IMMUNE REGULATION IN MULTIPLE MYELOMA: THE HOST-TUMOUR CONFLICT

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"You can discover what your enemy fears most by observing the means he uses to frighten you."

Eric Hoffer

(1902-1983)

Philosopher, USA

ABTRACT OF THESIS

There is little doubt that tumour-specific antigens exist for most human cancers though it is still unclear why these antigens do not cause tumour rejection. A successful immune response against a tumour is dependent on the detection of that tumour and the ability of the host immune system to mount an effective response. Clearly, malignant cells have evolved to evade the natural host defence mechanisms. Multiple Myeloma (MM) is a mature peripheral B cell malignancy with many defects in the host immune system including quantitative and qualitative abnormalities of both cell-mediated and humoral immunity. However, as yet, the mechanism(s) that induce these abnormalities of the immune system remain to be determined. Before immunotherapy can be applied on an entirely rational basis more knowledge is required as to why the immune system fails to identify multiple myeloma plasma cells as foreign.

The data presented in this thesis demonstrates that the malignant plasma cells of multiple myeloma are capable of suppressing the activation of T lymphocytes. The myeloma cells prevent activation of T cells from healthy donors by allo-antigen, mitogen and IL-2, mediated by the production of a soluble, immuno-suppressive factor. This factor was responsible for inducing cell cycle arrest and failure of the T cells to progress into the autocrine IL-2 autocrine pathway, which is of critical importance in the activation of T cells. To further investigate this interaction an *in vitro* model system was developed to examine the key stages of T cell activation and homeostasis.

Myeloma cells constitutively expressed high levels of TGF-β1 mRNA transcripts as detected by RT-PCR, which were translated into latent protein and secreted as detected by immunohistochemistry and ELISA, respectively. The reversal of the immuno-suppression induced by the myeloma cells using the specific TGF-β1 antagonist, Latency Associated Peptide, confirmed that TGF-β1 is a major factor in myeloma-associated suppression of T lymphocyte activation.

It was demonstrated that the myeloma cells prevent the T cells, upon activation, from up-regulating the surface expression of the α-chain of the IL-2R thus preventing the formation of the high-affinity receptor. The reduced expression of IL-2Rα resulted from altered transcription of the α-chain gene in response to re-stimulation of primary T cells with IL-2. When signalling events in primary T cells responding to re-stimulation with IL-2 was examined, myeloma cells inhibited the phosphorylation of both STAT3 and STAT5. However, using a novel IL-2-dependent T cell line (IDBL), which does not

require the expression of the high affinity IL-2R for its responses to IL-2, it was shown that these cells are insensitive to the myeloma-derived TGF-β, in terms of DNA synthesis and proliferation, despite demonstrating failure of phosphorylation of STAT5. It was demonstrated that phosphorylation of STAT3 was unchanged when IDBL cells were co-cultured with myeloma cell lines. However when the α-chain of the IL-2R was presented inter-cellularly *in trans*, this induced a susceptibility of the IDBL cells to HMCLs. This susceptibility resulted from failure of STAT3 phosphorylation associated with down-regulation of *bcl-2* and *pim-1* genes. As a consequence, the IDBL cells demonstrated reduced DNA synthesis and proliferation, similar to that seen with primary PBL, when IL-2Rα was presented in a an inter-cellular fashion.

Taken together, these features demonstrate that the presence of the α-chain of the IL-2R presented inter-cellularly *in trans*, whether from a transfectant in the case of IDBL cells or from $CD25^+$ T cells in the heterogeneous population of primary T cells, assists the susceptibility of activating T cells to myeloma-derived TGF-β resulting in growth arrest and failure to enter the autocrine IL-2 pathway. This suggests a role of the α-chain of the IL-2R in regulating the immune responses, here potentiating the T cell suppressive effects of myeloma-derived TGF-β1. The experimental data presented in this thesis has addressed the issue of the interaction of myeloma tumour cells with T lymphocytes, indicating a potential mechanism that may propagate the immune privilege of the tumour clone. This highlights areas that might be targeted by further research studies in anticipation of designing new therapeutic strategies.

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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.

Dr Gordon Cook

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I dedicate this thesis to Mr Thomas Hunter (1908-1980), who like many before and after him, suffered from Multiple Myeloma. R.I.P.

CHAPTER 1

Introduction and Review

Although there is little doubt that tumour-specific antigens exist for most human cancers, it is still unclear why these antigens do not cause tumour rejection. A successful immune response against a tumour is dependent on the detection of that tumour and the ability of the host immune system to mount an effective response. Clearly, malignant cells have evolved to evade the natural defence mechanisms resulting in growth and dissemination at the expense of the host. It is essential that the mechanisms employed by tumour cells to evade immune regulation be identified so that immunotherapy strategies can be designed which will realise their promise. In the context of Multiple Myeloma (MM), this universally fatal mature B cell malignancy could potentially be a target for directed immunotherapy and as such a better understanding of the basic immunology associated will greatly assist the search for novel treatment approaches.

1.1 MULTIPLE MYELOMA: CLINICO-PATHOLOGICAL FEATURES

Plasma cell dyscrasias are a heterogeneous group of disorders that are characterised by the clonal expansion of terminally differentiated B cells. These clonal plasma cells produce a monoclonal immunoglobulin (paraprotein ≡ M-component ≡ clonal idiotype) that can be detected in both serum and urine (Boccadoro & Pileri, 1995). In the malignant form, the expansion is uncontrolled and this is responsible for the clinical manifestations, namely bone marrow failure, lytic bone disease and renal failure. Overt MM is the commonest form of plasma cell dyscrasia. MM was first reported by Dr Samuel Solly in his communication to the Royal Medical Chirugical Society in London (Solly, 1844) though Dr William MacIntyre is frequently credited with the first description when he reported a case of light chain myeloma (MacIntyre, 1850). However, it wasn't until 1873 that the disease was referred to as *Multiple Myeloma* by Dr Rustizky to indicate the multiple nature of the bone marrow tumours (Tricot, 2000). In 1900 Dr Wright discovered that the homogeneous cellular infiltrate seen in the bone marrow of patients with multiple myeloma was in fact a tumour of plasma cells.

Multiple myeloma has an incidence of 3-4 new cases per 100,000 population per annum though this incidence demonstrates ethnic variation: the incidence is lowest in Chinese races at 1/100,000 and highest in European races at 4/100,000 (Reidel et al, 1991). Furthermore, the disease is commoner in Afro-American individuals compared to Caucasoid individuals with a male:female ratio of 3:2. In the 1960s, the disease

presented most commonly with back pain, reported in up to 70% of patients. Owing to increased awareness of the disease and availability of rapid biochemical analysis, less than 40% of patients now present with symptomatic bone disease and at least 20% of patients are now diagnosed whilst totally asymptomatic (Dimopoulos et al, 1992).

Multiple myeloma is an incurable disease, except in certain patients treated with allogeneic haematopoietic stem cell transplantation (Tricot et al, 1996). The survival of patients with MM is variable and can range from a few months to several years, though without therapy, the median survival is 7 months from diagnosis (Osgood, 1960). The median survival with treatment is 2.5-3 years but this may be prolonged in younger patients treated with dose intensive chemotherapy (Bersagel, 1995). However, only 3.5% of patients with multiple myeloma are alive at 10 years. The use of a number of clinical prognostic indicators reflecting tumour burden have been used to formulate a basic staging system, the Durie Salmon clinical staging system (Durie et al, 1982). This system, based on the presence of bone marrow failure, renal dysfunction and the extent of lytic bone disease, could separate patients prognostically in to early stage disease (low tumour burden) with a median survival of 5 years and those with advanced stage disease (high tumour burden) with a median survival of 15 months. The addition of biological variables such a β_2-microglobulin, CRP (indicator of IL-6 levels) and cytogenetic abnormalities may further refine this crude clinical staging system to allow the identification of those patients who will demonstrate the best response to conventional therapy (Davies et al, 1997). Mutations of oncogenes such as N- & K-Ras, and the tumour suppressor gene, p53, have limited prognostic predictability as these events are most frequently encountered in the advanced stages of disease and are relatively rare events at presentation. Altered expression of the cell cycle regulator, pRb, may be of significance, especially as this is located on chromosome 13. Abnormalities of chromosome 13 in MM are associated with a poor prognosis (Hallek, 1998). However, it is not clear whether the deletions of chromosome 13 are related to abnormalities of pRb as the regions involved in the deletions are quite extensive and may involve other tumour suppressor genes.

Plasma cells in Myeloma

The plasma cell infiltration of bone marrow (BM) is largely diagnosed through morphological examination of BM aspirates although increasingly the use of paraffin-

embedded biopsy sections, flow cytometry, *in situ* hydridisation studies and the use of magnetic resonance imaging are providing an better evaluation of the extent of marrow infiltration (Figure 1.1). The minimal diagnostic criterion is a plasma cell population of 10-15% of the total cell count (Kyle, 1992). However, the situation varies greatly amongst patients: 35% of patients have fewer than 30% BM plasma cells, 11% of patients have less than 10% and 3% had fewer than 5% (Bartl et al, 1995). This merely reflects the patchy nature of this disease and its dissemination throughout the marrow cavity. The plasma cell morphology demonstrates features that enable confident identification of atypical plasma cells: nuclear-cytoplasmic asynchrony, nuclear abnormalities including multiple nuclei and nucleoli and variations in size and cytoplasmic staining.

The exact origin of these clonal plasma cells is still the source of considerable debate and the existence of a "myeloma stem cell" has yet to be proven conclusively. Normal BM plasma cells are derived from B cells that have passed through the germinal centre of a lymphoid organ and undergo somatic hypermutation and class switching of their immunoglobulin (Ig) heavy chain genes (Davis et al, 1997). Following antigen-selection, only B cells with high affinity for the presented antigens survive, whereas the remaining B cell clones undergo apoptosis. The plasma cells of multiple myeloma demonstrate rearranged Ig genes that are extensively somatically hypermutated in a manner compatible with antigen selection, with no evidence that the process of hypermutation is continuing (Hallek et al, 1998). However, myeloma cells have a significantly lower rate of Ig secretion compared with normal plasma cells. It has been proposed that the development of malignant plasma cell expansions result from a multi-step transformation, which further clouds the issue of the putative "myeloma stem cell" (Hallek et al, 1998). It is still not clear whether the oncogenic events resulting in the development of MM occur after the normal maturation process which generates long-lived plasma cells (Ig gene rearrangement, somatic hypermutation and switch recombination) or in fact these events do not interfere with this process (Bakkus et al, 1994).

