein level (Ameglio et al, 1995).

Vascular endothelial growth factor (VEGF) has been detected in MM cells. This cytokine stimulates cells of the microenvironment to secrete IL-6 which is a major growth factor for MM (Danbar et al, 1998).

Interleukin-15 (IL-15), a cytokine which was recently discovered was found to share activities of IL-2. It was initially identified for its ability to induce T-cell proliferation. Proliferation of normal B cells has also been induced by IL-15 (Armitage et al, 1995). It is a member of the four-helix bundle cytokine family. IL-15 interacts with a heterotrimeric receptor that consists of the beta and gamma subunits of the IL-2 receptor as well as a specific, high-affinity IL-15alpha subunit (IL-15R $\alpha$ ). The IL-15R $\alpha$  is required for high-affinity binding but not signalling by IL-15 (Anderson et al, 1995b). Many tissues and cell types produce mRNA for IL-15 (Jonuleit et al, 1997; Blauvelt et al, 1996). Bone marrow stromal cell lines, primary human bone marrow stromal cells (Mrozek et al, 1996) and fetal intestinal epithelium

(Murray et al, 1998) also produce IL-15 which indicates a role in haematopoiesis. IL-15 may play a part in chronic inflammatory and autoimmune diseases as it operates as a potent chemoattractant for T-cells (Wilkinson & Liew, 1995). IL-15 is a proinflammatory type one cytokine (Khan & Kasper, 1996; Seder, 1996) although it has sometimes been observed as a type two cytokine stimulator (Mori et al, 1996; Ruckert et al, 1998). Abnormal IL-15 or IL-15R expression has been linked with lymphoid malignancies (Mezza et al, 1998; Trentin et al, 1996). MM cells expressed the IL-15alpha receptor (IL-15R $\alpha$ ) and this cytokine may be implicated in the autocrine propagation of this disease (Tinhofer et al, 2000).

Transforming growth factor-beta (TGF- $\beta$ ) was first identified by its ability to induce reversibly the phenotypic transformation of fibroblast cell lines (Roberts et al, 1981). TGF- $\beta$  is a pleiotropic cytokine that affects many cells, both normal and transformed. In culture TGF- $\beta$  induces cell proliferation of fibroblasts (Leof et al, 1986), osteoblasts (Centrella et al, 1987) and smooth muscle cells (Battegay et al, 1990). However in many cells TGF- $\beta$  inhibits proliferation, in that cell cycle arrest can occur in epithelial cells if TGF- $\beta$  is added during early G1 (Laiho et al, 1990). TGF- $\beta$  also has an immunomodulatory role; it inhibits cell proliferation of T lymphocytes, B lymphocytes, thymocytes, large granular lymphocytes, natural killer cells, and lymphocyte-activated killer cells (Kehrl et al, 1986a; Ristow, 1986; Rook et al, 1986; Kuppner et al, 1988; Wahl et al, 1988a; Ortaldo et al, 1991). The immunomodulatory response depends on the

differentiation state of the cell and cytokine exposure. Many cytokines are inhibited by TGF- $\beta$ , the effects and or the production of interferon- $\gamma$ , tumour necrosis factor- $\alpha$ , tumour necrosis factor- $\beta$ , interleukin-1, interleukin-2, interleukin-3 and the expression of the interleukin-2 receptor (Espevik et al, 1987,1988; Ohta et al, 1987; Ranges et al, 1987; Letterio and Roberts, 1998). The important effector functions in the TGF- $\beta$  induced immunosuppression is the effect of cell cycle arrest and the inhibition of cytokine production. However TGF- $\beta$  can also enhance immature lymphocyte proliferation (Cerwenka et al, 1994) and inhibit T-lymphocytes apoptosis (Cerwenka et al, 1996; Rich et al 1996). B-cell differentiation and immunoglobulin expression is also controlled by TGF- $\beta$ . Studies of TGF- $\beta$  have shown it to act as both tumour suppressor (Arteaga et al, 1990; Glick et al, 1993,1994; Pierce et al, 1995; Cui et al, 1996) and promoter (Sieweke et al, 1990; Welch et al, 1990; Steiner and Barrack, 1992; Arteaga et al, 1993). In multiple myeloma studies have shown the presence of TGF- $\beta$  mRNA in tumour cells and cell lines (Mathes et al, 1993; Klein, 1995; Cook et al, 1999). Multiple myeloma cells secrete higher levels of TGF- $\beta$  than normal B cells; also patients with MM produce higher levels of TGF- $\beta$  in bone marrow than controls (Urashima et al, 1996). High serum levels of TGF- $\beta$  have also been observed in patients with MM and may relate to the extent of myeloma bone disease (Kroning et al, 1997). The part played by TGF- $\beta$  in the pathogenesis is not clear, it may be involved in the survival of these myeloma cells or it may be involved in TGF- $\beta$  mediated immune suppression (Cook et al, 1999; Campbell et al, 2001).

### *Characteristics of the U266 Human myeloma cell line (HMCL)*

The myeloma cell line U266 is an extremely well characterised line described initially by Nilsson et al in 1970. Although unusual in being an IgE  $\lambda$  cell line, it expresses the major myeloma plasma cell characterising antigens on a high percentage of the cells. Thus, PCA-1 and CD44 (H-CAM) are 90-100% positive. CD38 and CD138 (Syndecan-1) are present on 50-90% of the cells, while B cell lineage markers such as CD10, CD19, and CD20 are present in less than 10%. However, the fact that not all U266 cells are positive for CD38 / CD138 suggests a degree of heterogeneity within the cell population, and it was for this reason that the development of sub-clones of the line might produce some variation in characteristics between individual clones.

### **AIMS**

The growth of multiple myeloma plasma cells may depend on complex interactions between the tumour cells, an assortment of cytokines and the bone / bone microenvironment. Although IL-6 is an essential paracrine / autocrine growth factor in MM, the part played by other cytokines in the pathogenesis of MM is not clear.

In addition to its phenotypic characteristics, U266 grows readily in tissue culture, and it was therefore identified as a robust, available, and appropriate model for myeloma plasma cells in these experiments.

Recent work in our laboratory has explored the relationship between TGF- $\beta$  produced by MM cells and the suppression of anti-plasma cell T cell responses. These anergic T cells can have their responses reinstated by exposure to IL-15, following which they are once again able to respond to, and enter the autocrine cycle of, IL-2. Clinical studies are planned that will explore the effect of IL-15 at restoring T cell anti-myeloma activity in vivo, by stimulating T cells with CD3 and IL-15 ex vivo before re-infusing them to patients. Therefore it was important to explore the effect of IL-15 and IL-6 against myeloma plasma cells. In addition, the potent anti myeloma agent Dexamethasone was included in the experiments to observe the possible impact of a therapeutic agent on the cytokine effects.

## CHAPTER TWO

### METHODS

## **METHODS**

### **Tissue Culture Techniques**

#### *Maintenance and Growth of Cell Lines*

U266 human myeloma cell lines were grown in suspension in RPMI 1640 medium with 10% foetal calf serum and 1% glutamine (RPMI<sup>+</sup>), in a humidified incubator at 37 °C in an atmosphere of 5% (v/v) CO<sub>2</sub>. Every 3-4 days cells were counted using a haemocytometer and set up at  $1 \times 10^6$  cells per flask ( $1 \times 10^5$  cells /ml) with fresh RPMI<sup>+</sup>.

#### *Storage and recovery of frozen cells*

Cells were washed by centrifugation for 5 minutes at 100g in a MSE Centaur 2 centrifuge. The pelleted cells were resuspended in 90% FCS / 10% DMSO at a concentration of  $5 \times 10^6$ - $1 \times 10^7$  cells / ml and aliquoted into 1ml Nunc cryotubes. The cryotubes were allowed to freeze slowly overnight in polystyrene (about 1°C/min) to -70°C after which they were transferred to liquid nitrogen. Nunc cryotubes were thawed by placing in a waterbath at 37°C, the cells were transferred to a universal container, fresh RPMI<sup>+</sup> was added and cells washed as described to remove DMSO. The cells were then

resuspended in RPMI<sup>+</sup> and seeded in a 25cm<sup>2</sup> tissue culture flask, and incubated at 37°C, 5% CO<sub>2</sub>

#### *Testing for Mycoplasma*

The adherent fibroblast cell line NRK49, was seeded in 60mm dishes at 0.25 x10<sup>4</sup> cells / ml in 4ml of fresh RPMI<sup>+</sup>. In addition, conditioned media (2ml) which had been in contact with the cells for a minimum of 2 days was added to the dishes. One dish of NRK49 cells was set up in parallel with fresh RPMI<sup>+</sup> as a control. The NRK49 cells were then incubated for 3-4 days at 37°C. The medium was removed and the cells washed twice with 2.5ml of PBS and then 2.5ml of fixative (3 volumes methanol SS: 1 volume glacial acetic acid) was slowly added to each dish. Cells were then washed twice more with 5ml of fixative for 10 minutes each wash. The dishes were inverted and left to drain. To each dish, 5ml of PBS containing 0.05ug / ml Hoechst 33258 stain was added and left for 10 minutes at room temperature. The stain was discarded and the dishes washed twice with distilled water. The fixed cells were examined for mycoplasma using a fluorescence microscope with a water immersion lens. Hoescht 33258 is a stain for DNA; negative cells have only the nuclei stained whereas positive cells have staining within the cytoplasm, thus confirming contamination with mycoplasma.

## RNA ISOLATION

### *RNeasy*<sup>®</sup>

Approximately  $1 \times 10^6$  cells were lysed in RLT-mercaptoethanol buffer (350 $\mu$ l). The lysate was loaded onto a QIAshredder<sup>®</sup>. The column was then spun sitting on a 2ml collection tube for 2 minutes at 21,250g, and the homogenized lysate collected. An equal volume of 70% ethanol was added (350 $\mu$ l) and mixed well by pipetting. This sample was then applied to an RNeasy<sup>®</sup> mini spin column sitting on a 2ml collection tube which was spun for 15 seconds at 10,840g. The flow-through was discarded. RW1 buffer (700 $\mu$ l) was pipetted onto the RNeasy<sup>®</sup> column which was centrifuged at 10,840g for 15 seconds. The RNeasy<sup>®</sup> column was transferred onto a new 2ml collection tube, ethanol re-constituted RPE buffer (500 $\mu$ l) was pipetted onto the column and this was centrifuged at 10,840g for 15 seconds, and again the flow-through was discarded. RPE buffer (500 $\mu$ l) was again pipetted onto the column and centrifuged at 21,250g for 2 minutes to dry the RNeasy<sup>®</sup> membrane. The RNeasy<sup>®</sup> column was transferred to a new 1.5ml collection tube, RNase free water was added (30 $\mu$ l) onto the column and the assembly was then centrifuged at 10,840g for 1 minute. The optical density of the eluted RNA was measured at 260nm. The quantity of RNA was calculated as follows. RNA (5 $\mu$ l) was diluted with distilled water (495 $\mu$ l) and absorbance ( $A_{260}$ ) was measured in a 1ml cuvette using a BioQuest

spectrophotometer (Cecil CE2501). An absorbance ( $A_{260}$ ) of 1 was equivalent to 40 $\mu$ g / ml of RNA, the concentration (in  $\mu$ g per ml) of RNA in each sample was calculated by absorbance ( $A_{260}$ ) x 40 x dilution factor. Therefore the total amount of RNA in each sample equaled the product of concentration multiplied by the original volume of the sample (ml).

#### *Trizol®*

Approximately  $10^6$  cells were lysed in 1ml of Trizol® for 5 minutes at room temperature, which permits the complete dissociation of nucleoprotein complexes. Chloroform was added (0.2ml chloroform per 1ml of Trizol) then the sample tubes were capped securely, shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2-3 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C. Centrifugation separated the mixture into a lower pink chloroform phase, a protein interphase, and a colourless aqueous phase. The RNA remained in the aqueous phase which was transferred to a fresh tube and precipitated by the addition of 0.5ml of isopropyl alcohol per 1ml of Trizol reagent. The samples were incubated at room temperature for 10 minutes followed by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was discarded and the pellet washed once with 1ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at 7,500g for 5 minutes at 4°C. The RNA pellets were then air dried and resuspended in RNase free water. Incubation for 10 minutes at 55°C assisted RNA dissolution.

## **RT-PCR**

### *cDNA Synthesis*

Oligo (dT) 12-18 primer (0.5 $\mu$ g of primer per 5 $\mu$ g of RNA) was hybridized to the poly (A) tail of mRNA to be used as a primer for first strand cDNA synthesis with reverse transcriptase. This mixture (22  $\mu$ l of RNA solution + 2 $\mu$ l of primer) was heated to 70°C for 10 minutes, and then quickly chilled on ice. The tube was centrifuged at 10,840g for 10 seconds. The following mixture was added to the tube: 8 $\mu$ l 5x First-Strand Buffer, 4 $\mu$ l 0.1M DTT, 2 $\mu$ l dNTP (10mM each dATP, dGTP, dCTP, and dTTP), 2 $\mu$ l Reverse Transcriptase (Superscript™ II). The tube was then equilibrated at 20°C for 5 minutes. The temperature was then raised to 42°C, which was the optimal temperature for the enzyme activity for 50 minutes then raised to 95°C for 5 minutes to inactivate the enzyme. The cDNA produced was then amplified in a polymerase chain reaction.

### *Polymerase Chain Reaction*

This method was used to amplify cytokine and receptor sequences, 2 $\mu$ l of cDNA was mixed with 48 $\mu$ l of PCR mix (containing 20mM Tris-HCl, 50mM KCl; 2.5mM MgCl<sub>2</sub>; 0.2mM of each of dATP, dCTP, dTTP, and dGTP; 0.25 $\mu$ M of the cytokine primer pairs (Table: 2); 0.4 units Taq polymerase). PCR amplification was carried out using a Hybaid programmable thermocycler. The samples were denatured at 95°C for 3

minutes, then 30 cycles of 95°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute, followed by a final 5 minute extension at 72°C. The products of PCR samples were run on 2% agarose gels containing 10 µg / ml ethidium bromide with molecular weight markers to determine the size of the products. A reaction was considered positive if a band of the appropriate size was visible and negative control lanes remained clear (including a no RT control).

### *Primers*

Most primers used in these studies were published sequences (Kotake et al, 1996). Primers were selected which would result in a cDNA product which spanned introns to ensure that the product was not the result of DNA contamination.

Table: 2 Oligonucleotide PCR primer sequences

CYTOKINE	FRAGMENT SIZE (bp)	SEQUENCE
$\beta$ -Actin-5' $\beta$ -Actin-3'	548	GTGGGGCGCCCCAGGCACCA CTCCTTAATGTCACGCACGACGATTTC
IL-1 $\alpha$ -5' IL-1 $\alpha$ -3'	421	GTCCTCTGAAATCAGAAATCCTTCTATC CATGTCAAATTTCACTGCTTCATCC
IL-1 $\beta$ -5' IL-1 $\beta$ -3'	802	ATGGCAGAAGTACCTAAGCTCGC ACACAAATTGCATGGTGAAGTCAGTT
IL-2-5' IL-2-3'	266	ACTCACCAGGATGCTCACAT AGATAATCCATCTGTTCAGA
IL-4-5' IL-4-3'	456	ATGGGTCTCACCTCCCAACTGCT CGAACACTTTGAATATTTCTCTCTCAT
IL-6-5' IL-6-3'	628	ATGAACTCCTTCTCCACAAGCGC GAAGAGCCCTCAGGCTGGACTG
IL-10-5' IL-10-3'	352	TCTCAAGGGGCTGGGTCATATCCCA ATGCCCAAGCTGAGAACCAAGACCCA
IFN- $\gamma$ -5' IFN- $\gamma$ -3'	495	ATGAAATATACAAGTTATATCTTGGCTTT GATGCTCTTCGACCTCGAAACAGCAT
TNF- $\alpha$ -5' TNF- $\alpha$ -3'	695	ATGAGCACTGAAAGCATGATCCGG GCAATGATCCCAAAGTAGACCTGCCC
TGF- $\beta$ -5' TGF- $\beta$ -3'	181	ACCATGATCGTGCGCTCTGCAAGTGCAGC AAGGAATAGTGCAGACAGGCAGGA
IL-15-5' IL-15-3'	393	TGGAAACCCCTTGCATAGCCAGCTCTT CTGTATCATGAATACTTGCATCTCCGGACTC
IL-15R-5' IL-15R-3'	538 439	GCGGGGATCCACGTGCCCTCCCCCAT GTCCGTGGAA CCACAAGCTTGTGCGCTGTGGCCCTGTG GATACACAC

## CELL STUDIES

### *Cell Proliferation*

Cell proliferation was measured by the amount of  $^3\text{H}$ -labelled thymidine incorporated into the replicating DNA of cultured cells. Cells were set up in 25cm<sup>2</sup> flask at  $1 \times 10^5$  cells / ml in RPMI<sup>t</sup>. The cytokine (IL-15) or glucocorticoid to be assessed was added, they were then aliquoted (200µl) per well in RPMI<sup>t</sup> onto a microtitre plate (96 well) and cells incubated at 37°C in a humidified incubator for 3 days. Then 0.5µCi of  $^3\text{H}$ -labelled thymidine was added to each well, after 24 hours the plates were harvested onto filter paper by automatic cell harvester. The incorporation of  $^3\text{H}$ -labelled thymidine was measured by counting in a Packard Matrix beta-counter.

### *Cell Cloning*

U266 cells were seeded at  $0.2 \times 10^3$  cells / ml in 25mm<sup>2</sup> dishes containing RPMI<sup>t</sup> / 0.9% Methocult. The plates were incubated at 37°C in a humidified incubator and observed for the formation of colonies. The colonies were then removed from the semi-solid medium, numbered and separately grown in fresh RPMI<sup>t</sup>. This step was repeated and these cells were known as cloned myeloma cells (Brown et al, 1995).

### *Annexin V Affinity Assay*

The externalisation of phosphatidylserine (PS) is one of the earliest events of programmed cell death (Martin et al, 1995). Annexin V was used to detect PS externalisation (Engeland et al, 1998). PS occurs early in cell death and remains until cell necrosis. Annexin V was used with a vital dye propidium iodide (PI) therefore identifying three populations of cells: live cells (An<sup>-</sup>/PI<sup>-</sup>), early apoptotic cells (An<sup>+</sup>/PI<sup>-</sup>) and late apoptotic/necrotic cells (An<sup>+</sup>/PI<sup>+</sup>). The Annexin V-affinity assay was used for the detection of apoptosis. Cells were washed with PBS for 5 minutes (x2) at 140g using a MSE Centaur 2 centrifuge. Annexin V, (Catalogue Number 65874X from BD Biosciences) (5µl) and 5µl of 100µg / ml propidium iodide were added and incubated in the dark at room temperature for 15 minutes. They were resuspended in 1x Annexin V Binding Buffer (10<sup>6</sup> cells / ml; R & D Systems). Cells were analysed within an hour of the incubation. Controls were set up as follows, cells were resuspended in Binding Buffer only, cells resuspended in Annexin V alone, cells in propidium iodide alone, analysis was carried out using Cell Quest software (Becton Dickinson).

### *Cell cycle assays*

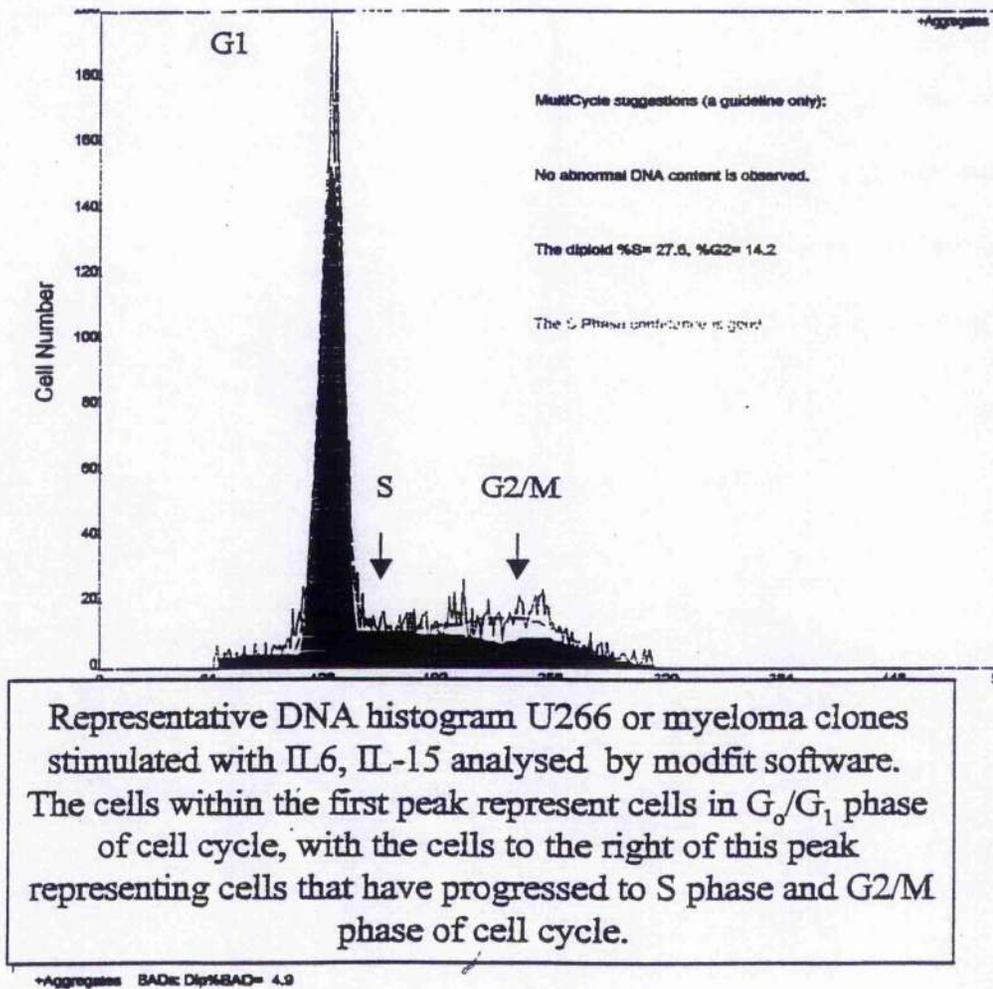
The cell cycle stage of cell nuclei was determined, using the stain propidium iodide (PI). The amount of PI absorbed is directly proportional to the amount of DNA in the nucleus. PI staining was carried out using the

DNACon3 kit (Dako) according to the manufacturer's instructions. Briefly, DNACon3 tubes were re-constituted with the supplied PI solution to provide a cell lysis and staining solution. (This contains cell lysis buffer to give a free suspension of nuclei; RNase to stop non-specific binding of PI to RNA; and a chromatin stabilizer to help make staining more uniform). Approximately  $10^6$  cells per sample were incubated in re-constituted DNACon3 solution for one hour with vigorous vortexing (x4 during this period of time). Samples were then directly analysed on a FACScan using Cell Quest software (Becton Dickinson). Nuclei were gated to exclude debris and analysed on a linear FL-2 scale (fig.3). PI staining generated characteristic cell cycle histograms depicting cell nuclei in G<sub>0</sub>/G<sub>1</sub>, S phase and G<sub>2</sub>/M phases of the cell cycle. Cell cycle analysis was carried out using Wincycle for Windows Software (Coulter Immunotech). This programme combines 6 different algorithms to estimate cell cycle status of the sample – a sample result is contained in figure: 3

#### *Mixed Lymphocyte Reactions (MLR)/Mitogen stimulation of T cells*

A proliferative response is generally observed when lymphocytes of 2 individuals of different HLA types are co-cultured. This results from the reaction of T cells of an individual to major histocompatibility (MHC) antigens on the other individual's cells which were measured by tritiated thymidine uptake. It has been observed that U266 cells were extremely poor at inducing MLR reactions, as soluble factors produced by the U266 cells

Figure :3 Histogram of cell cycle



suppresses the T cell proliferation induced (Cook *et al* 1999). Also, soluble factors from HMCL suppress the proliferation of T cells induced by the mitogen Concanavalin A (Con A)(Cook *et al*, 1999). To test whether clones which expressed differing cytokine mRNA species generated during this project had differing effects on T cell activation, clones were assessed for their ability to provoke MLR responses and to suppress Con A-induced proliferation.