MM is a slow growing tumour and using the incorporation of DNA precursors (^{3}[H]thymidine or bromodeoxyuridine), a labeling index can be calculated as the percentage of plasma cells actively entering S phase of cell cycle (Kyle, 1992). Using this technique, only 1-3% of plasma cells are cycling compared to much higher values

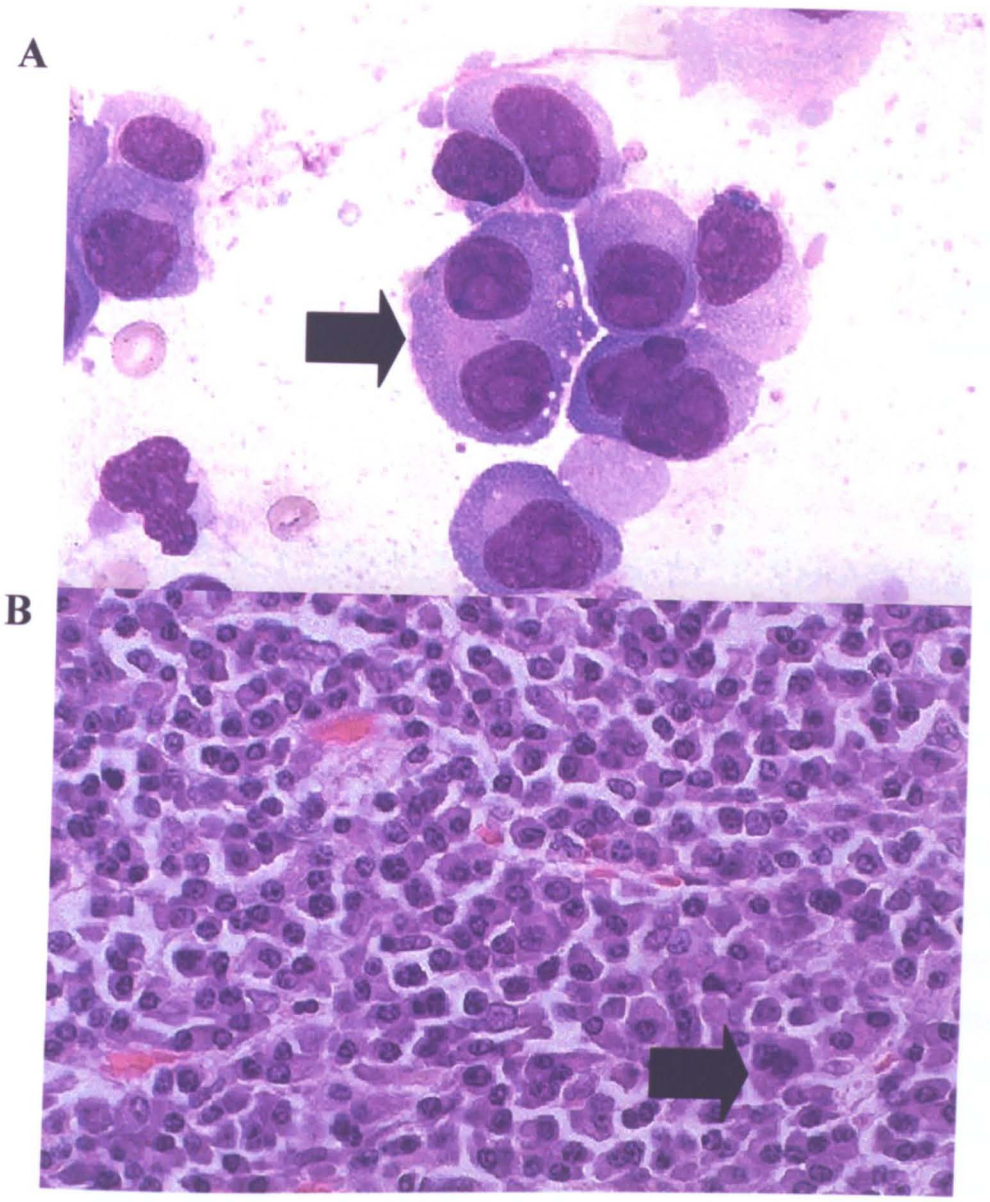


Figure 1.1. Bone marrow plasma cell infiltrate in multiple myeloma. The morphology of bone marrow aspirate (A- Geimsa, X400) and histology of paraffin-embedded marrow trephine biopsy (B- H&E, X200) demonstrate atypical plasma cells with bi-nucleate forms (arrow) and prominent nucleoli easily identified.

detected in reactive plasma cell expansions. However, as a biological variable it has not been shown to correlate with survival (Davies et al, 1997). Perhaps of more interest is the role of programmed cell death or apoptosis. Apoptosis plays a pivotal role in the normal development of B cells, especially for the deletion of autoreactive B cells (Hallek et al, 1998). *Bcl-2* is a membrane protein associated with the outer membrane of mitochondria and nuclei and regulates apoptosis (Korsmeyer, 1992). Over-expression of *bcl-2* in cancer cells can result in chemoresistance and protection from apoptosis and recent work has shown the existence of several *bcl-2*-related proteins in cancer cells that can inhibit (Bcl-X_L, Mcl-1, NR-13, A1, Bcl-W) or enhance (Bax, Bcl-X_S, Bak, Bad) apoptosis (Gajewski, 1996).

Expression of *bcl-2* is thought to play an important role in B-cell malignancies, especially in follicular lymphoma, where more than 80% of the patients have the translocation t(14;18), which results in overexpression of *bcl-2* (Hermine et al, 1996). This translocation occurs at lower frequency (0% to 15%) in MM, but despite this an over-expression of *bcl-2* is seen in the majority of fresh plasma cells from patients with MM and in human myeloma cell lines (Pettersson et al, 1992; Ong et al, 1995). High levels of *bcl-2* protein are likely to mediate the resistance of MM cells to apoptosis induced by dexamethasone, IL-6 deprivation or other drugs (Tian et al, 1996). However, when bcl-2 expression was examined in relationship to patient survival, no correlation was evident (Ong et al, 1995).

Adhesion Molecules and Other Cell-Surface Antigens

The differentiation of lymphoid precursor cells into mature B lymphocytes is accompanied by characteristic changes of cell-surface antigens. The application of high resolution, multiparameter flow cytometry has been used to identify and characterize normal plasma cells in the human BM. Plasma cells exist in at least two different subpopulations, early lymphoplasmacytoid cells and late mature plasma cells (Terstapen et al, 1990). These two populations appeared phenotypically different, but both strongly express CD38. Mature, normal plasma cells show a very heterogeneous immunophenotype in that they can express early B-cell antigens (CD19, CD20, CD10), myeloid antigens (CD13, CD33), HLA-DR, common haematopoietic antigens (CD45), and adhesion molecules (CD11b, CD11c).

The neoplastic cells of MM probably follow a similar maturational pattern to the normal counterpart. Although the MM "stem cell" is unknown, there is evidence for cell clones

in patients with MM that show the immunophenotype of early B-cell precursors, which express CD38 at high levels, as well as CD10, CD34, CD19, and CD20 (Boccadoro & Pileri, 1995). Myeloma cells show a similar heterogeneity in their immunophenotype as their normal counterparts, according to their differentiation stage. Immature myeloma cells ($CD38^{++}/CD45^{low}/CD49e^{-}$) appear to express the same clonal idiotype as mature cells ($CD38^{++}/CD45^{-}/CD49e^{+}$). Whether CD34 is expressed on MM cells remains controversial and published studies indicating the expression of the stem cell antigen on clonally-related cells in myeloma balance those reports which state that MM cells do not express CD34 and this may be explained by technical differences (Davies et al, 1997).

Syndecan-1 (CD138) is a heparan sulfate-bearing proteoglycan present on the surface of myeloma cells where it mediates myeloma cell-cell and cell-extracellular matrix adhesion (Cook et al, 1997). It has been reported that some cells demonstrate striking localization of syndecan-1 to a single small membrane protrusion (uropod), with the remainder of the cell surface being mostly negative for syndecan-1 (Borset et al, 2000). In addition to syndecan-1, several other cell surface molecules localize specifically to the uropod, including CD44 and CD54. The functional importance of these observations are speculative but these molecules may be involved in mediating cellular migration, cell-cell and cell-matrix interactions and attachment of heparin-binding proteins, like hepatocyte growth factor or osteoprotegerin (Dhodapkar & Sanderson, 1999; Borset et al, 2000). Myeloma cells also shed syndecan-1 and this soluble form has been shown to induce apoptosis of HMCLs and high serum levels correlate with a poor prognosis, suggesting a regulatory role of syndecan-1 in the growth of myeloma cells (Dhodapkar et al, 1998; Seidl et al, 2000).

It is believed that myeloma cells originate outside the BM and give rise to plasma cells upon migration, using expression of unique adhesion molecules to interact with the marrow microenvironment (Vidriales et al, 1996). Despite the similarity of most antigens expressed on myeloma cells and normal plasma cells, some of the antigens detectable on myeloma cells are rather unique. For example, the adhesion molecule CD56, normally found on NK cells is the 140-kD isoform of N-CAM, is highly expressed on some myeloma cells but not on normal mature plasma cells (reviewed by Cook et al, 1997). It seems to mediate adhesion of myeloma cells to each other (homotypic adhesion). The expression of adhesion molecules and other cell-surface molecules such as CD49d, CD54, CD44, CD58, ICAM-1 and CD29 are important for

the communication of MM cells with the BM micro-environment. The interaction of MM cells with BM stromal cells (BMSCs), mediated by these adhesion molecules results in IL-6 secretion by BMSCs from both normal and MM patients resulting from up-regulation of IL-6 gene transcription conferred by NF-κB binding to the IL-6 promoter (Chauhan et al 1996). One of the cell-surface molecules triggering IL-6 secretion in MM cells is CD40, a member of the TNF receptor superfamily that is expressed by various lymphoid malignancies (Banchereau et al, 1994). Activation of CD40 induces the clonogenic growth and enhances the survival of MM cells *in vitro* (Tong et al, 1994). Stimulation of CD40 by its ligand, CD40L, stimulates the secretion of IL-6 from MM cells and cell lines, suggesting the possibility for induction of IL-6-mediated autocrine MM cell growth (Tong & Stone, 1996). However, the source of CD40 stimulation in the BM stroma remains unknown; expression of CD40L seems to be restricted to T lymphocytes, but its expression on BMSCs has not been investigated.

Cytokines and growth factors.

The proliferation, differentiation, and function of lympho-haematopoietic cells is regulated by a complex network of growth factors and cell surface molecules which establish a fine-tuned communication between stromal cells and lympho-haematopoietic precursors in the BM (Sieff, 1990). These growth factors bind to specific cell-surface receptors that belong to different families, the receptor tyrosine kinases and the haematopoietic cytokine receptors (Miyajima et al, 1992). The nature of the biological response to any growth factor is defined by the tissue or lineage distribution of growth factor receptors and by distinct transmembrane signalling events in which tyrosine kinases play a pivotal role.

The malignant plasma cells in MM are localized in the bone marrow (BM) in close association with stromal cells, and are rarely found in other locations. The pathogenesis of MM depends on the presence of growth factors that support the survival, proliferation, and differentiation of MM cells in the BM during the different disease stages (Klein, 1995). The cytokines involved in MM pathogenesis are similar to those mediating the proliferation of normal early plasma cells (plasmablasts), and their differentiation to mature plasma cells. Interleukin-6 (IL-6) is of particular importance during this process.

Interleukin-6 (IL-6) - IL-6 is a cytokine that has pleiotropic effects on haematopoietic and non-haematopoietic cells (Hirano et al, 1990). It induces purified B cells to differentiate into Ig-secreting plasma cells (Klein, 1995). The evidence that IL-6 is involved in MM pathogenesis was established by the following experimental and clinical findings: (1) IL-6 could induce *in vitro* growth of myeloma cells freshly isolated from patients, (2) myeloma cells spontaneously produced IL-6 and expressed IL-6R, (3) anti-IL6 antibodies inhibited the growth of MM cells or cell lines *in vitro*, and (4) treatment of MM patients with MoAbs to IL-6 showed some anti-tumour effect (Zang et al, 1992; Klein, 1995). IL-6 supports the survival and/or expansion of MM cells not only by stimulating cell division, but also by preventing programmed cell death (apoptosis) which can be induced by serum starvation, or by treatment with dexamethasone and anti-Fas antibodies (Lichtenstein et al, 1995; Chauhan et al, 1997). Similarly IL-6R antagonists, which block the activation of MM cells by IL-6, act as pro-apoptotic factors for MM cells (Demartis et al, 1996). There is still some controversy about the source of IL-6 provided as a MM growth factor during disease progression. Some investigators have found IL-6 to be produced by the tumour itself in an autocrine manner, but stronger evidence supports the notion of paracrine IL-6 secretion by the tumour microenvironment in the BM (Klein, 1995). IL-6 is probably produced in large amounts by BM stroma cells (BMSC), osteoblasts, and osteoclasts (Caligaris-Cappio et al, 1992). The IL-6 secretion by BMSCs seems to be regulated by cytokines like IL-1β secreted by the tumour as antibodies to IL-1β or the use of the IL-1β receptor antagonists were able to block the IL-6 production in short-term cultures of BM cells from MM patients (Klein, 1995). The synergistic interaction of IL-6 and TGF-β on the maintenance of the malignant clone is discussed below.

IL-6 Receptor (IL-6R) - Stimulation of cells by IL-6 requires binding to the IL-6R which is composed of at least two subunits, the α– and β-chains (IL-6Rα & IL-6Rβ or gp130, respectively). Binding of IL-6 to IL-6Rα induces the tyrosine phosphorylation and dimerisation with gp130 (Klein, 1995). gp130 is the common β-subunit shared by the receptors for ciliary neurotropic factor (CNTF), oncostatin M (OSM), leukemia inhibitory factor (LIF), IL-11, cardiotrophin 1, and IL-6 and is essential for transmitting their respective signals. Accordingly, these six cytokines share some biological functions with IL-6, and some MM cell lines respond to LIF, OSM, or CNTF if the appropriate receptor α-chain is expressed. In some MM patients, IL-11 levels may also

be elevated in the BM, but the effects of IL-11 and of the other three cytokines on the growth of MM cells *in vivo* remain uncertain and have not been studied in the same depth as the effects of IL-6 (Klein, 1995). Another potential mechanism contributing to the growth and expansion of MM is the agonistic effect of the soluble IL-6Rα that enhances the sensitivity of myeloma cell lines to the effects of IL-6 (Gaillard et al, 1993). Soluble IL-6Rα is generated by receptor shedding from the cell membrane or by alternative RNA splicing. While high serum levels of IL-6 were shown to predict a poor prognosis or to reflect an active disease in MM the prognostic importance of serum IL-6R is somewhat lacking (Pulkki et al, 1996).

Interleukin-15 (IL-15) – IL-15 is a monocyte/macrophage-derived T cell stimulatory cytokine that can enhance cytotoxic T cell proliferation, regulate NK cell survival and regulate proliferation and differentiation in pre-activated B cells (reviewed by Ma et al, 2000). IL-15 has also been shown to inhibit anti-Fas and dexamethasone-induce apoptosis of T cells. IL-15 not only shares many biological properties of IL-2 but also signals through the β and γ_c chains of the IL-2R though utilises its own high affinity α chain (IL-15Rα). Some investigators have recently shown that fresh MM plasma cells constitutively express IL-15Rα and IL-15 (Tinhofer et al, 2000). Using monoclonal antibody blocking experiments, the authors demonstrated that autocrine IL-15 prevented spontaneous apoptosis and augmented cell survival in IL-6-depleted fresh MM plasma cells and cell lines. Furthermore, the authors reported that IL-15 treatment of fresh MM plasma cells reduced anti-Fas and chemotherapy-induced (doxorubicin and vincristine) apoptosis but not that associated with dexamethasone treatment. As such, IL-15 could potentially play a role in the cytokine milieu that supports clonal expansion and survival of the malignant cells in multiple myeloma.

Other Stimulatory Cytokines - Granulocyte colony-stimulating factor (G-CSF) is a haematopoietic growth factor with structural homology to IL-6 (Cosman et al, 1990). Moreover, the G-CSF receptor shares some homology with gp130. Both G-CSF and IL-6 induce activation of NF-IL-6, a transcription factor involved in the synthesis of IL-6 and G-CSF is a potent growth factor for freshly explanted myeloma cells and MM cell lines (Klein, 1995). The mechanism of action by which G-CSF mediates MM growth is unknown. Granulocyte-macrophage-CSF (GM-CSF), IL-3, stem cell factor (SCF), tumour necrosis factor-α (TNF-α), hepatocyte growth factor (HGF), and insulin-like growth factor 1 and 2 (IGF-1 and IGF-2) are also potential MM growth factors, because

they were shown to stimulate growth and/or specific intracellular signalling events of MM cells or cell lines *in vitro*, often in a synergistic manner with IL-6 (Hallek et al, 1998).

Inhibitory Cytokines - Some growth factors commonly found in the BM microenviroment inhibit the growth of MM cells. For example, Interferon-γ (IFN-γ) was reported to inhibit IL-6-dependent proliferation of fresh MM cells (Klein, 1995). It did not affect the endogenous IL-6 production by MM cells, but IFN-γ can interfere with IL-6 transmembrane signalling, resulting in enhanced apoptosis. Interestingly, IFN-γ also inhibits cytokine-mediated bone resorption, which is a recurring clinical problem resulting in significant morbidity and mortality in MM (Klein, 1995). IFN-β and IFN-α were both shown to inhibit the proliferation of the MM cell line U266 (Berger et al, 1997). These effects are mediated, at least in part, by a down-modulation of the IL-6R and as a consequence, IFN-β reduces the IL-6-dependent tyrosine phosphorylation and activation of several signalling proteins, including Ras.

Taken together, a variety of growth factors promote and some inhibit the growth of myeloma cells *in vitro* and/or *in vivo*. It is difficult to clarify the functional relevance of these redundant growth factor effects on MM cells. Moreover, it seems important to emphasize that the expression of the various cytokine receptors may vary among individual patients, and also among different tumour cells in a given patient. Nevertheless, IL-6 clearly seems to be the most important stimulatory factor of these cytokines, because the biologically active IL-6 concentrations found *in vitro* and *in vivo* are 500- to 5,000-fold higher than the concentrations of other MM growth factors (Klein, 1995).

1.2 IMMUNE REGULATION IN MULTIPLE MYELOMA

There is a high incidence of infection-related mortality in patients with myeloma and the pathogenesis of tumour-associated immune deficiency is important. Furthermore, clearly there is a breakdown in the normal regulatory control of the malignant B cell compartment that is a hallmark of disease progression. Attempts by the host immune system to regain control have been suggested by the observed better prognosis

associated with higher serum levels of interleukin-2 (Cimino et al, 1990). None-the-less evidence exists to suggest that phenotypic and functional changes occur in T-cells, B-cells, macrophages and NK/LAK cells (Mellstedt et al, 1982; Lauria et al, 1984; Massaia et al, 1988; Wen et al, 1990). This suggests that any interaction between the host immune system and the MM cells may be flawed at a fundamental level.

Destruction of immune targets requires the activation of efficient, immune-competent T cells. Such an induction is postulated to require a two-signal system for efficient activation (Geppert et al, 1990). Following the interaction of cellular adhesion molecules and their ligands, the initial signal (Signal 1) is generated by T cell recognition of antigenic peptides (Ag) presented with major histocompatibility complex molecules (MHC) via T cell receptors (Bjorkman et al, 1987). Following this Ag/MHC-restricted signal, a second non-MHC-restricted signal (Signal 2) is generated via co-stimulatory pathways, which determines whether the TCR complex engagement results in functional activation or clonal anergy (Geppert et al, 1999). Several co-stimulatory pathways may exist but one important pathway is the interaction of CD80 and CD86 expressed on B cells and antigen presenting cells (APC) with their ligands CD28 and CTLA-4, expressed on T cells (Linsley et al, 1991; Schwartz, 1992). Providing that this second or co-stimulatory signal is received then signalling from the TCR-CD3 complex (particularly via CD3ζ chains) is transmitted from the cell membrane to protein tyrosine kinases (especially $p56^{lck}$ and ZAP70, reviewed by Robey & Allison, 1995). These act on many intra-cellular pathways culminating in the up-regulation of new genes required by T cells to become activated, including proto-oncogenes (*pim-1, c-fos, c-myc*) and cytokines involved in the immune response e.g. IL-2 and its receptor complex (IL-2R$\alpha\beta\gamma_c$), IL-3, IL-4, IL-5, IL-6 (Ullman et al, 1990).

Clearly, tumour cells can interfere with this activation process at any stage either by a single method or an orchestrated manipulation of the recognition and effector mechanisms (Figure 1.2). I will review the current published literature in discussion of how myeloma tumour cells may interact with the host immune system. This forms the rationale behind the experimental studies presented in this thesis.

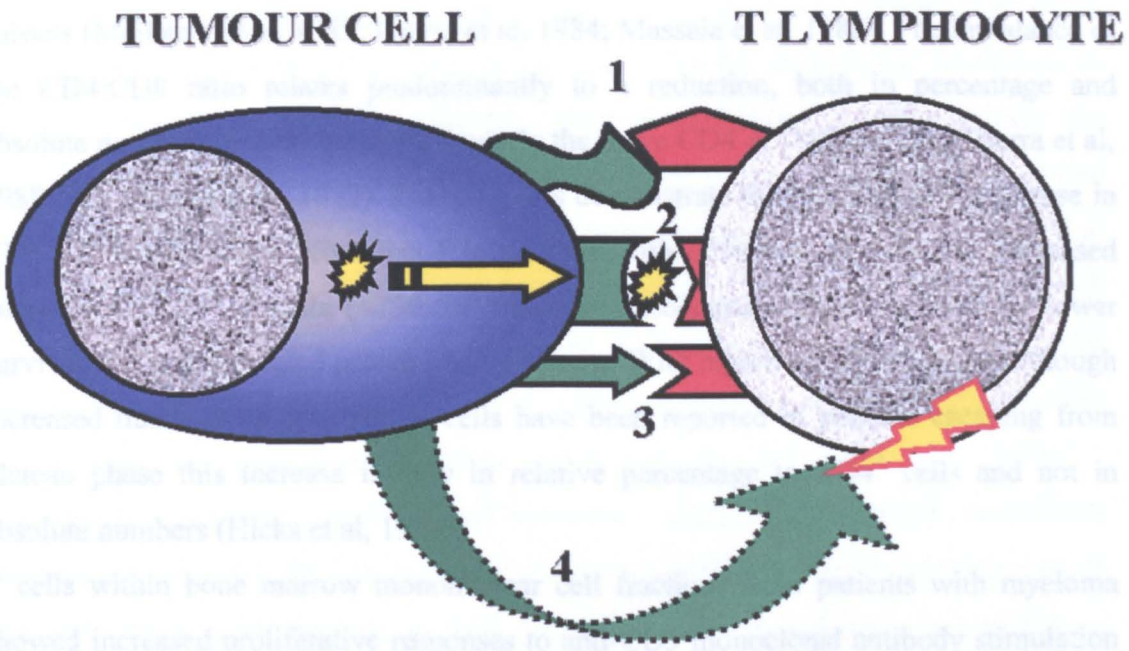


Figure 1.2. Interactions between tumour cells and the host immune system. Potential defects in the recognition of tumour cells as immune targets: **1** - Defects in inter-cellular adhesion. **2** - Lack of MHC molecules, defective antigen processing/presentation, absent/silent antigens (Signal 1). **3** - Absence of co-stimulatory molecules (Signal 2). **4** - Mediation of apoptosis or prevention of activation by soluble factors or cytokines.