Peripheral blood mononuclear cells (PBMNC) were separated from the peripheral blood of normal, healthy volunteers by centrifugation over ficoll-hypaque, followed by 4 washes in PBS. PBMNC were re-suspended in RPMI<sup>t</sup> at  $4 \times 10^6$  cells / ml for MLR and  $8 \times 10^6$  cells / ml for Con A assays. Stimulator MM clones were treated with  $50 \mu\text{g} / \text{ml}$  Mitomycin C (Sigma) for 30 minutes at  $37^{\circ}\text{C}$  followed by 3 washes in RPMI<sup>t</sup>. For MLR reactions,  $100 \mu\text{l} / \text{well}$  of responder PBMNC were seeded in wells of a 96 well plate.  $100 \mu\text{l}$  of stimulator MM clones were then added to the wells to give concentrations of responder: stimulators of 1:5-1:160. For Con A stimulation, wells were set up with  $50 \mu\text{l}$  of responder: stimulators of 1:5-1:160. For Con A stimulation, wells were set up with  $50 \mu\text{l}$  of PBMNC in an identical manner to the MLR plus  $100 \mu\text{l}$  of Con A at  $5 \mu\text{g} / \text{ml}$  (Final Con A concentration =  $2.5 \mu\text{g} / \text{ml}$ ). Proliferation was assessed by the uptake of  $0.5 \mu\text{Ci}$  of tritiated thymidine to the cultures during the last 18 hours of culture. Cells were harvested onto fibreglass membranes and counted using a Packard Matrix counter. Con A-induced proliferation was assessed at 3

days, MLR reactions were assessed at 5 days, although Con A Control wells were added to MLR plates and assessed at 5 days.

### **TGF- $\beta$ 1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)**

The detection of active TGF- $\beta$ 1 in supernatants of U266 HMCLs was analysed using a Promega ELISA kit. U266 cell suspensions were centrifuged at 100g for 5 minutes, washed in PBS (x3) and resuspended at a concentration of  $1 \times 10^6$  cells / ml in fresh RPMI<sup>-</sup>. RPMI<sup>+</sup> alone was set up as a control. The U266 cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% (v/v) CO<sub>2</sub> for 3 days. The cell suspensions and controls were centrifuged at 100g for 5 minutes and supernatants were removed, acidified for 15 minutes at room temperature, then neutralized as this assay was designed to measure biologically active TGF- $\beta$ 1. All samples were diluted with sample buffer to obtain readings within the range of the ELISA kit (16-1000pg / ml). These samples were analysed following the manufacturer's instructions. The results were expressed minus control samples.

### **STATISTICAL ANALYSIS**

All statistical analysis of experimental data was carried out with Microsoft Excel analysis software (Microsoft Office 2000). Where data represents more than 3 observations, this was presented as the mean +/- standard deviation of the mean. Two-sample student t-test was used for comparison

of data groups. The statistical significance of difference between two data groups was expressed as the probability or  $p$  value. Statistical significance was indicated if the  $p < 0.05$ , with the greater significance being indicated to the lesser  $p$  value.

## CHAPTER THREE

### RESULTS

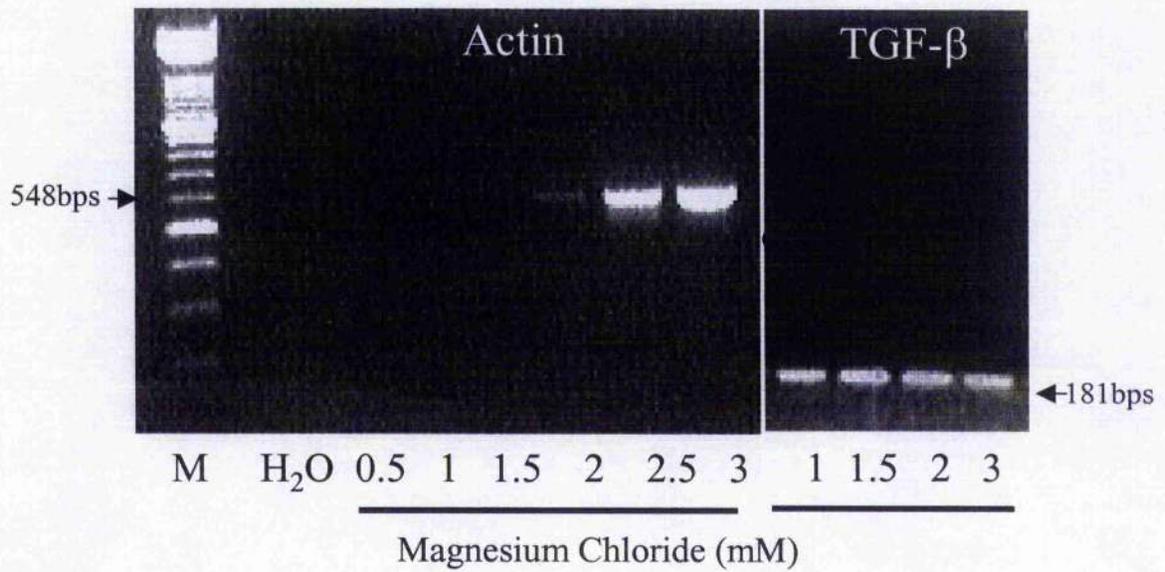
## **Cytokine profiling of myeloma clones**

Positive control RNA was prepared from peripheral blood mononuclear cells (PBMNC) cultured in the presence of PHA/PMA for 4-6 hours. The cells were harvested, mRNA was isolated using RNEasy<sup>®</sup> or Trizol, and cDNA prepared as described in Materials and Methods. Optimisation of the PCR reaction was initially achieved by titration of the magnesium chloride (MgCl<sub>2</sub>) concentration in a specific PCR for actin using the positive control cDNA (Fig 4). From this titration, 2.5 mM MgCl<sub>2</sub> concentration was then used in all subsequent PCR reactions. All primer sets were tested in specific PCR reactions with positive control cDNA.

The cell line U266 (which was mycoplasma negative) was cloned as previously described. The mRNA from the clones was studied using RT-PCR. Twenty-three clones (M1-M23) were isolated. Ten clones were examined. These ten were chosen because they exhibited the most vigorous growth within the clones isolated. The clones were examined for the expression of the following cytokines (Table 3): interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, interferon gamma (IFN $\gamma$ ), tumour necrosis factor-alpha (TNF- $\alpha$ ), transforming growth factor-beta (TGF- $\beta$ ), IL-15 and IL-15 alpha receptor (IL-15R $\alpha$ ). The clones were found to exhibit different cytokine profiles. M5 produced mRNA for IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, and TGF- $\beta$  (fig 5), whilst the M6 clone did not (fig 6), this clone was only positive for actin. M8 clones were positive for cytokines

IL-1  $\alpha$ , IL-4, and TGF- $\beta$ . M1 clones were positive for cytokines IL-6 and TGF- $\beta$  (Fig 7). All clones constantly transcribed TGF- $\beta$  mRNA with the exception of M6 which, after remaining negative for TGF- $\beta$  for six weeks, became positive for TGF- $\beta$  mRNA (fig 8). All the clones that were examined transcribed mRNA for IL-15 $\alpha$  receptor (fig 9). All clones were regularly examined and with the exception of M6 retained their cytokine profile throughout these experiments.

Figure 4 : Optimisation of MgCl<sub>2</sub> concentration



**Figure 4 :** Optimization of PCR reaction was achieved by titration of magnesium chloride in a specific PCR for actin using positive cDNA. A 2.5mM concentration of magnesium chloride was used in all subsequent PCR reactions

Figure 5 : Cytokine profiling by PCR of Clone M5

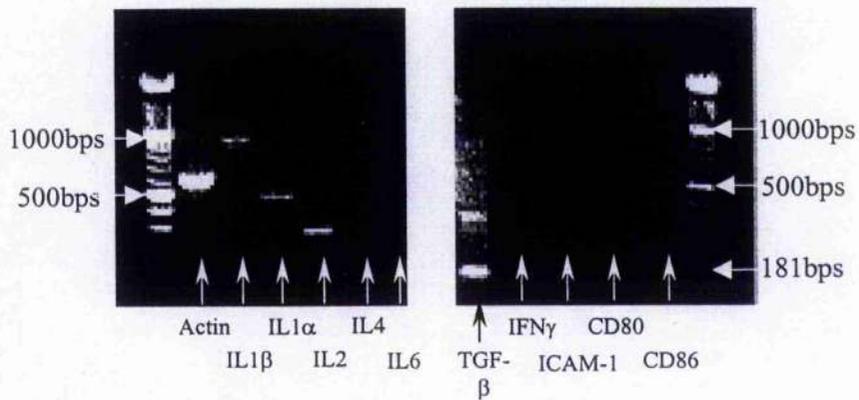


Figure 5 : Clone M5 expressed mRNA transcripts for Actin, IL-1a, IL-1-β, IL-2 and TGF-β

Figure 6 : Cytokine profiling by PCR of clone M6

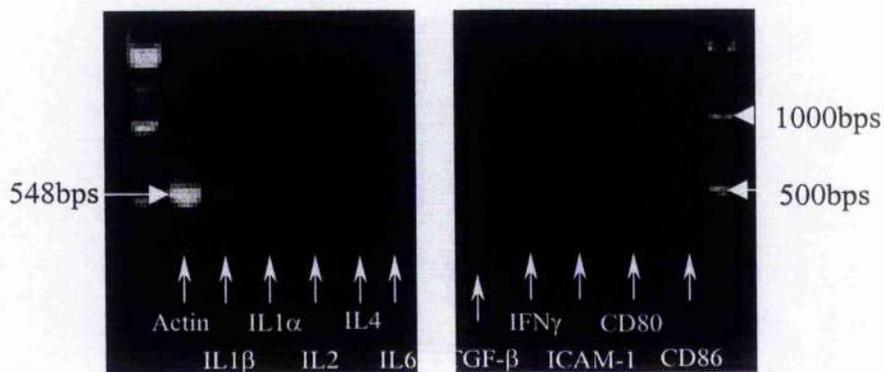


Figure 6: Clone M6 expressed mRNA transcripts for Actin

Figure 7 : cytokine expression of IL-6 in clone M1

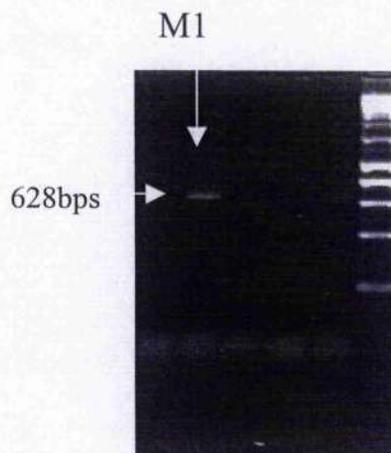


Figure 7 : Clone M1 expressed mRNA transcripts for IL-6

Figure : 8 Myeloma clone M6 after six weeks

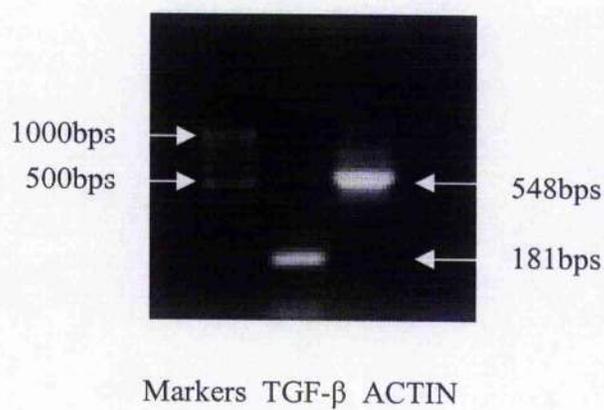
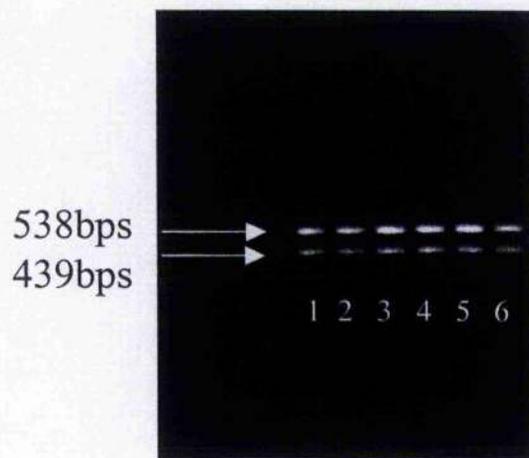