EVADING DETECTION

T Lymphocytes - Ineffective Policemen?

Patients with multiple myeloma have altered distribution of CD4 and CD8 lymphoid subsets (Mellstedt et al, 1982; Lauria et al, 1984; Massaia et al, 1988). The imbalance of the CD4:CD8 ratio relates predominantly to a reduction, both in percentage and absolute numbers of $CD4^+$ cells particularly the naive $CD4^+/CD45RA^+$ cells (Serra et al, 1988; San Miguel et al, 1992). These reports demonstrate that the observed decrease in $CD4^+$ T cells is more pronounced in patients with advanced disease and decreased numbers of $CD4^+$ T cells ($<700x10^6$/litre) are associated with a significantly lower survival rate and increased probability of relapse. With regard to $CD8^+$ T cells, although increased numbers of cytotoxic T cells have been reported in patients escaping from plateau phase this increase is only in relative percentage to $CD4^+$ cells and not in absolute numbers (Hicks et al, 1989).

T cells within bone marrow mononuclear cell fractions from patients with myeloma showed increased proliferative responses to anti-CD3 monoclonal antibody stimulation compared with healthy, age-matched controls with demonstrable anti-plasma cell activity. This was thought to imply an immunoregulatory role for such T cells (Massaia et al, 1993). Certainly, peripheral blood lymphocytes from patients with myeloma have been shown to be capable of proliferative responses to autologous tumour cells and with high effector:target cell ratios cytotoxicity could be demonstrated (Mackenzie & Paglieroni, 1977; Paglieroni & MacKenzie, 1979). The idiotypic immunoglobulin may be regarded as a tumour specific antigen and as such, proliferation and differentiation of idiotype-expressing malignant B cells may accordingly be under regulation by idiotypic reactive T cells. In animal plasmacytoma models, T cells can be shown to regulate the growth of the plasma cells by specific recognition of the idiotype antigen (Bergenbrant et al, 1993). Furthermore, evidence of T cell tumour recognition is suggested by the growth of T cell clones by stimulation with IL-2 and $F(ab')_2$ fragments derived from autologous idiotype, and the production of cytokines such as interferon-γ, IL-2 and IL-4 by peripheral blood T cells after stimulation with autologous idiotype (Osterborg et al, 1991).

Several investigators have studied oligoclonal peripheral blood T cell expansions in patients with MM. In one study, using monoclonal antibody immunostaining of TCR β chain usage, 79% of patients demonstrated oligoclonal T cell expansions that were

stable over a median follow-up of 18 months (Raitakari et al, 2000). The TCR β chain usage was random amongst the 21 variable regions studied. When the phenotype of these expanded clones was examined, the T cells were found to be predominantly $CD3^+/CD8^+/CD57^+CD28^-$ cells expressing high levels of perforin but normal levels of both Fas and *bcl-2*. Other groups have demonstrated that the clonal $CD8^+$ T cells do in fact restrict the TCR β chain usage to Vβ3, Vβ5.2 or a combination of both though this was more commonly seen in patients with low tumour burden i.e. after treatment and with early stage disease (Yi et al, 1997B). However, this same group demonstrated that these oligoclonal $CD8^+$ cell expansions did not demonstrate idiotypic reactivity though non-expanded Vβ22 restricted $CD8^+$ cells did. The antigen specificity of the clonal $CD8^+$ cells remain uncharacterised but may represent chronic antigenic stimulation by other tumour associated antigens. It has previously been shown that $CD8^+/CD28^-$ T cells in the peripheral blood of HIV-infected patients and normal individuals preferentially express CD57 and contained active cytolytic T cells which demonstrate expanded clones as determined by TCR Vβ usage and this is thought to result from chronic antigen exposure (Mugnaini et al, 1999).

The presence of these oligoclonal T cells and idiotype-reactive T cells have been identified as an independent prognostic factor (Halapi et al, 1997). The inability to generate "tumour reactive" T cells *in vitro* whilst clinical evidence fails to demonstrate that these cells have an ability to keep the disease under control *in vivo* serves to suggest that tumour cells are often antigenic but not immunogenic. The ability to recognise and eliminate tumour cells may be compromised in cancer patients by many factors including inadequate antigen presentation and tumour-derived immunosuppressive factors. T cells of patients with myeloma have abnormalities in signalling molecules particularly PKC-α and NFκB (Bianchi et al, 1997) and enhanced susceptibility to apoptosis results from increased surface Fas expression and reduced intracellular bcl-2 levels (Massaia et al, 1995). Taken together, these features represent a fundamental defect in the T cells. In order to achieve the maximal effect from immunotherapy strategies, a fuller understanding of the biological interplay between myeloma tumour cells, which presumably cause these defects and immune effectors is mandatory.

Altered Co-stimulation Molecules – The unseen enemy

T cell activation is accomplished with specific antigen presentation (Signal 1)

associated with co-stimulatory (Signal 2) cell-cell interaction (Schwartz, 1989; Geppert et al, 1990). Tumour cells may evade detection by disrupting this process either at the cell-cell interaction, antigen processing/presentation or co-stimulation phases. Myeloma tumour cells express an array of surface adhesion molecules involved in both cell-stroma and cell-cell interaction (Cook et al, 1997). In a study in this lab, a panel of human myeloma cell lines demonstrated surface expression of both HLA-ABC and HLA-DR (Table 1.1) similar to studies of MHC surface expression on freshly isolated cells from patients with multiple myeloma (San Miguel et al, 1991; Yi e tal, 1997). However, significant surface expression of the co-stimulatory molecules CD80 and CD86 was not detected nor was the expression of CD80 and CD86 mRNA transcripts detected by RT-PCR (Table 1.1). These results are similar to those reported by other groups though variable expression has been reported between human myeloma cell lines (HMCL) and fresh tumour cells from patients which might represent the level of maturation or the state of activation (Gray & Woodlief, 1994; Pellat-Deceunynck et al, 1994; San Miguel et al, 1995).

Interestingly, plasma cells from myeloma patients, plasmacytomas and HMCL demonstrate constitutive surface expression of CD28, a molecule found on the majority of CD4 T cells and approximately half of CD8 T cells (Pellat-Deceunynck et al, 1994; Robillard et al, 1998). Myeloma cells express a similar density of CD28 antigen to that of normal T cells and it has been demonstrated to be able to bind B7-Ig chimeric proteins (Zhang et al 1998). Blockade of CD28 signalling failed to alter the proliferation, survival, differentiation or surface expression of antigens and cytokine receptors by myeloma cells. However, the CD28 molecule was demonstrated to be functionally active by binding of the p85 sub-unit of PI-3K to CD28 being triggered by CD80-transfected cells. Whether CD28 has a role in auto-stimulation of the tumour clone or contributes to a down-regulation of the immune control of the malignant plasma cells *in vivo* remains to be elucidated. However, fresh tumour cells from patients with early and plateau disease are $CD28^-$ compared with fresh tumour cells from patients with aggressive disease that were $CD28^+$ (Pellat-Deceunynck et al, 1994). The relevance of these findings *in vivo* and their effect on the host's ability to generate competent immune responses remains unclear.

Can Myeloma Cells Present Antigen?

The myeloma-derived idiotype (Id) is a tumour specific antigen that can be recognised

Cell Line	CD80 mRNA	CD86 mRNA	Surface antigen expression					
			CD80	CD86	CD28	CD40	HLA I	HLA II
JIM-3	Neg	Neg	N	N	++	++	+++	N
JIM-1	Neg	Neg	N	N	+++	++	+++	++
JJN	Neg	Neg	N	N	++	++	+++	+++
U266	Neg	Neg	N	N	+++	++	+++	+++
RPMI-8226	NT	NT	N	N	++	++	+++	+++
EJN	NT	NT	N	N	++	++	+++	++

Table 1.1. Expression of surface molecules involved in providing T cell co-stimulation (Signal 2) by human myeloma cell lines using monoclonal antibody immunostaining and flow cytometry (from Cook et al, 1997). The presence of CD80 and CD86 mRNA as detected by RT-PCR using cell line-derived total RNA. **Key:** N- <20% cells positive, +- 20-40% cells positive, ++- 40-70% cells positive, +++- 75-100% cells positive, NT- not tested.

by antigen-specific T cells in the form of peptides bound to MHC molecules (class I and II). Professional antigen presenting cells such as dendritic cells can efficiently present the Id (Dabadghoa et al, 1997). However, despite the high production of Id by the clonal plasma cells, a clinically significant naturally occurring immune response has rarely been demonstrated. In addition to the variable surface expression of co-stimulatory molecules and the potential for an immunologically hostile microenvironment (see below), MM tumour cells may function as poor antigen presenting cells. Several studies have shown that fresh MM tumour cells can induce a poor allogeneic MLR which was augmented by anti-CD28 MoAb or pre-treating the tumour cells with CD40L or TNFα and interferon-γ (Quing et al, 1997; Schultze, 1997). The T cell proliferative responses arose predominantly from the $CD8^+$ lymphocyte compartment and were considerably reduced compared to standard MLR responses.

The ability of malignant plasma cells to process and present recall antigens (purified protein derivative and tetanus toxoid) to autologous T cells with the generation of proliferative responses and IFN-γ secretion has been demonstrated (Quing et al, 1997). However, when MM cells were used alone to stimulate autologous T cells, no proliferative response directed towards the tumour cells was demonstrated despite the addition of anti-CD28 MoAb (Schultze, 1997). This unresponsiveness was partly reversed when the tumour cells were pre-activated with CD40L then used to stimulate an autologous reaction. These features suggest that myeloma tumour cells have the capacity, if stimulated appropriately, to process and present antigens that can be recognised by T cells, at least *in vitro*. This implies that T cells, especially tumour-specific T cells, may play a role in the *in vivo* tumour regulation of growth and differentiation. However, the fact that the majority of tumour cells are not destroyed in patients with MM suggests that at least *in vivo*, this attempt at regulation may indeed be hampered either by insufficient co-stimulatory molecules *in vivo* or that specific peripheral tolerance has been induced, as has been observed in other human B cell malignancies (Cardoso et al, 1996).