Figure 8 : Clone M 6 after six weeks now produced mRNA transcripts of TGF- $\beta$

Figure 9 : Expression of IL-15 $\alpha$  receptor  
by PCR of myeloma clones



**Figure 9 :** All clones expressed mRNA transcripts of IL-15  $\alpha$  receptor  
2 bands the smaller product is a splice variant lacking exon 3

1 - M1    4 - M6  
2 - M3    5 - M7  
3 - M5    6 - M8

**Table : 3 Summary of cytokine profiling of myeloma clones**

Clone	IL-1 $\alpha$	IL-1 $\beta$	IL-2	IL-4	IL-6	IL-10	INF $\gamma$	TGF $\beta$	TNF $\alpha$	Actin	IL-15
M1	-	-	-	-	+	-	-	+	-	+	-
M2	-	-	-	-	-	-	-	+	-	+	-
M3	-	-	-	-	-	-	-	+	-	+	+
M5	+	+	+	-	-	-	-	+	-	+	-
M6	-	-	-	-	-	-	-	-	-	+	-
M7	-	-	-	-	-	-	-	+	-	+	-
M8	+	-	-	+	-	-	-	+	-	+	-
M9	-	-	-	-	-	-	-	+	-	+	-
M16	-	-	-	-	-	-	-	+	-	+	-
M19	-	-	-	-	-	-	-	+	-	+	-

### **Effects of myeloma clones on Immunosuppression**

Clones M5 and M6 displayed different patterns of cytokine mRNA expression (fig 5 and fig 6 respectively). M6 was the only clone that did not express TGF- $\beta$ . These clones were tested for their ability to suppress ConA and MLR reactions as previously described. M5 at a ratio of 1:80 (MM: PBMNC) showed moderate suppression of ConA induced proliferation (fig 10). The higher the concentration of M5 cells the lower the level of induced proliferation in the MLR (fig 11). In contrast, clone M6 did not suppress ConA induced proliferation (fig 10), but permitted a normal MLR reaction (increased levels of stimulators increased the level of proliferation) (fig 11). Therefore clone M6 which did not express TGF- $\beta$  by PCR neither suppressed ConA nor MLR induced proliferation in T cells, whereas the TGF- $\beta$  producing M5 suppressed ConA and at high concentration of stimulator reduced proliferation.

Clones M3 and M9 (fig 12) did not express any of the T cell stimulating cytokines as expressed by clone M5 (IL-1 $\alpha$ , IL-1 $\beta$ , IL-2). M3 expressed mRNA for IL-15 and TGF- $\beta$ , whilst M9 expressed mRNA for TGF- $\beta$ .

When M3 was tested for its ability to suppress Con A and MLR reactions, M3 suppressed Con A proliferation but failed to induce an MLR response (figs 13, 14). However M9 suppressed (51%) Con A proliferation at a ratio of 1:5 (fig 14). Therefore a MM clone (M3), which expressed mRNA for IL-15 and TGF- $\beta$  was an efficient T cell inhibitor whereas a clone (M9) which did not co-express mRNA for IL-15 and TGF- $\beta$  was a poor T cell inhibitor.

**Figure 10: Effects of Clone M5, M6 on Con A induced proliferation**

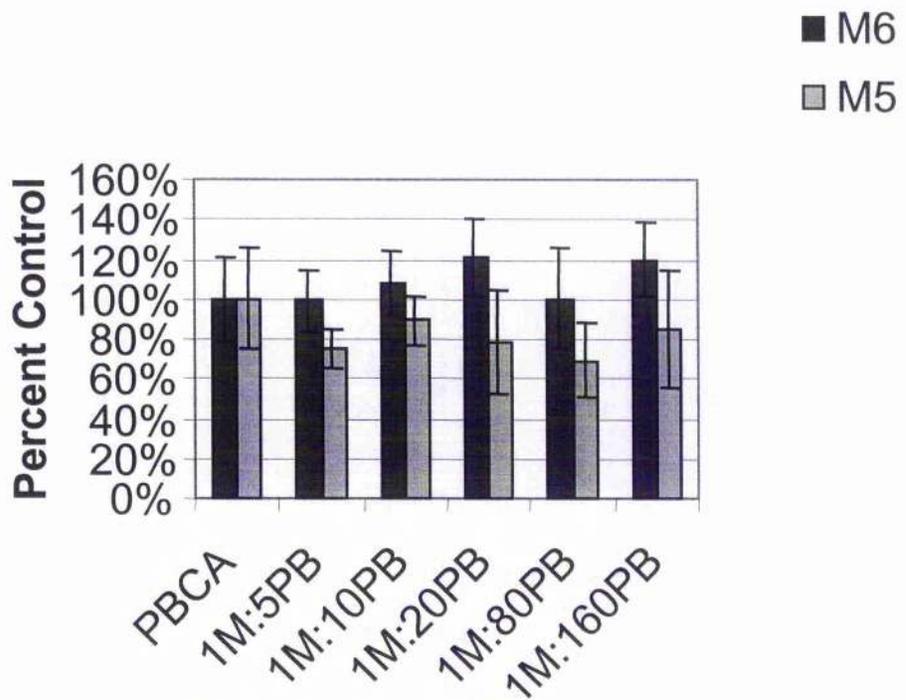
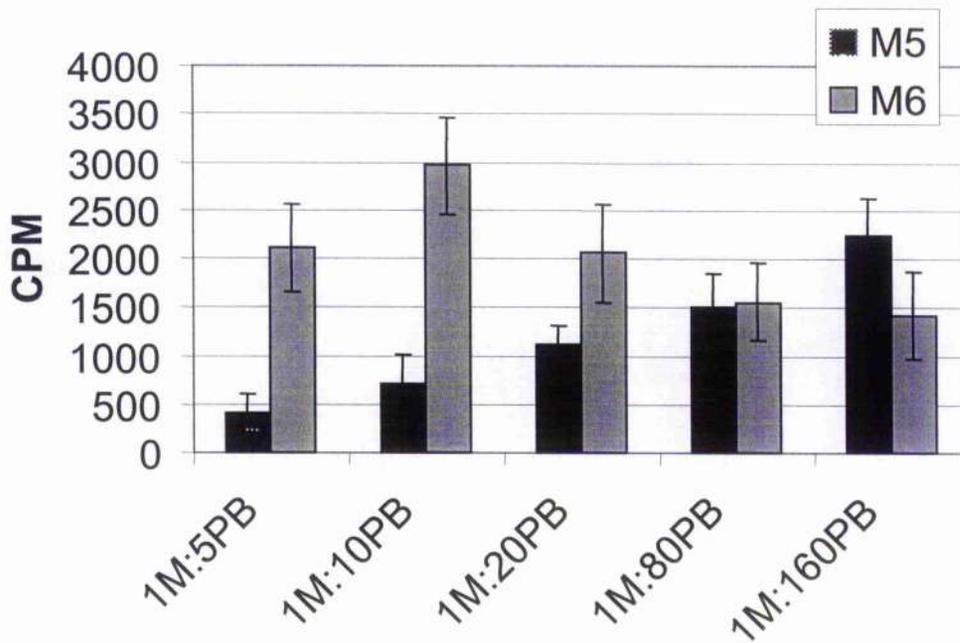


Figure 10: Clone M5 which expressed TGF- $\beta$  shows moderate suppression of Con A induced proliferation whereas no suppression was seen with Clone M6 which did not express TGF- $\beta$

KEY PBMC = peripheral blood mononuclear cells: Con A  
 1M(myeloma clones):5PB = ratio of clones;peripheral blood mononuclear cells

**Figure 11: Clone M5, M6, MLR response with PBMC from a normal donor**



**Figure 11:** Clone M6 demonstrates a modest MLR response to PBMC from a normal donor after 5 days whereas with clone M5 the higher concentration of myeloma cells the lower the level of induced proliferation in the MLR.

Key 1M:5PB – the ratio myeloma cells :PBMC

Figure 12 : Cytokine profile of clones M3 and M9

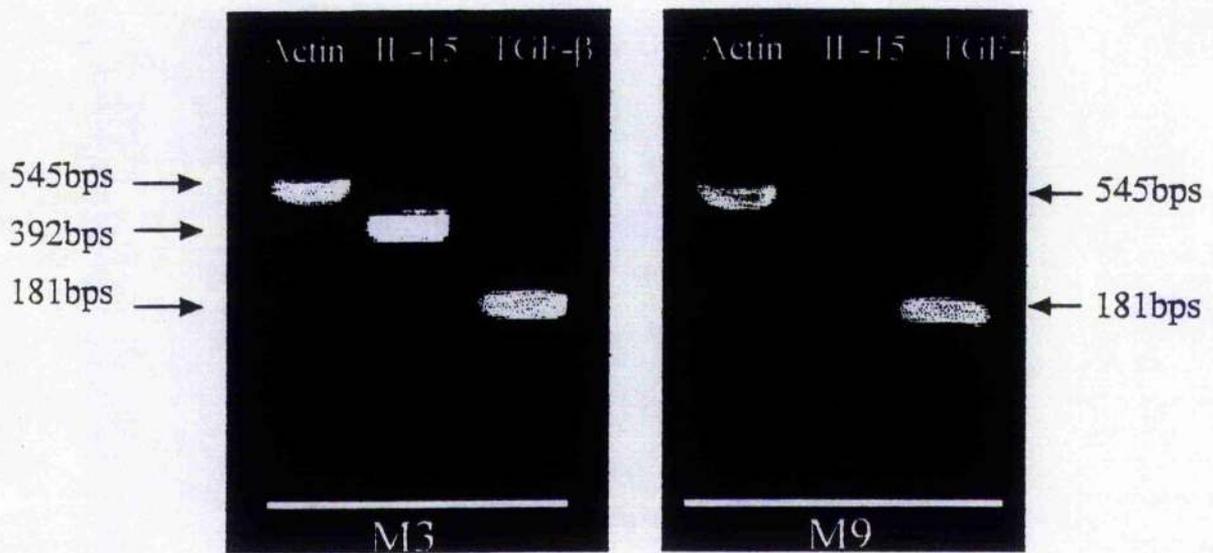
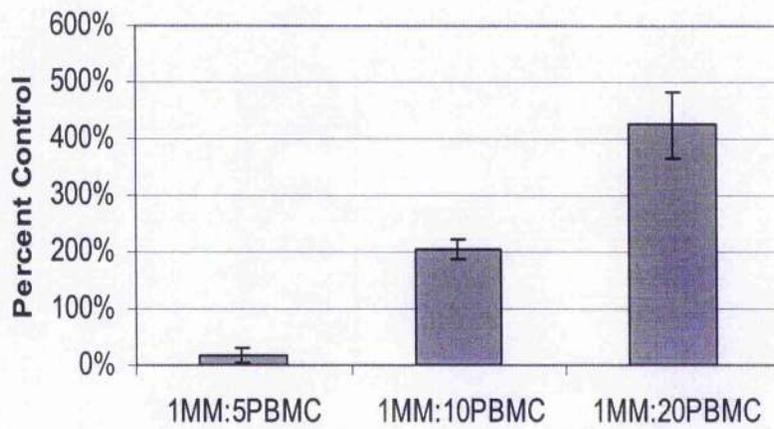