Antigen Silencing

Assuming there is a clonal origin and a series of genetic modifications involved in the development of neoplastic cells it is reasonable to expect that tumour cells express multiple cellular proteins that are distinctive from the tissues from which they are

derived. However, cytotoxic T lymphocytes (CTL) are not active *in vivo* against most of these epitopes despite the association of these antigens with MHC molecules (Chen, 1998). This results in limited T cell responses to a reduced number of antigens out of a larger available pool - a phenomenon known as "immunodominance". Silent tumour antigens are natural peptides presented in association with MHC molecules that are not immunogenic during the clonal expansion of the tumour cells. Despite these antigens failing to be immunogenic *in vivo*, they are fully competent targets for CTL lysis (Koeppeb et al, 1993).

Many factors contribute to antigen silencing including altered TCR ligand peptide affinity, TCR occupancy thresholds, levels of co-stimulatory and adhesive molecules and the status of the T cells (naïve *vs.* memory). When taken together, a "compensation model" of CTL activation is evident: weak antigenic signals may require a larger degree of adhesion/co-stimulation than stronger antigenic signals. This, coupled with a potentially immunologically hostile environment may provide a route for evading immunological surveillance. With respect to multiple myeloma, little if any data exist regarding the existence of silent antigens. If present then these might provide a useful source of antigenic material to stimulate CTL responses *in vitro* particularly if the amino acid structure can be subtly modified to improve their immunogenicity and dendritic cells are employed as professional antigen presenting cells (Lippford et al, 1995; Paglia et al, 1996). This is particularly important as silent antigens work in the induction phase, not in the effector phase of the immune response and thus pre-activated CTLs against these antigens may operate with improved efficiency as has been shown in a murine lymphoma model (Johnston et al, 1996). Further research in myeloma is needed to identify silent antigens, if such exist, which might provide suitable targets for the design of immunotherapy strategies.

ATTACK IS THE BEST FORM OF DEFENCE.

Tumour-derived Cytokines and Immunosuppression

An array of cytokines and immune-modulating agents have been reported by many investigators in the field of tumour immunobiology (Czarniecki et al, 1988). In particular, B cells can produce a large number of cytokines both in disease and health which can direct the immune system into a pro-inflammatory or a humoral response

(reviewed in Pistola, 1997). These include interleukin 10 (IL-10), transforming growth factor beta (TGF-β), Fas and FasL, vascular endothelial growth factors (VEGF), and MUC-1. The effects of the cytokines are summarised in Table 1.2.

I. IL-10

This is a pleiotropic cytokine produced by B cells, monocytes, macrophages and keratinocytes which inhibits synthesis of the pro-inflammatory cytokines IL-1, IL-6, IL-8, IL-12, TNF-α and GM-CSF (Huhn et al, 1996). Originally identified in mouse T helper cells, murine IL-10 was referred to as CSIF (Cytokine Synthesis Inhibitory Factor) and was shown to be a product of Th_2 T helper cells which inhibited cytokine production by Th_1 cells (Fiorentino et al, 1989). Murine and human IL-10 share 73% amino acid homology including hydrophobic leader sequences and although huIL-10 is active on murine T cells, muIL-10 appears not to be significantly active in human cells (Geissler, 1996). It is interesting to note that IL-10 proteins and cDNA reveals a strong predicted amino acid sequence homology (84%) to BCRFI, an as yet uncharacterised open reading frame in the Epstein-Barr viral genome. It has been proposed that that the BCRFI gene represents a cytokine gene that assists the virus to evade or attenuate the host defences (Viera et al, 1991). Multiple roles have been suggested for IL-10 including inhibition of monocytes/macrophages and T cell effector function. However, IL-10's effects are not all inhibitory since it can enhance the viability and proliferation of B cells and differentiation (Huhn et al, 1996). IL-10 is the most potent inducer of immunoglobulin secretion in various B cell activation systems *in vitro* (Rousset et al, 1992).

IL-10 is spontaneously secreted by a variety of human tumours and local secretion by tumour cells can render themselves insensitive to CTL lysis (Becker et al, 1994; Matsuda et al, 1994). In multiple myeloma the role of IL-10 is less clear. Normal B cells differentiating to Ig-secreting B cells lose IL-10 mRNA transcripts, though terminally differentiated plasma cells retain expression of the IL-10R (Mathes et al, 1995). Some investigators have demonstrated that IL-10 is a growth factor for malignant plasmablastic cell lines and that IL-10-dependent MM cell lines can be generated (Lu et al, 1995). This growth promoting activity is independent of IL-6, though these IL-10-dependent MM cell lines demonstrated a plasmablastic morphology

Effect	TGF-β	IL-10	VEGF
Inhibition of T-cell growth	+	-	+
Inhibition of CTL differentiation	+	+/-	+
Inhibition of T cell cytokine production	+	+	-
Induction of T-cell anergy	+	-	-
Shift in the T_{h1}-T_{h2} balance towards Th2	+	+	-
Down-regulation of adhesion/co-stimulatory molecules	+	+	-

Table 1.2. The effects of cytokines on immune effector cells. Key: TGF-β, transforming growth factor beta; T_h, T helper lymphocyte; VEGF, vascular endothelial growth factor.

and low Ig secretion. This suggests that these cell lines were more primitive than the mature malignant plasma cells seen in the disease *in vivo* and the addition of IL-10 to the culture system did not induce terminal maturation of these cell lines. These same investigators, using a sensitive ELISA assay, demonstrated a low level of serum IL-10 in patients with stable disease (3.3%) compared to a higher level of detection in patients with terminal phase disease and plasma cell leukaemia (60%). Similarly, other investigators have demonstrated that serum IL-10 levels are higher in patients with multiple myeloma compared with control patients and that serum IL-10 levels correlate with the paraprotein level (Ameglio et al, 1995). Thus the role of IL-10 in the pathogenesis and immune dysfunction in MM remains uncertain. However, this cytokine may be involved in the later phases of the disease associated with widespread dissemination of tumour cells and deterioration of clinical status.

II. TGF-β

Transforming growth factor-beta (TGF-β) was originally purified from platelets and found to mediate anchorage-independent growth of fibroblasts and play a key role in the growth and differentiation of epithelial cells (reviewed in Letterio & Roberts, 1998). TGF-β is a pleiotropic cytokine that can affect a wide variety of tissues and cells. There are five isoforms of TGF-β (TGF-β1-5) which signal through the same serine-threonine kinase receptors (TGF-βR I and II). TGF-β has effects on all of the mediators of the immune response –T cells, B cells, monocytes/macrophages and dendritic cells. TGF-β is a potent immunosuppressive cytokine and influences all stages of T lymphocyte development and differentiation through to their activation and proliferation, the exact effect being determined by the stage of maturation of the T cell. A subset of T helper cells (Th_3) produce TGF-β (Chen et al, 1994; Bridoux et al, 1997; Letterio & Roberts, 1998) upon activation, in addition to IL-4 and IL-10, and these may represent a sub-group of cells capable of suppressing the inflammatory response to limit tissue damage. Exogenous TGF-β inhibits T cell proliferation by down-regulating IL-2 mediated-signals mediated through reduced tyrosine phosphorylation of proteins of 120, 100, 85, 75 and 50 kDa and inhibition of IL-2 mediated phosphorylation of the retinoblastoma susceptibility gene product, which is pivotal in the progression of cell cycle (Wahl et al, 1989; Fontana et al, 1989; Ahuja et al, 1993; Becker et al, 1994). However, in contrast to these inhibitory functions, a growing body of evidence is being produced to suggest

that TGF-β can also enhance T cell growth, especially those with naïve phenotypes and promote effector function through enhancement of cytokines and inhibition of apoptosis (Kerhl et al, 1986; Cerwenka et al, 1996). TGF-β suppresses normal B cell proliferation and immunoglobulin production, induces apoptosis of pre-B cells mediated through interactions with stromal cells and suppresses haematopoiesis through antagonism of stem cell factor (Heinrich et al, 1995; Letterio & Roberts, 1998). TGF-β has been reported to account for the profound suppressive effects on normal haematopoietic stem cells and B cell function associated with malignancy (Dernynck et al, 1987).

In multiple myeloma, several studies have shown TGF-β mRNA in tumour cells and derived cell lines (Mathes et al, 1993; Klein, 1995; Mathes et al, 1995; Urashima et al, 1996). Some investigators have shown that the level of TGF-β secreted by MM cells is greater than normal B cells and CD40L-activated B cells and that BM stromal cells from MM patients secrete more TGF-β than BM stromal cells from normal control subjects (Urashima et al, 1996). Other groups have demonstrated high serum levels of TGF-β in patients with MM using ELISA assays and there is a suggestion that serum levels correlate with the extent of myeloma bone disease (Hang et al, 1995; Kroning et al, 1997). In contrast to its effects on normal B cells, TGF-β does not decrease the proliferation of MM cells and may even augment IL-6 secretion and related proliferation (Urashima et al, 1996). The exact role of TGF-β in the pathogenesis is unclear, although data is emerging that indicates TGF-β is a major cytokine involved in the maintenance and survival of the malignant clone in myeloma. This may be mediated through autocrine/paracrine IL-6 and other, as yet, poorly understood interactions with the stromal elements in the bone marrow micro-environment. It is also possible that TGF-β contributes to both the identified cellular and humoral immune deficiencies associated with this disease as well as the bone marrow failure associated with high tumour burden (Boccadoro & Pileri, 1995).

III. Fas/Fas ligand

Fas (Apo-1/CD95) is a member of the TNF receptor/nerve growth factor receptor family and it regulates apoptosis through interaction with its ligand, FasL, which is also a member of the TNFR/NGFR family and can act both in the membrane bound and soluble forms (Oehm et al, 1992; Suda et al, 1993; Nagata & Goldstein, 1995). Fas is present on the cell surface of a variety of cells including activated T cells. FasL is

constitutively expressed in sites of immune privilege such as the eye, the spleen and at a low level in the thymus and more recently it has been described on the surface of tumour cells (Tanaka et al, 1996). The recognised role of Fas/FasL in the immune system lies in the process of acquisition of self tolerance through clonal deletion of thymocytes and T cell mediated cell killing as part of the host defence against virally infected or transformed cells (Alderson et al, 1995; Rensing-Ehl et al, 1995). Recently it has been shown that FasL engagement inhibited CD4+ T-cell proliferation, cell-cycle progression and IL-2 secretion *in vitro* and prevented superantigen-mediated CD4+ T-cell expansion in a murine model (Desbarats et al, 1998). Thus with the acquisition by tumour cells of FasL, the active suppression of tumour-specific Fas^+ T cells might be an active mechanism of escape from immune surveillance. However this "counter attack" or immune privilege mediated by Fas/FasL interactions in immune evasion by human cancers is more complicated than first thought and is rapidly becoming an established area of tumour immunology research. Disarming this counter-attack might offer a potential for therapeutic intervention (reviewed in O'Connell et al, 1999).

In myeloma, some investigators have demonstrated the expression of both Fas and its ligand, FasL on the surface of myeloma cell lines (Villunger et al, 1997). The FasL was shown to be functionally active in inducing apoptosis in Fas-sensitive T-cell acute lymphoblastic leukaemia cell line. One question is raised by these and other similar findings: why do the tumour cells which are $Fas^+/FasL^+$ not commit autocrine "suicide" or juxtacrine "fractricide"? It is possible that this does not happen because of an intrinsic resistance to Fas-mediated apoptosis. Many mechanisms have been proposed to account for this e.g. expression of antagonistic soluble Fas, failure to establish death-inducing signalling complexes (DISCs), altered regulation and function of the caspases, the exact pathways involved remain uncertain (Alderson et al, 1995; Nagata, 1996; O'Connell et al, 1999). The understanding of Fas resistance and counterattack is fundamental to designing interventional strategies to disarming this system for effective cancer management and we await the developments in this field with anticipation.

IV. Vascular Endothelial Growth Factors (VEGF)

VEGF is a 34-42 kDa cytokine produced in large amounts by most tumours and previously recognised mainly for its angiogenic properties (Toi et al, 1996). It stimulates the proliferation of endothelial cells and thus has a pivotal role in tumour neovascularisation. More recently it has been shown to have a profound inhibitory

effect on haemopoiesis (Gabrilovish et al, 1996). In particular, VEGF have been shown to responsible for defective dendritic cell (DC) maturation in vivo. VEGF binds to haemopoietic progenitor cells through specific binding to its receptor, Flt-1 which blocks activation of the transcription factor NF-κB (Oyama et al, 1998). NF-κB is composed of 50-65 kDa subunits which bind to a 10 bp motif in the promoter sequence in responsive genes and include p50, p52, p65 (RelA), cRel and RelB. It has been shown that reduced DC generation results from targeted disruption of RelB (Weigh et al, 1995).

VEGF mRNA transcripts have been detected in both fresh MM tumour cells and HMCL (Danbar et al, 1998). Interestingly, the investigators demonstrated that when HMCL were cultured with excess rhIL-6, there was an increase in the level of VEGF mRNA, and when VEGF was added to cultures of human bone marrow stromal cells there was an increase in IL-6 production. Thus MM tumour cells may produce VEGF to stimulate the stromal cells to produce the growth and differentiating agent, IL-6, for the malignant clone and in addition, this cytokine may serve to "disarm" the potent antigen-presenting DC from stimulating a host reaction. Despite this *in vivo* effect on progenitor cells, DCs can be generated from the peripheral blood DC-progenitor cells of patients with MM and that these DCs are both functionally and phenotypically similar to those generated from healthy donors (Tarte et al, 1997). This is an area of the biology of multiple myeloma that warrants further investigative research.

V. Muc-1

Cell surface mucins play a significant role in cell-cell communication (Parry et al, 1990). The muc-1 mucin is a high molecular weight glycoprotein consisting of a core protein with highly branched carbohydrate side chains and is expressed on the apical surface of a number of epithelial cells, including malignant cells (Zotter et al, 1988). In malignant cells, there exists variable glycosylation of the core protein and a high level of expression of this under-glycosylated muc-1 is associated with high metatstatic potential and poor prognostic index in epithelial malignancies (Kobayashi et al, 1992). Muc-1 is a ligand for ICAM-1 and high levels of expression may induce immunosuppression or anergy by interaction with its ligand on T cells (Regimbald et al, 1996). Furthermore, muc-1 expressed and shed by breast cancer cells has been shown to

induce apoptosis in T cells which may be rescued by IL-2 (Gimmi et al, 1996; Agrawal et al, 1998).

Myeloma cells, both fresh cells and HMCLs, express muc-1 which can be up-regulated by dexamethasone (Treon et al, 1999). They can also produce a soluble variant that is capable of suppressing an alloantigen T cell response. It has been shown that HLA-unrestricted CTL can be generated from PBMNC of some MM patients which directly recognise the under-glycosylated form of muc-1 and demonstrated direct cytotoxic capability against muc-1^+ MM and breast cancer cell lines (Noto et al, 1997). However, it is worthy of note that this phenomenon was demonstrated in only 2 of the 6 patients tested which may suggest that muc-1 may limit the generation of adequate populations of immune effector cells. The data currently available on the pathogenic role of muc-1 in MM is limited and the role that muc-1 plays in immuno-suppression remains to be clarified.

1.3 TUMOUR TOLERANCE- The "Danger" of "Self" ignorance.

Illustrated above are many potential mechanisms that may allow evasion of immune regulation of the malignant clone, which either work alone or in orchestration. How then do these facts fit with the theories/paradigms of balancing immunity and tolerance in the human immune system? Whether you are a "Danger theorist" (Matzinger, 1998) or a "self-nonself" proponent (Janeway, 1992), clearly the host's immune system can not or will not identify the tumour as an immunological threat and deal with it appropriately, as it would with infection and parasitism. In the expanded "self-nonself" model of immune responses, it is believed that antigen drives the immune response. As such, immunological tolerance is ensured by eliminating or inhibiting specific reactive lymphocytes and this results from a balance of antigen presentation (Goodnow, 1996). Not all self-reactive lymphocytes are eliminated and many are retained with a wide variety of conditional and potentially flexible restraints, namely reduced or increased antigenic triggering thresholds, inhibition of their immune effector function and constraints of the migratory capacity. The antigen presentation is further tuned by the relative amount and avidity of antigen, the timing of antigenic exposure and the presence of co-stimulation, either by cell-surface moleucles e.g. CD80/CD86 or cytokines e.g. IL-2 (Goodnow, 1996).

Proponents of the "self-nonself" model of immunity and tolerance firmly believe that immune responses are antigen driven and that tumourogenic events are frequent but evolutionary distinct antigens reacting with pattern recognition receptors of the immune cells results in effective eradication i.e. immune surveillance (Matzinger, 1998). The idea of tumour growth therefore results from a break-down in this immune surveillance, either related to antigen delivery (in terms of quantity, avidity, timing, etc). As such, in the context of MM, if the idiotype is a tumour-specific antigen capable of generating immune responses, then there is chronic exposure of the host immune effector cells to high levels of antigen, which may "de-sensitise" or tolerise the immune response. This may be suggested by the lack of immune dysfunction seen in monoclonal gammopathy of undetermined significance (MGUS) where the idiotype concentration is considerably lower compared with MM (Boccadoro & Pileri, 1995). In addition to alterations in tuning of antigen-driven responses, under the auspices of the expanded "self-nonself" model, defects in immune surveillance can also arise from loss of tumour antigen or by suppressing the effector arm of the immune response, mediated by cytokines like IL-10 and TGF-β .

However, "Danger theorists" maintain that the central drive to the immune system, whether in response to tumour, infection or parasitism, results from the appropriate presentation of signal 2 in context of suitable antigens, resulting in an effective immune response. In terms of malignant cell growth, the lack of necrotic cell death within the tumour clone, fails to provide the appropriate signal 2 stimulation and therefore, a lack of immune response (P Matzinger, 2000, personal communication). In MM, as indicated above, there is a lack of cell-surface co-stimulatory molecules on the myeloma cells that prevents satisfactory APC function. Similarly, if the tumour cells secrete VEGF, this limits the differentiation and maturation of professional APCs. However, this does not adequately explain either the tumour-associated immune dysfunction or the significance of tumour cell-derived immune modulating cytokines. In respect to the latter, the production of cytokines such as IL-10 and TGF-β are inherent properties of the cells from which the malignant clone has evolved and are not escape mechanisms adapted by the tumour cells to survive a potentially hostile enviroment (P Matzinger, 2000, personal communication). However, the former aspect, namely the tumour-associated immune dysfunction seen with many malignancies, is not explained under this model. However, if IL-2 is considered as a signal 2 (Goodnow, 1996), TGF-β

can inhibit IL-2–mediated stimulation of T cells and may represent a block to the appropriate delivery of Signal 2 to ensure a useful immune response.

It therefore may not be possible to account for tumour immune tolerance under one paradigm or another, a fact which may have its roots in the diverse interplay of the innate and adaptive immune systems which have evolved in man (Vance, 2000). It is likely that a number of tumour mechanisms are involved in the evasion of immune regulation by myeloma cells. In the field of immunotherapy, the key issue is which of the mechanisms, if any, exerts the major tolerising effect and can this be reversed or even circumvented. Therefore, central to this is a greater understanding of the influence that myeloma cells may exert on immune effectors including at the molecular level.

THESIS AIMS

The aim of the work presented in this thesis is to establish what influence myeloma cells exert on the functional capacity of T cells. In the investigation of this interaction, I will present evidence to support the theory that myeloma cells inhibit the activation of T cells through the production of a soluble immunosuppressive factor, TGF-β1, and that this prevents activation of T cells by interfering with entry into the IL-2 autocrine pathway. The work presented in this thesis may assist in the design of novel treatment strategies using immune effector cells in attempting to eradicate the disease or at least, maintain long-term disease-free survival for these patients.

CHAPTER 2

General Materials and Methods

GENERAL MATERIALS AND METHODS

All solutions used in this thesis are detailed in appendix 1.

2.1 TISSUE CULTURE

All cell culture work was performed under sterile conditions with all manipulation of cell suspensions being conducted under laminar flow. All plastics were sterile. Cell lines were maintained in ventilated tissue culture flasks in a humidified incubator with 5% CO_2. Cell suspensions were replenished with fresh complete medium (CM) every 3-4 days. For human T cell lines, every 10 days non-viable cells were removed by density centrifugation (Ficoll-Hypaque; Lymphoprep, Nycomed, Norway). Cells were maintained at 10^4-10^5 cells/ml by sub-culturing. When HMCLs were used in the experimental studies, they were treated with mitomycin-C to prevent proliferation but to preserve protein synthesis. Cells were pelleted from suspension by centrifugation at 1,500G for 5 minutes. The cells were re-suspended in 50 μg/ml of mitomycin-C (Sigma, UK). The cells were incubated at 37^0C for 30 minutes before being washed x3 in CM.

Peripheral blood mononuclear cells (PBMNC) and peripheral blood lymphocytes (PBL) were isolated fresh from healthy volunteers, after informed consent. Venous blood was collected into preservative-free heparin or sodium citrate and immediately layered onto Ficoll-Hypaque that had been allowed to equilibrate with room temperature. The samples were then separated by centrifugation at 1500G for 30 minutes (no brake). The buffy coat was removed from the packed red cell/plasma interface by pipetting and isolated PBMNC were then washed x3 in PBS with the last 2 washes employing centrifugation at a speed of 900G for 5 minutes to remove contaminating platelets. Cells were then counted and re-suspended at the appropriate concentration in CM. When PBL isolation was required, the above procedure was performed and isolated PBMNC re-suspended in CM were plated in 75cl culture flasks and incubated at 37^0C for 2 hours. After this time period, non-adherent cells were removed and contained >90% lymphocytes, both B and T cells. When PBMNC were used as stimulators in MLR reactions, they were treated with mitomycin-C as above except the mitomycin-C was used at a concentration of 25 μg/ml.

PROLIFERATION ASSAYS

Proliferation of cultured cells was assessed by ^{3}[H] thymidine uptake (reviewed by Hellerstein, 1999). Proliferation assays were cultured in quadruplicate in 96-well microtitre plates. Proliferation was assessed by the addition of 0.5μCi of tritiated thymidine ([^{3}H]-dThd) in the last 18 hours of incubation. Incorporated radiolabel was assessed by harvesting the 96 well microtitre plate onto nitrocellulose filters (Packard Instruments, The Netherlands) using a Micromaster 196 cell harvester (Packard Instruments) and analysed using a Packard Matrix 96 counter.