Figure 12 : RT-PCR for the expression of Actin, TGF- $\beta$  and IL-15 mRNA transcripts from the two clones M3, M9. M3 expressed mRNA transcripts for Actin, TGF- $\beta$  and IL-15. M9 expressed mRNA transcripts for Actin and TGF- $\beta$

Figure 13 : Clone M3 response to MLR with PBMCs from a normal donor

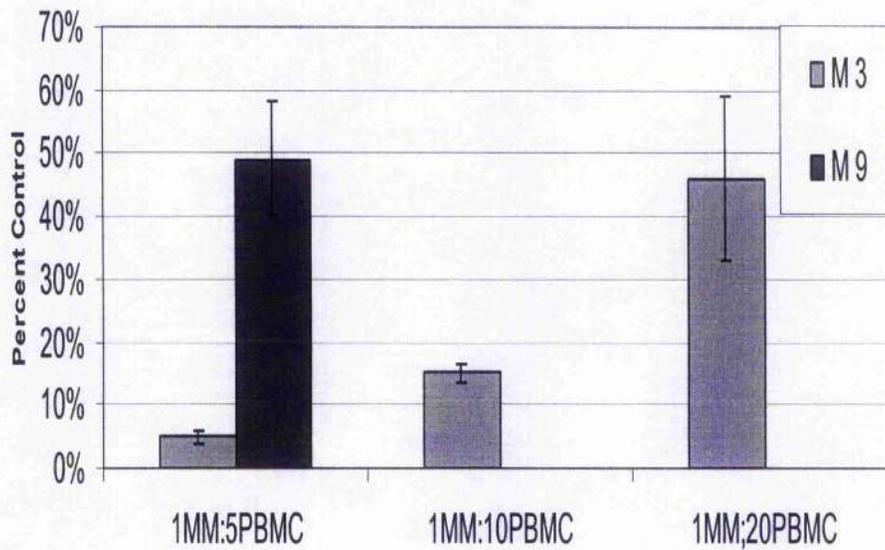


**Figure 13 :** Clone M3 which expressed mRNA transcripts for IL-15 and TGF- $\beta$  failed to induce an MLR response at the highest concentration of myeloma clones.

Key : 1MM:5PBMC

Ratio of myeloma clones to PBMCs

Figure 14 : Effects of M3 and M9 on Con A induced proliferation



**Figure 14 :** Clone M3 suppressed ConA proliferation 95% at a concentration of 1 myeloma cell : 5 peripheral blood mononuclear cells whereas clone M9 at the same concentration suppressed ConA proliferation 50%

**Effects of exogenously added IL-6 or IL-15 alone or in combination with dexamethasone, on proliferation, cell cycle progression and apoptosis**

***The effects of exogenously added IL-15 on proliferation.***

The U266 cells were set up as previously described in "Materials & Methods". Various concentrations of the cytokine IL-15 (0.01ng / ml-10ng / ml) were assessed for an ability to increase U266 proliferation, a significant difference was noted with 0.1ng / ml and 10ng / ml (fig 15), 10ng / ml of IL-15 was used in the following experiments as this was the concentration used in the T cell experiments (Campbell et al, 2001).

***Effects of exogenously added IL-6 or IL-15 alone or in combination with dexamethasone, on proliferation.***

***U266***

The cell line U266 was dexamethasone sensitive, that is dexamethasone decreased proliferation of the cells (figs 16, 17). IL-15 alone did significantly decrease proliferation (fig 16,  $p = 1.01E-05$ ). IL-6 alone did

increase proliferation (fig 16, NS). IL-6 and IL-15 protected against sensitivity to 50  $\mu\text{g}$  / ml dexamethasone (fig 16) but not at 100  $\mu\text{g}$  / ml dexamethasone (fig 17).

#### *Myeloma clone M1*

This clone was resistant to dexamethasone, that is dexamethasone did not decrease proliferation of the M1 clone (fig 18). IL-6 did not increase proliferation (fig 18) whilst IL-15 did significantly increase ( $p < 0.005$ ) proliferation. The effect of IL-15 in combination with dexamethasone was to increase proliferation (fig 18). IL-6 in combination with dexamethasone potentiated proliferation of this clone (fig 18) but was not statistically significant.

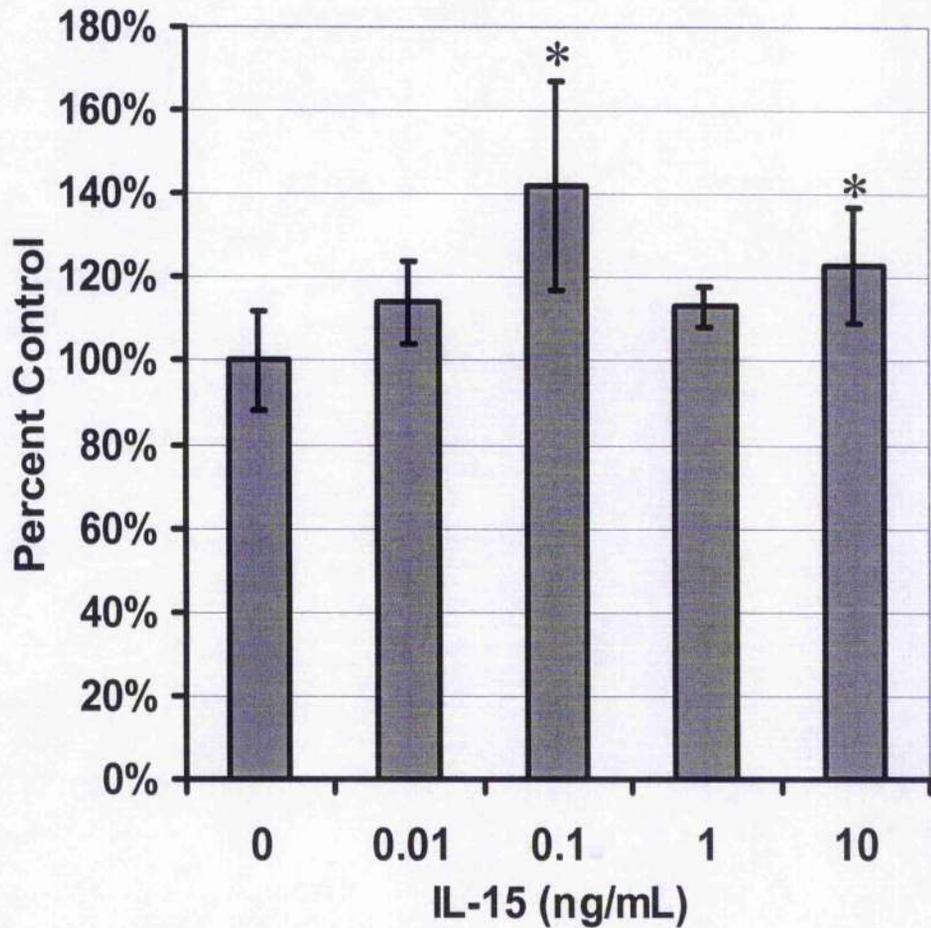
#### *Myeloma clone M3*

This clone was resistant to dexamethasone, that is no decrease in proliferation was seen (fig 19). IL-15 slightly increased proliferation (fig 19). IL-6 reduced proliferation (fig 19, NS). IL-15 in combination with dexamethasone significantly decreased proliferation (fig 19,  $p = 0.033$ ), as which IL-6 in combination with dexamethasone (fig19,  $p=0.0011$ ).

*Myeloma clone M16*

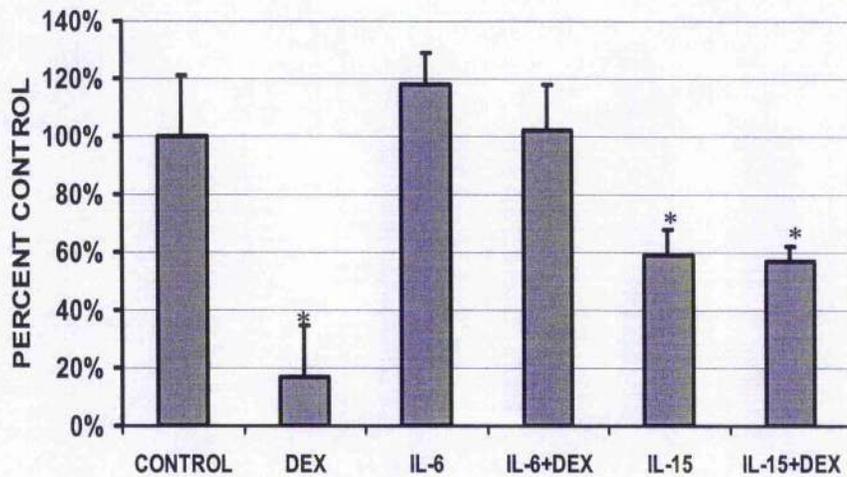
This clone was resistant to dexamethasone at 50µg / ml but not at 100µg / ml (fig 21, p=0.011). IL-15 in combination with dexamethasone did not affect resistance to dexamethasone (figs 20, 21). IL-6 in combination with dexamethasone had no effect on dexamethasone resistance (fig 21).

Figure 15 : Effects of exogenously added IL-15 on U266 proliferation *in vitro*



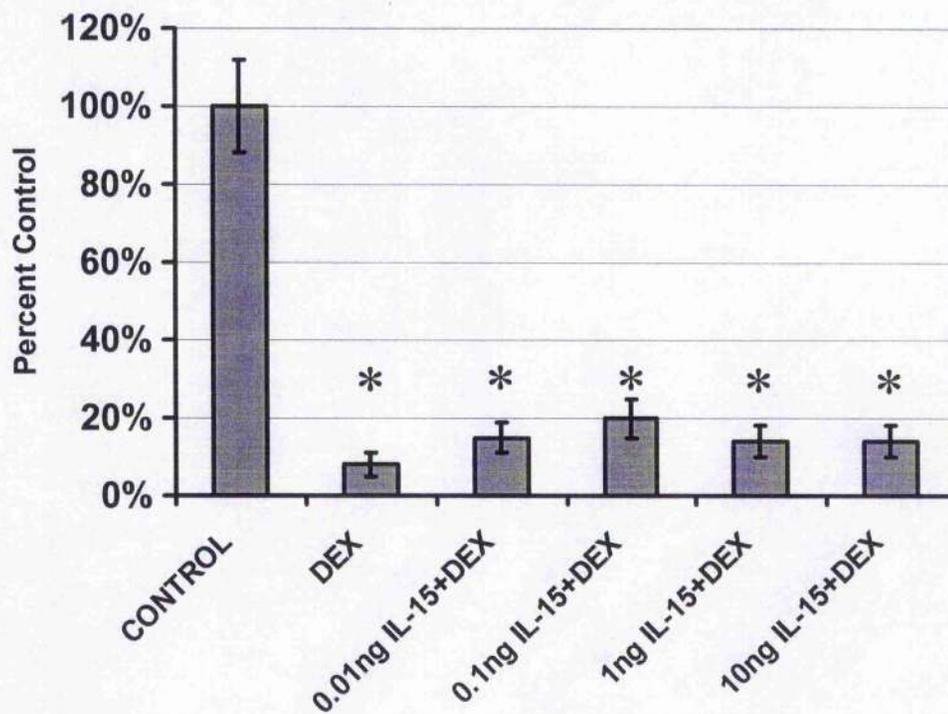
**Figure 15 :** The proliferation of U266 myeloma cells were significantly increased by the addition of IL-15 at concentrations of 0.1ng / ml ( $p = 0.015$ ) and 10ng /ml ( $p = 0.026$ ) with respect to the control 0ng /ml of IL-15.

Figure 16: Effects of IL-6 / IL-15 in combination with 50 $\mu$ g / ml of Dexamethasone on U266 proliferation *in vitro*



**Figure 16 :** U266 cells were sensitive to dexamethasone (50 $\mu$ g / ml) that is dexamethasone significantly decreased proliferation ( $p = 6.5E-11$ ). IL-15 alone significantly decreased proliferation ( $p = 0.001$ ). IL-6 and IL-15 protected against dexamethasone ( $p = 0.001$ ) sensitivity

Figure 17 : Effect of exogenously added IL-15 in combination with Dexamethasone on U266 proliferation *in vitro*

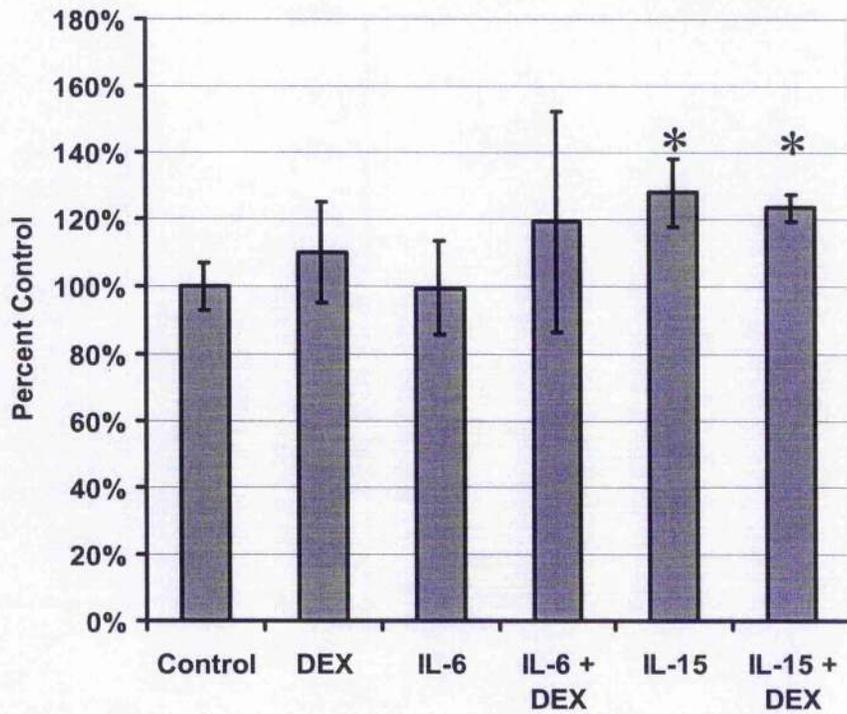


**Figure 17 :** The cell line U266 was sensitive to dexamethasone alone ( $p < 0.005$ ) or in the presence of various concentrations of IL-15. Dexamethasone ( $100\mu\text{g} / \text{ml}$ ) decreased proliferation significantly ( $p < 0.005$ ) with respect to the control.

DEX =  $100\mu\text{g} / \text{ml}$

Fig 18 : Effect on *in vitro* proliferation of the clone M1 in response to exogenously added IL-6, Dexamethasone, IL-15, or

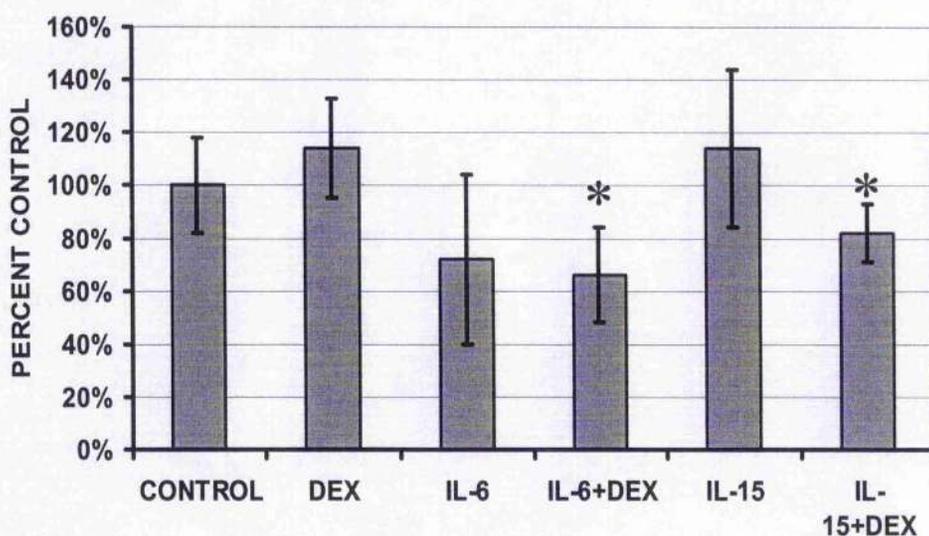
IL-6 / IL-15 in combination with Dexamethasone



**Figure 18 :** Clone M1 was resistant to dexamethasone, that is dexamethasone (50 $\mu$ g / ml) did not decrease proliferation. IL-6 did not increase proliferation, whilst IL-15 did significantly increase proliferation ( $p < 0.005$ ) with respect to the control.

DEX = 50 $\mu$ g / ml

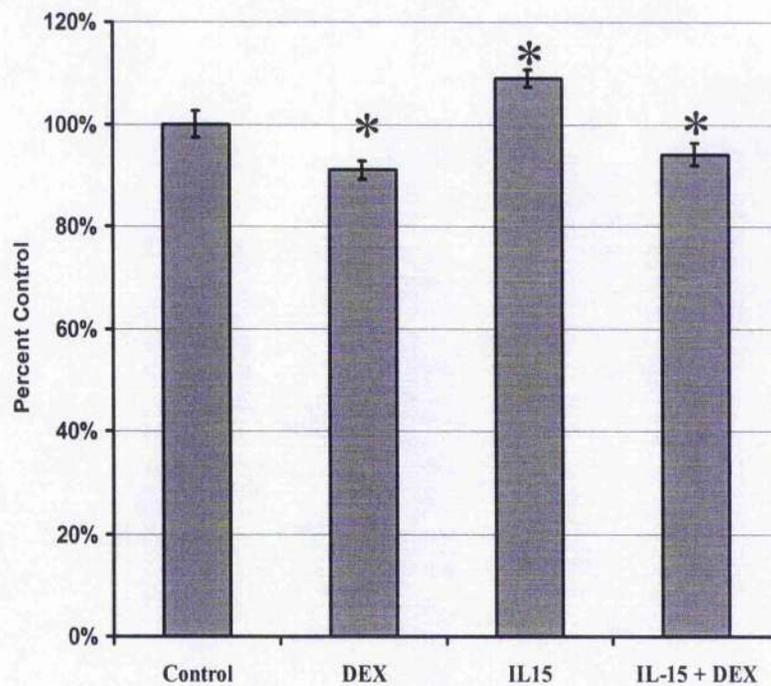
Figure 19 : Effect on *in vitro* proliferation of the clone M3 in response to exogenously added IL-6, Dexamethasone, IL-15, or IL-6 / IL-15 in combination with Dexamethasone



**Figure 19 :** Clone M3 is resistant to dexamethasone. that is no decrease in proliferation . IL-6 in combination with dexamethasone significantly decreased proliferation ( $p = 0.001$ ). IL-15 in combination with dexamethasone significantly decreased proliferation ( $p = 0.033$  ).

DEX=50 $\mu$ g /ml

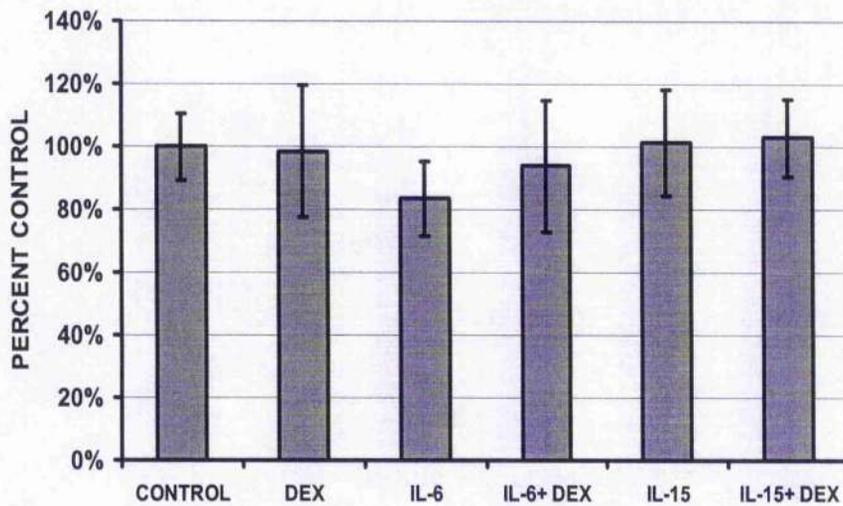
Figure 20 : Effect of exogenously added Dexamethasone and IL-15 alone, or in combination, on *in vitro* proliferation of the M16 clone



**Figure 20 :** Clone M16 was sensitive to dexamethasone (100 $\mu$ g / ml), that is proliferation was significantly decreased ( $p=0.001$ ). Exogenous IL-15 significantly increased proliferation ( $p= 0.0068$ ) but did not protect against the effects of dexamethasone.  
p values calculated with respect to control

DEX = 100 $\mu$ g / ml

Figure 21: Effect on *in vitro* proliferation of the clone M16 in response to exogenously added IL-6, Dexamethasone, IL-15, or IL-6 / IL-15 in combination with Dexamethasone



**Figure 21 :** Clone M16 was resistant to dexamethasone ( 50 $\mu$ g / ml), that is no decrease in proliferation. Exogenous IL-6, IL-15 alone or in combination with dexamethasone had no significant effect on proliferation of this clone

**TABLE : 4 Profile of myeloma clones examined in cell studies**

	ACTIN	IL-6	IL-15	TGF- $\beta$
U266	+	-	-	+
M1	+	+	-	+
M3	+	-	+	+
M16	+	-	-	+

## Cell Cycle

### *Effects of exogenously added IL-6 or IL-15 alone or in combination with dexamethasone, on cell cycle and apoptosis*

#### *U266*

When exogenous IL-6 alone was added to the U266 cells, the proportion of cells in G<sub>1</sub> (-15%) stage decreased while cells in S (+15%) and G<sub>2</sub> (+12%) stage increased (table: 5) thus supporting the proliferation results, with a slight decrease in apoptosis (increase of -4% on control). No change in the proportion of cells in G<sub>1</sub> (+2%), S phase and G<sub>2</sub> (0%) was shown with exogenous IL-15 alone (Table: 5). Exogenous IL-6 in combination with dexamethasone reduced the amount of cells in G<sub>1</sub> significantly ( $p = 0.007$ ) and increased the cells in S phase ( $p = 0.007$ ) that was indicative of proliferation (table: 5). Exogenous IL-15 in combination with dexamethasone (100µg / ml or 200µg / ml) showed a slight reduction in G<sub>1</sub> and an increase in S phase (Table: 5).

#### *Clone M1*

The clone was dexamethasone resistant. The cell cycle stages of the clone were not significantly altered with the various concentrations of dexamethasone (Table: 6). Exogenous IL-6 alone did not change G<sub>1</sub> phase nor S phase (-3%) of the cell cycle of the M1 clone (Table: 6). When exogenous IL-15 was added alone, it decreased the proportion of cells in G<sub>2</sub> (-60%) phases. Dexamethasone in combination with IL-6 variably altered

the proportion of cells in S phase and only reduced the proportion of cells in G<sub>2</sub> phase (-20%) at the dexamethasone concentration of 100µg / ml. IL-15 in combination with dexamethasone more than doubled the proportion of cells in G<sub>2</sub> phase (from 60% to 140%), but decreased the proportion of cells in S phase (-22%) and depending on the concentration of dexamethasone used increased (+9%) or reduced (-8%) the proportion of cells in G<sub>1</sub>. No significant differences were observed possibly due to insufficient data, rather than that there were no significant differences.

### *Clone M3*

This clone was resistant to dexamethasone. When this clone was treated with 100µg / ml dexamethasone alone (table: 7). There were minor changes in the proportion of cells in G<sub>1</sub> (reduced from 73% to 68%) and in the proportion of cells in S phase (increased from 24% to 32%). A slight increase was observed in the proportion of cells in G<sub>2</sub> stage (+33%) of the cell cycle with the addition of IL-6 (NS). The addition of IL-15 did not change the proportion of cells in G<sub>1</sub> (-4%) or S phase (+12%). IL-6 in combination with dexamethasone had no significant effect on the proportion of cells in cell cycle. At the 100µg / ml dexamethasone concentration the proportion of cells in G<sub>1</sub> was not altered (-6%) while the proportion of cells in G<sub>1</sub> at the 200µg / ml dexamethasone was slightly decreased (-14%). The combination of IL-15 and dexamethasone (100µg /ml or 200µg / ml) reduced the proportion of cells in G<sub>1</sub> and slightly increased the proportion of cells in S phase (+12%).