2.2 FLOW CYTOMETRY

PBMNC from normal healthy donors, T cell lines and human myeloma cell lines were phenotyped by direct immunofluorescence using a FACScan flow cytometer (Becton Dickinson). The methodologies employed were based on those reported described by Shapiro (1988). Cells were analysed by single and double immunofluorescence staining using direct immunoconjugates of either fluorescence isothiocyanate (FITC) or R-Phycoerythrin (PE). Details of the specific monoclonal anti-human antibodies (MoAb) used in FACS analysis are detailed in Table 3.1

Cells to be analysed by immunofluorescence staining were isolated from the specific culture conditions and washed three times in cold PBS. Cells were re-suspended at a concentration of 0.5-1x10^{7}/ml. For single parameter staining 50μl of cell suspension (5x10^{5} cells) was incubated with the appropriate quantity of MoAb at 4^{0}C in the dark for 30 minutes. For background/non-specific antibody uptake an appropriate isotype negative control was added instead of the test MoAb. The samples were then washed once in PBS and re-suspended in 200μl of PBS before analysis. If the cells were not immediately analysed, the cells were re-suspended in Cell Fix (Becton Dickinson), which contained 1% w/v paraformaldehyde, and stored at 4^{0}C in the dark until analysed. Cells for double staining were treated as above except that appropriate quantities of each MoAb were added to the 50μl of cell suspension. For compensation adjustments when calibrating the flow cytometer, samples stained with only one direct immunoconjugate MoAb and the opposite isotype immunoconjugate control MoAb e.g. anti-human CD3-FITC MoAb and PE-conjugated isotype control MoAb were added to the cell suspension.

The flow cytometer (FACScan, Becton Dickinson) is equipped with a single argon laser that emits a 488nm wavelength beam. Cells pass through the laser beam in a single stream as a result of a hydraulic pressure and fluid sheath system. As the cell passes through the laser beam, changes occur in the laser radiation which are measured by two detectors which detect light scatter and three photomultiplier tubes (PMTs) which detect fluorescent signals (Figure 2.1). This detection system measures the cell size (forward scatter; FSC), cytosolic complexity/granularity (side scatter; SSC) and fluorescence (FL1, FL2, FL3). As the cell enters the laser beam, the light is refracted around the cell with the degree of refraction correlating directly with the cell size i.e the greater the laser beam refraction, the larger the cell (FSC). Cytosolic organelles and granules cause the laser beam to be refracted at 90^0 to the incident laser axis (SSC) and the amplitude of the side scatter reflects the degree of cell granularity.

There are at least nine commercially available fluorochromes conjugated to MoAb for flow cytometry analysis. The exhibit variable absorbance and emission spectra (440-750 nm). The two most commonly used fluorochromes are fluorescence isothiocynate (FITC; 389 kDa with an absorbance maximum at 495 nm, emission maximum 520 nm when excited by 488 nm light) and phycoerythrin (R-PE; 240kDa with an absorbance maximum at 564 nm, emission maximum 576 nm when excited by 488 nm light). Detectors were set at appropriate wavelengths to measure the emitted light from the excited fluorochromes (FITC: FL1; R-PE: FL2). Before analysis of each set of samples, the cytometer was first calibrated using negative controls. The mononuclear cell suspension was prepared as described above. Whilst in acquisition mode, FSC vs. SSC (size vs. granularity) dot plots were obtained using linear scales allowing dead cells, residual RBCs and cellular debris to be gated out. The fluorescence emitted from the excited immunoconjugate fluorochromes was measured using a $\log_{10}$ scale with FL1 and FL2 detectors being set typically at 580-620 meV and 500-540 meV, respectively.

When performing simultaneous, multi-colour, immunofluorescence analysis using a flow cytometer, intrinsic spectral overlap of the different fluorochromes used, if uncorrected, will lead to emission of a given fluorochromes into an inappropriate detector. This lack of compensation of spectral overlap can result in misinterpretation of data. However, compensation for this spectral overlap can be achieved by electronic subtraction of the unwanted signal. FITC is detected as a green signal by the FL1 detector when a 488nm wavelength argon laser is used to excited the fluorochromes and R-PE is measured as an orange signal by FL2 detector. The peak emission of FITC is

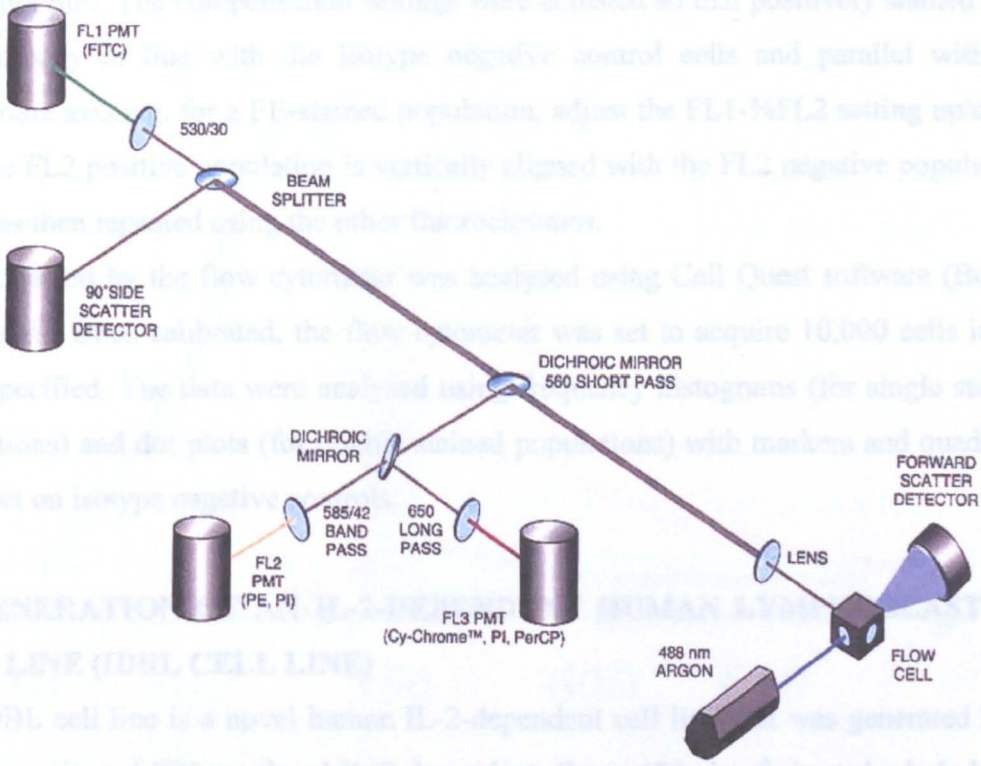


FIGURE 2.1. Diagrammatic representation of a single laser flow cytometer with five parameters of detection (modified from R&D Systems Product catalogue, UK).

530 nm and R-PE is 575 nm and a significant amount of orange fluorescence is present in the FITC emission and some green fluorescence is present in the orange emission (Figure 2.2). Compensation allows the electronic subtraction of these spectral overlaps. Compensation was set using isotype negative immunoconjugate-stained cells to set the background autofluorescence to within the first decade on a log scale. Next cells stained with single immunoconjugates were acquired by the cytometer whilst monitoring a 2-colour dot plot. The compensation settings were adjusted so that positively stained cells were directly in line with the isotype negative control cells and parallel with the appropriate axis e.g. for a PE-stained population, adjust the FL1-%FL2 setting up/down until the FL2 positive population is vertically aligned with the FL2 negative population. This was then repeated using the other fluorochromes.

Data collected by the flow cytometer was analysed using Cell Quest software (Becton Dickinson). Once calibrated, the flow cytometer was set to acquire 10,000 cells in the gates specified. The data were analysed using frequency histograms (for single stained populations) and dot plots (for double stained populations) with markers and quadrants being set on isotype negative controls.

2.3 GENERATION OF AN IL-2-DEPENDENT HUMAN LYMPHOBLASTOID CELL LINE (IDBL CELL LINE)

The IDBL cell line is a novel human IL-2-dependent cell line that was generated from mitogen-activated PBL rendered IL-2 dependent. From 450mls of citrated whole blood (from a donor provided and virally screened by The Scottish National Blood Transfusion Service) PBMNC were prepared using Ficoll-Hypaque and centrifugation at 1500G for 30 minutes. The PBMNC were removed by careful pipetting of the interface layer and washed x3 in PBS, the second and third centrifugation steps were performed at 900G to remove contaminating platelets. The PBMNC were then counted and 2 aliquots of 10^7 cells (to be used as autologous antigen presenting cells/stimulators) were re-suspended in cryopreservation medium and stored in the vapour phase of liquid N_2 until required. The remaining PBMNC ($2x10^8$ cells) were re-suspended at $1x10^6$ cells/ml in CM containing Con A 5μg. The PBMNC were cultured at $37^0C/5\%CO_2$ for 7 days when the medium was replaced with CM and rhIL-2 20U/ml and cultured for a further 7 days. After this period, viable cells were recovered by

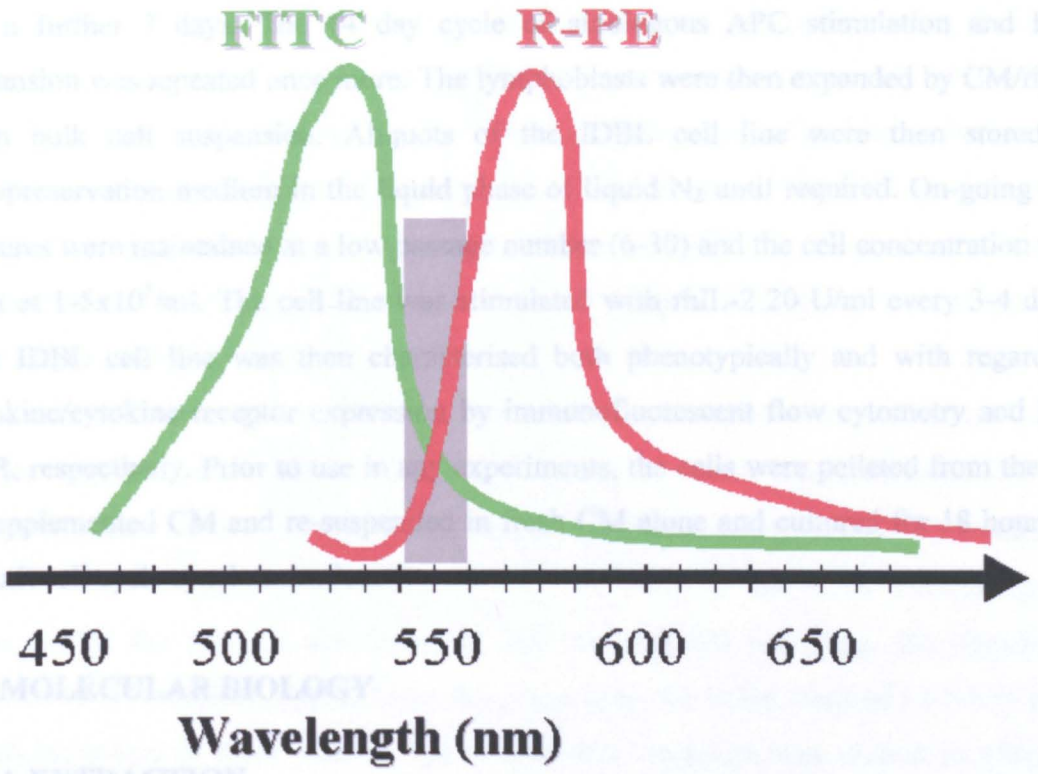


FIGURE 2.2. Emission spectra of FITC and R-PE demonstrating spectral overlap and the need for electronic detector compensation.