### *Clone M16*

The clone was resistant to dexamethasone. The various concentrations of dexamethasone (Table: 8) increased the proportion of cells in S phase. After the addition of IL-6 to the clone, the proportion of cells in S phase (+20%) increased with no change in G<sub>1</sub> but a reduction in G<sub>2</sub> phase (-31%). The addition of IL-15 alone to the clone induced no change in G<sub>1</sub> (-3%) but increased the proportion of cells in S phase (+65%) with a decrease in G<sub>2</sub> phase (-94%) cells (Table: 8). The addition of IL-6 in combination with dexamethasone, increased the proportion of cells in S phase at 50µg / ml and 100µg / ml dexamethasone (+6%, +55% respectively) with a reduction in G<sub>2</sub> (50%-100%). The combination of IL-15 with dexamethasone 50µg / ml showed no change the proportion of cells in G<sub>1</sub> (+2%) or S phase (-6%) with an increase in G<sub>2</sub> (+50%).

### *Apoptosis*

Apoptosis of the clones was not increased by the use of dexamethasone. When dexamethasone was used in combination with IL-6 or IL-15 apoptosis was variable.

**Table: 5 Effects of exogenous IL-6 or IL-15 alone or in combination with dexamethasone on U266 cells**

	G <sub>1</sub>	S	G2/M	Apoptosis
Dex 0	52	33	16	24
IL-6	44(-15%)	38(+15%)	18(+12%)	23(-4%)
IL-15	53(+2%)	33(0%)	15(-6%)	27(+12%)
Dex 50*	59	28	14	29
IL-6	44(-25%)	39 (+39%)	18(+28%)	26 (-10%)
IL-15	46(-22%)	38 (+36%)	15(+7%)	14 (-52%)
Dex 100	61	24	16	23
IL-6	44(-28%)	37 (+54%)	19 (+19%)	20 (-13%)
IL-15	57(-7%)	30 (+25%)	14 (-13%)	23 (0%)
Dex 200	62	25	14	24
IL-6	43(-30%)	38 (+52%)	20 (+43%)	40 (+65%)
IL-15	57(-8%)	29 (+16%)	8 (-43%)	29 (+21%)

\* Dex 50 = Dexamethasone 50µg / ml

† Percentage cells in G<sub>1</sub> (percentage change with respect to control)

**Table 6: Effects of exogenous IL-6 or IL-15 alone or in combination with dexamethasone on cell cycle of the clone M1**

	G <sub>1</sub>	S	G <sub>2</sub> /M	Apoptosis
Dex 0	62	34	5	18
IL-6	62 (0)	33 (-3%)	6 (+20%)	16 (-11%)
IL-15	62 (0)	32 (-6%)	2 (-60%)	19 (+6%)
Dex 50	62	38	5	16
IL-6	63 (+2%)	33 (-13%)	5 (0)	17 (+6%)
IL-15	59 (-5%)	32 (-16%)	10 (+100%)	18 (+13%)
Dex 100	63	32	5	18
IL-6	63 (0)	33 (+3%)	4 (-20%)	17 (-6%)
IL-15	58 (-8%)	31 (-3%)	12 (+140%)	23 (+28%)
Dex 200	60	36	5	22
IL-6	63 (+5%)	33 (-8%)	5 (0)	15 (-32%)
IL-15	65(+8%)	28 (-22%)	8 (+60%)	25 (+14%)

Dex 50 = 50µg / ml

**Table 7: Effects of IL-6 or IL-15 alone or in combination with dexamethasone on the cell cycle of the clone M3**

	G <sub>1</sub>	S	G <sub>2</sub> /M	Apoptosis
Dex 0	73	24	3	24
IL-6	73 (0)	23 (-4%)	4 (+33%)	6 (-75%)
IL-15	70 (-4%)	27 (+12%)	3 (0)	3 (-87%)
Dex 50	73	24	3	27
IL-6	73 (0)	23 (-4%)	4 (+33%)	8 (-70%)
IL-15	75 (+3%)	23 (-4%)	3 (0)	8 (-70%)
Dex 100	68	32	0	12
IL-6	64 (-6%)	36 (+13%)	0	9 (-25%)
IL-15	66 (-3%)	33 (+3%)	2	5 (-58%)
Dex 200	63	26	11	9
IL-6	73 (+16%)	23 (-12%)	4 (-64%)	15 (+67%)
IL-15	61(-3%)	29 (+12%)	10 (-9%)	5 (-44%)

**Table 8: Effects of dexamethasone in combination with IL-6 or IL-15 on the cell cycle of the clone M16**

	G <sub>1</sub>	S	G <sub>2</sub> /M	Apoptosis
Dcx 0	64	20	16	15
IL-6	65 (+2%)	24 (+20%)	11 (-31%)	19 (-27%)
IL-15	66 (+3%)	33 (+65%)	1 (-94%)	13 (-13%)
Dex 50	61	34	4	10
IL-6	62 (+2%)	36 (+6%)	2 (-50%)	15 (+50%)
IL-15	62 (+2%)	32 (-6%)	6 (+50%)	24 (+140%)
Dex 100	62	27	11	12
IL-6	58 (-6%)	42 (+55%)	0	14 (+17%)
IL-15	67 (+8%)	32 (-19%)	0	8 (-33%)
Dex 200	65	35	0	9
IL-6	65 (0)	35 (0)	0	8 (-11%)
IL-15	67(+3%)	31 (-11%)	2	7 (-22%)

## **TGF- $\beta$ ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)**

U266 cells were examined for cytokine mRNA transcripts by RT-PCR. Cytokine mRNA transcripts for TGF- $\beta$ 1 were observed. The supernatants from the U266 cells after 3 days culture were examined for the production of TGF- $\beta$ 1 protein by ELISA. This cell line produced significant quantities of TGF- $\beta$ 1 when compared to the control which was RPMI<sup>+</sup>. U266 cells secreted  $11 \pm 1$  ng / ml of TGF- $\beta$ 1, RPMI<sup>+</sup> had a background content of  $4 \pm 1$  ng / ml of TGF- $\beta$ 1 ( $p < 0.05$ ).

## **CHAPTER FOUR**

### **DISCUSSION**

## DISCUSSION

### Cytokine profiling of Myeloma clones

Multiple myeloma is a monoclonal proliferation of plasma cells, although the characteristics of the cell of origin have yet to be identified. It has been suggested that small terminal deoxynucleotidyl transferase negative CD10+ lymphoid cells of the bone marrow or post-germinal centre may be implicated. There have been many attempts to identify the factors that are responsible for the growth and survival of myeloma cells. Although myeloma cells produce some of these factors, others are produced and secreted by cells of the local microenvironment. Factors secreted by myeloma cells include IL-1 $\beta$ , IL-6, TGF- $\beta$ , TNF- $\beta$  granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF)(Soutar et al, 1996). Human myeloma cell lines may be of some assistance in identifying cytokines which are significant in the progression of the disease, particularly those cytokines which are important in immunosuppression and tumour expansion.

Although HMCL may be useful in the study of the biology of MM, doubt exists as to their usefulness in dissecting events that occur within the bone marrow microenvironment as these HMCLs were created from patients with advanced disease, which is extramedullary. These HMCLs have an altered phenotype and biology compared with malignant plasma cells harvested from bone marrow of patients (San Miguel et al, 1995) and may be unrepresentative of myeloma cells in the early stages of disease. The down

regulation of the surface antigen CD56 (NCAM) and VLA-5 is linked with extramedullary dissemination. While HMCLs are not exactly representative of primary myeloma cells, they share basic characteristics such as Ig gene re-arrangement and production of cytokines that make them a useful tool in the development of methods to study myeloma cells.

This project involved the cloning of the U266 cell line to obtain different phenotypes in order to further examine the role of cytokines in suppression / proliferation / development of myeloma.

In this study we investigated the cytokine profiles of several myeloma clones from the IL-6 independent cell line U266. This cell line, which was thought to be monoclonal in origin (Nilsson et al, 1970), produced clones that exhibited different cytokine profiles. These clones expressed mRNA transcripts for a range of T-cell activating and modulating cytokines. The U266 clone M5 expressed mRNA for the T cell stimulating cytokines IL-1 $\alpha$ , IL-1 $\beta$  and IL-2 (fig 5). M8 clones were positive for the cytokines IL-1 $\alpha$ , IL-4 and TGF- $\beta$ . M1 expressed mRNA for IL-6 and TGF- $\beta$ (fig 7). M3 expressed transcripts for IL-15 and TGF- $\beta$  (fig 12). All clones, with the exception of clone M6, constantly transcribed TGF- $\beta$ . These results demonstrate heterogeneity within the U266 myeloma clone population as regards to cytokine production (figs5-7) and hopefully provide a way of examining the effects of individual cytokines on myeloma cells.

Controversy exists as to the production of IL-6 and dependency of the U266 myeloma cell line on exogenous IL-6 (Barut et al, 1992; Schwab et al, 1991; Diamant et al, 1996). In this study IL-6 mRNA transcripts were detected in one clone only whereas the others remained negative (fig7). These discrepancies between this and other studies may be accounted for by changes in the cytokine profiles with time in culture or in the use of different methods of detection that may involve differences in sensitivity.

IL-1 $\beta$  is not produced by normal plasma cells but is found in the majority of MM patients (Donovan et al, 1998). IL-1 is believed to be important in the pathogenesis of MM (Torcia et al, 1996). Torcia demonstrated that osteoclast activating factor (OAF) activity of culture supernatants from unfractionated bone marrow cells of MM patients correlated with the IL-1 $\beta$  content. Also the use of the IL-1 receptor antagonist, sIL-1R type I or II, and neutralizing anti-IL-1 $\beta$  antibodies eliminated OAF activity. These results indicate that OAF activity of myeloma cells in humans is related to IL-1 $\beta$ . Interestingly IL-1 $\beta$  increases the expression of adhesion molecules and induces paracrine IL-6 production. These adhesion molecules may be responsible for myeloma cells remaining in the bone marrow where the production of IL-1 $\beta$  and the paracrine production of IL-6 ultimately lead to osteolytic lesions. Adhesion molecules may also support the growth and survival of myeloma cells within this microenvironment. In these experiments only one clone co-expressed IL-1 $\alpha$  and IL-1 $\beta$ (fig 5) and another expressed IL-1 $\alpha$ . It is possible that these differences in expression

may depend on the different stages of maturation. The U266 myeloma cells are extramedullary and therefore at a stage of development where down regulation of adhesion molecules might be expected and therefore little or no expression of IL-1 mRNA would be anticipated.

IL-15 is a cytokine that shares similar biological activities with IL-2 (Grabstein et al, 1994). Proliferation of cytotoxic T cells (Grabstein et al, 1994) and survival of NK cells was stimulated by IL-15 (Carson et al, 1997). Further studies have revealed a proliferation and differentiation promoting function of IL-15 in preactivated human B cells (Armitage et al, 1995). The constitutive expression of IL-15 $\alpha$  receptor (IL-15R $\alpha$ ) in myeloma cell lines and in plasma cells of MM patients has been observed (Hjorth-Hansen et al, 1999). Furthermore IL-15 transcripts in myeloma cell lines have been detected (Tinhofer et al, 2000). These findings may confirm the existence of an autocrine IL-15 loop as a method of tumour expansion in MM. IL-15 therefore could play an important role in the clonal expansion and survival of malignant cells in MM. In this study IL-15 transcripts of mRNA were expressed in one clone only (fig 12). However, all clones expressed the IL-15R $\alpha$  (fig 9). The presence of two amplification products 538bp and 439bp shown figure 9 is consistent with the existence of two alternatively spliced mRNA isoforms characterized by the presence or absence of exon 3 (+99bp). IL-15 receptor contains the  $\beta$  and  $\gamma$  chain of the IL-2 receptor and a specific  $\alpha$  chain (Giri et al, 1995). The IL-15R $\alpha$  is a high affinity binding receptor that transduces signals only in the presence of the

IL-2 / IL-15R $\beta$  and  $\gamma$  chain. Due to the high affinity of IL-15R $\alpha$  for IL-15 it has been suggested that IL-15 R $\alpha$  may act as a molecular sink for excess IL-15 or may associate receptors yet to be identified (Anderson et al, 1995; Kennedy et al, 1996). In this context the IL-15 receptor found on myeloma cells may enhance the suppression of T cells by capturing IL-15. Blocking IL-15 activity inhibits the expansion of memory CD8<sup>+</sup> T cells which play an important role in the long term protection against pathogens and tumours.

MM cells secrete TGF- $\beta$  which has suppressive effects on B, T, NK cells and macrophages. TGF- $\beta$  also up regulates IL-6 secretion from stromal cells (Urishima et al, 1996). Autocrine Il-6 can be induced through the activation of CD40 with CD40 ligand (CD40L) in MM (Urashima et al 1995). Thus TGF- $\beta$  may be helpful to maintain the malignant clone directly in addition to its immunosuppressive effects. Apart from M6 all clones expressed mRNA transcripts for TGF- $\beta$ . However, after six weeks, in culture M6 also expressed mRNA transcripts for TGF- $\beta$ (fig 8).

### **Effects of Myeloma clones on Immunosuppression**

Immune surveillance may fail owing to suppression of immune responses by MM cells. Many factors have been implicated in this suppression, for example Fas ligand, sMUC-1 and TGF- $\beta$ , as mediators of T cell suppression (reviewed Cook G, Campbell J. 1999). T lymphocytes are strongly regulated by TGF- $\beta$ . T cells that are activated secrete TGF- $\beta$  and

exogenous TGF- $\beta$  inhibits IL-2 dependent proliferation (Kerhl et al, 1986a). Effector functions of activated cells are suppressed by TGF- $\beta$ 's inhibitory effects on the secretion of an assortment of cytokines. Therefore TGF- $\beta$  inhibits the effects and / or the production of interferon- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , IL-1, IL-2, IL-3 and the expression the IL-2 receptor (Espevik et al, 1987,1988; Ohata et al, 1987; Ranges et al, 1987; Letterio & Roberts, 1998). A regulatory role in the differentiation of T cells may be performed by TGF- $\beta$  as TGF- $\beta$  can induce the expression of CD8. Although the later stages of differentiation is inhibited by TGF- $\beta$  and therefore inhibits the generation of CD4 CD8 cells (Suda & Zlotnik, 1992; Takahama et al, 1994; Mossalayi et al 1995). While the generation of T<sub>H1</sub> cells, a subset of helper T cells with a characteristic cytokine secretion pattern was originally observed to be promoted by TGF- $\beta$  (Swain et al 1991), it has now been implicated as regulator of T<sub>H2</sub> differentiation of cells and a inhibitor of T<sub>H1</sub> differentiation of cells (Schmitt et al, 1994; Strober et al, 1997).

Tumour cells of MM patients directly inhibit the autocrine IL-2 growth pathway in T cells. TGF- $\beta$  secreted by the tumour was responsible for the lack of T cell response (Cook, et al, 1999). In this study we have observed that clones M3 and M5, which expressed transcripts for mRNA of TGF- $\beta$ , suppressed the MLR at high concentration (1MM: 5PBMNC), with suppression diminishing as the MM cells were diluted with PBMNCs. This was in contrast to M6 which had not expressed mRNA for TGF- $\beta$  nor suppressed the MLR. In the Con A induced proliferation experiments,

clones M3, M5 and M9 all suppressed proliferation unlike clone M6 that neither expressed mRNA for TGF- $\beta$  nor suppressed Con A induced proliferation. Interestingly the degree of suppression was much stronger in the clone M3 that also co-expressed IL-15. In this context this may indicate that IL-15 enhances the TGF- $\beta$  suppression of T cells. Clones therefore that co-expressed these two cytokines were better T cell inhibitors.

## **Cell studies**

### ***Cell proliferation***

Although IL-6 is thought to be a major growth factor in multiple myeloma (Klein, et al, 1995) other cytokines may contribute to the expansion of these malignant cells. Many cells and tissues constitutively express IL-15 mRNA but it is not usually expressed in T, B or Natural killer (NK) cells (Bamford et al, 1996; Doherty et al, 1996), although it may be expressed by neoplastic T cells (Yamada et al, 1998; Dobbeling et al, 1998). Myeloma cell lines express the  $\alpha$  receptor for IL-15, which may play a role in the proliferation and or survival of these cells (Tinhofer et al, 2000). This study examined the effects of exogenously added IL-6 or IL-15 alone or in combination with dexamethasone on proliferation of U266 cells and myeloma clones.

### **The effects of exogenously added IL-15 on U266 proliferation**

Proliferation of U266 cells were significantly increased by the addition of IL-15 at concentrations of 1ng / ml ( $p < 0.05$ ) and 10ng / ml ( $p < 0.05$ ) with

respect to control 0ng / ml of IL-15 (fig 15). This data may suggest a role for IL-15 in the proliferation of the neoplastic plasma cell.

**The effects of exogenously added IL-6 or IL-15 alone or in combination with dexamethasone on proliferation**

IL-6 did increase proliferation in the U266 cells (fig 16) although not significantly. Exogenous IL-6 promotes cell proliferation by phosphorylation of the retinoblastoma protein (pRB). When pRB is dephosphorylated it binds E2F which prevents cells from leaving G1 thus preventing cell growth (Urashima et al, 1997). However IL-6 did protect against the effects of dexamethasone. Recent studies have demonstrated that IL-6 is an anti-apoptotic factor for myeloma cells. It prevents spontaneous, drug induced, and Fas induced apoptosis (Lichtenstein et al, 1995, Hardin et al, 1994, Chauhan et al, 1997). Interestingly a significant decrease ( $p = 0.001$ ) was observed in proliferation of these U266 cells when IL-15 was added. IL-15 also protected the cells from the anti proliferative effects of dexamethasone at 50 $\mu$ g / ml (fig 16), but not at 100 $\mu$ g / ml (fig 17). These results may be of some significance as multiple myeloma clinical trials are planned, which will investigate the use of IL-15 ex vivo to protect T cells from the immunosuppressive effects of myeloma plasma cells. The removal of all IL-15 from reinfused cells would be of major importance as IL-15 may act as a survival / growth factor for myeloma cells (Hjorth-Hansen et

al, 1999; Meazza et al, 1998), as well as having toxic effects for the recipient.

The myeloma clone M1, which produces transcripts of IL-6 mRNA, was resistant to the effects of dexamethasone but a significant increase ( $p=0.0056$ ) in proliferation was observed with the addition of IL-15 (fig18). The clone M3 (expressed IL-15 mRNA) was also resistant to dexamethasone but both IL-15 and IL-6 in combination with dexamethasone potentiates a significant reduction ( $p=0.001$ ,  $p=0.03$ , respectively) in proliferation (fig19). M16 was resistant to dexamethasone at  $50\mu\text{g} / \text{ml}$  (fig 21), but not at  $100\mu\text{g} / \text{ml}$  (fig 20) Interestingly IL-15 increases proliferation. These different responses may be due to the different cytokine profiles exhibited, cell surface receptors, or other soluble factors. The fact that clones examined were resistant to dexamethasone may be due to the cloning process that selected for those clones showing vigorous growth. By choosing these clones we may have a more resistant type of cell. It is of note that two clones had a significant increase in proliferation in response to exogenous IL-15. These results require further investigation. Investigators have already demonstrated the presence of IL-15 mRNA and protein in the microenvironment of plasma cells (Mrozek et al, 1996) which may suggest the possibility of paracrine stimulation.

In order to further investigate the effects of exogenous IL-6 and IL-15, cell cycle experiments were carried out. Cell cycle and apoptosis assays were

performed on the U266 clones with IL-6 or IL-15 alone or in combination with dexamethasone. These cell cycle studies confirm the proliferation results on U266. The dexamethasone sensitivity did not result in cell death by apoptosis, but rather G1 arrest which were reversed significantly with the addition of IL-6. Although dexamethasone is considered to be apoptotic, no such apoptotic effect was observed. It has been reported that IL-15 may protect the cells from apoptosis (Hjorth-Hansen et al 1999) but this has not been demonstrated, as these cells are resistant to apoptosis.

### **Conclusions**

We have demonstrated that myeloma clones producing TGF- $\beta$  mRNA transcripts (produced significantly high levels of TGF- $\beta$  protein) can suppress T cell proliferation. Suppression of IL-2 T cell responses may be mediated by TGF- $\beta$  since it prevents T cell proliferation by inhibiting the phosphorylation of Jak-1 kinase activation of STAT 5 transcription factor (Bright et al, 1997). This prevents T cells from entering the IL-2 autocrine growth pathway. The combination of IL-15 and TGF- $\beta$  enhances suppression of T cell proliferation. The myeloma clones expressed mRNA transcripts for IL-6, IL-15, IL-15R and TGF- $\beta$ . Due to these findings the effects of exogenous IL-6, IL-15 and dexamethasone on proliferation, cell cycle and apoptosis of the myeloma clones were investigated.

IL-6 and IL-15 did protect the U266 cells from the effects of dexamethasone at 50 $\mu$ g/ml but not at 100 $\mu$ g/ml. IL-6 did not induce any significant

proliferation. In contrast, IL-15 induced proliferation of the clone M1 (expresses IL-6, TGF- $\beta$  mRNA transcripts) and M16 (expresses TGF- $\beta$  mRNA transcripts). However, M16 did not always proliferate in the presence of IL-15, other soluble factors may be involved. Interestingly exogenous IL-15 in combination with dexamethasone significantly decreases proliferation in the clone M3 (expresses IL-15, TGF- $\beta$  mRNA transcripts) that is resistant to the effects of dexamethasone. The role of IL-15 is complex and seems to be dependent on what other cytokines /factors are present.

Further studies in the interrelationship of IL-6 / IL-15 / TGF- $\beta$  are needed in both cell lines and fresh MM cells obtained from patients.

The use of immunotherapy in MM is becoming more attractive as the value of donor lymphocyte infusions (DLI) after allogeneic BMT, and the beneficial effect of the immuno-modulating agent thalidomide, is becoming more apparent. TGF- $\beta$  induced immunosuppression can be overcome by stimulating pre-transplant T cells with IL-15, after which the T cells are again responsive to IL-2. However, before the use of agents such as IL-15 can be considered for in vivo use, it is essential to clarify the possible direct effect this and other agents may have on the MM cells.

MM is a clonal expansion of malignant plasma cells in the bone marrow. Complex interactions between the malignant cells and the cells of the microenvironment (e.g. osteoblasts, osteoclasts and stromal cells) control the growth of these MM cells. Although IL-6 is a major growth factor in this microenvironment, several local growth factors are deposited in bone by

osteoblasts, these factors are released as the bone is reabsorbed. Bone marrow stromal cells and myeloma cells produce TGF- $\beta$ . This factor is also deposited in bone and released when it is reabsorbed. IL-6 production is stimulated by TGF- $\beta$ . Therefore the bone derived TGF- $\beta$  may also contribute to the production of IL-6. Another local factor, Insulin like growth factor-1 (IGF-1), is deposited in bone; IGF-1 supports the growth of myeloma cells (Jelinek et al, 1997). In addition dexamethasone induced apoptosis is prevented by IGF-1 (Xu et al, 1997).

It has been suggested that reabsorbing bone and the cells involved in that process may be important in generating the appropriate environment for the proliferation and survival of malignant plasma cells. Improving our understanding of these interactions may provide us with opportunities to identify new therapeutic approaches to treating MM.

Thus, although the data presented in this study provide valuable preliminary information; further studies are required.

Ideally, these should include experiments in which the role of the bone marrow microenvironment is investigated.

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