density gradient centrifugation. An aliquot of cryopreserved PBMNC, to be used a stimulators, were thawed in a 37^0C water bath and the cells were washed x3 in warm CM. The stimulator cells were then incubated in 25μg/ml mitomycin-C for 30 minutes at 37^0C. The cells were washed x3 in warm CM and re-suspended in CM containing Con A 5μg, and the recovered viable lymphoblasts were added to the stimulators. The IL-2 stimulated lymphoblasts and the autologous APCs were co-cultured with Con A for 7 days when the medium was replaced with CM and rhIL-2 20 U/ml and cultured for a further 7 days. The 14 day cycle of autologous APC stimulation and IL-2 expansion was repeated once more. The lymphoblasts were then expanded by CM/rhIL-2 in bulk cell suspension. Aliquots of the IDBL cell line were then stored in cryopreservation medium in the liquid phase of liquid N_2 until required. On-going cell cultures were maintained at a low passage number (6-30) and the cell concentration was kept at 1-5x10^5/ml. The cell line was stimulated with rhIL-2 20 U/ml every 3-4 days. The IDBL cell line was then characterised both phenotypically and with regard to cytokine/cytokine receptor expression by immunofluorescent flow cytometry and RT-PCR, respectively. Prior to use in any experiments, the cells were pelleted from the IL-2-supplemented CM and re-suspended in fresh CM alone and cultured for 18 hours to permit cell cycle synchronisation.

2.4 MOLECULAR BIOLOGY

RNA EXTRACTION

The reliability of methodologies to detect gene mRNA transcripts are dependent on many factors including the quality and quantity of RNA extracted. Total RNA from bulk cell populations of interest was extracted using the TRIzol reagent (Gibco BRL) for total RNA extraction. For all RNA work, glassware, tubes, aqueous solutions and pipette tips were autoclaved before use. Cell suspensions were pelleted at 10000 rpm in a microfuge and the supernatant decanted. The pellets were re-suspended in TRIzol, 1ml of reagent per 5-10 x10^6 cells and vortexed for 60 seconds. The homogenised samples were incubated on ice for 10 minutes to allow complete dissociation of nucleoproteins and then 0.2 mls of chloroform was added per 1 ml of TRIzol. The tubes were capped and shaken vigorously for 15 seconds and the mixture was then allowed to separate for 5 minutes at room temperature. The samples were centrifuged at 11000 rpm for 5 minutes. Following centrifugation, the mixture separated into a lower red, phenol-

chloroform phase, an interphase of protein and a colourless upper aqueous phase containing nucleic acids. The volume of the aqueous phase was approximately 60% of the volume of TRIzol added and this aqueous phase was carefully removed without disturbing the interphase layer and transferred to a separate eppendorf tube. Isopropanol (0.5 mls per 1ml of TRIzol used) was added to precipitate the RNA. The sample was agitated and incubated at room temperature for 10 minutes. The samples were centrifuged at 11000 rpm for 5 minutes. The precipitated RNA often formed a gel-like pellet and after the supernatant was decanted, this pellet was re-suspended in 1ml of 75% ethanol per 1ml TRIzol used. The samples were mixed completely before being centrifuged at 7500 for 5 minutes. The supernatant was removed and the pellet was then air-dried (for approximately 30 minutes) at room temperature. The RNA was dissolved in 20 µl of RNase-free water and was then heated at 65^0C for 10 minutes to disable secondary structures and the samples were then spun and placed on ice prior to determining the yield and quality of RNA.

The yield and purity of total RNA was assessed by spectrophotometry (Sambrook et al, 1989). An absorbance reading of 1 at 260nm (A_{260}) represents 40µg/ml of total RNA (or single stranded DNA) and the concentration of RNA (µg/ml) can be determined by multiplying the A_{260} value obtained by 40. The purity of the RNA solution can be assessed by the ratio of absorbance at 260 nm and 280 nm (A_{280}- the absorbance maximum for proteins). Typically the A_{260}/A_{280} ratio was in the range of 1.65-1.95 in all RNA isolations in these studies. 5µl of total RNA solution was diluted in 495µl of distilled water for spectrophotometry. The integrity of the total RNA was also examined by agarose gel electrophoresis under denaturing conditions (Sambook et al, 1989). Denaturing gels were prepared using 1.2g of agarose, 10mls 10xMOPS and 85mls of ultrapure H_20 which was then heated in a microwave. Following heating the mixture was allowed to cool to "hand-hot" temperature when 5 mls of 37% formaldehyde solution was added and the mixture was allowed to set in a suitable gel-sized tank. RNA samples, 5-10 µg in 12 µl were mixed with 13µl of Northern Loading buffer and 1µl of 1mg/ml ethidium bromide and heated to 65^0C for 15 minutes to denature. Following denaturing, the samples were pulse-spun in a microfuge and chilled on ice before loading into the wells of the denaturing gel. The gels were run using 1x MOPS buffer at 18-24V over 4-6 hours (until the dye front had traversed 2/3 of the gel). The gel

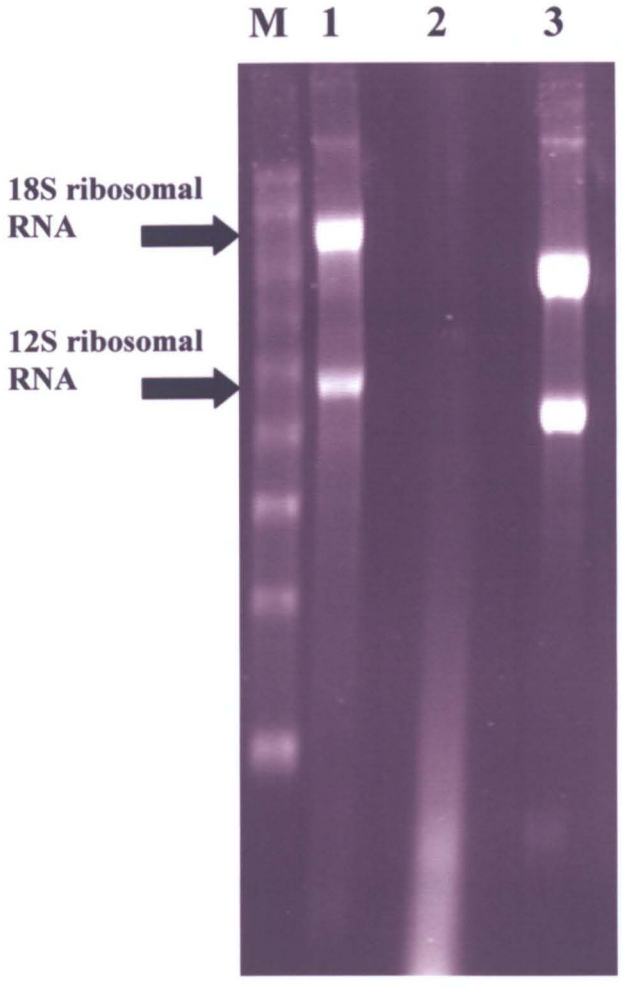


FIGURE 2.3. Verification of total RNA quality by gel electrophoresis under denaturing conditions. Lane 1 represents RNA extracted from PBL stimulated with Con A for 72 hours and lane 2 represents RNA from resting PBL that has degraded. Lane 3 represents RNA extracted from a Chinese hamster ovary cell line indicating species-specific differences in the size of the ribosomal bands.

image was captured using UViphoto software. A representative example demonstrating the 18S and 12S ribosomal RNA bands is illustrated in Figure 2.3 image captured using UViphoto software.

cDNA SYNTHESIS

First strand cDNA synthesis was carried out by reverse transcription using the Superscript system (Life technologies Ltd), following the manufacturer's instructions. 5μg of RNA in 12μl of RNase-free H_2O was decanted into a 200μl PCR tube and 1μl of 500 μg/ml olio $dT_{12\text{-}18}$ primer was added. This reaction mixture was heated to 70^0C for 10 minutes using a PCR Express thermal cycler (Hybaid, UK), pulse spun in a microfuge and chilled on ice. The resulting mixture was then reverse transcribed by the addition of the following:

4 μl x10 1st stage buffer (Gibco, UK)
2 μl 0.1M Dithiothreitol (DTT)(Gibco, UK)
1 μl of 100mM dNTP
1 μl of Superscipt II reverse transcriptase (Gibco, UK)

The mixture was then incubated at 42^0C for 50 minutes in the PCR express machine and the reaction was terminated by denaturing the reverse transcriptase by heating the mixtures to 90^0C for 5 minutes, before being pulse spun and chilled on ice. The cDNA was either used immediately for downstream PCR amplification or stored at -20^0C until required.

PRIMER DESIGN AND VERIFICATION

Almost all the primer pairs used in these studies were either published sequences with ideal reactions conditions or in-house primer pairs with previously identified reaction conditions. However, I wished to amplify a larger IL-2Rα sequence so that I could discriminate the IL-2Rα PCR product from the IL-2Rγ_c product by a significant size variation and as such, needed new sense and anti-sense primers. Similarly, published primer pairs were not available for the human *pim-1* oncogene. Therefore, primer pairs for both these loci had to be designed. Sense and anti-sense primer sequences were

designed using cDNA sequences of the gene of interest on Entrez Browser Database. This revealed the following sequences:

IL-2Rα
5' Primer: 5'-GGGTTGATGTCATGACTGTAG
3' Primer: 5'-GCTGCTGAATCTTCTGACTC
Amplifies a **781 bp** product.

Pim-1
5' Primer: 5'-GTCCAAAATCAACTCGCTT
3' Primer: 5'-CGGCGACAGGCTGTGGA
Amplifies a **918 bp** product.

Using cDNA synthesised from total RNA isolated from mitogen-activated peripheral blood lymphocytes (PBL), the PCR conditions were optimised for both gene primer pairs in terms of the annealing temperatures and $MgCl_2$ concentration. The melting temperatures (Tm) were predicted to range from 55^0C to 65^0C and an annealing temperature of 60^0C was found to be suitable for both gene primer pairs. The optimal $MgCl_2$ concentration was demonstrated to be 2.5µl of 50mM solution per 50 µl reaction (Figure 2.4).

Confirmation of specificity of PCR products was performed using enzyme restriction digest. Amplified products were generated using cDNA from mitogen-activated PBL. The products were visualised by 2% agarose gel electrophoresis and the products were extracted from the gel (see below). Suitable restriction enzymes were selected based on restriction site analysis of the PCR products using Primer Detective software. The *pim-1* PCR product was restriction digested by the enzyme BamH I which cut the product into 3 fragments: 51, 241 and 626 bps in length. Gel extracts were re-suspended in RNAse-free H_2O and the cDNA quantified by spectrophotometry. 2µg of cDNA was placed in a sterile PCR tube with 1µl of BamH I (Gibco), 2µl of x10 Enzyme buffer (Gibco) with sufficient RNAse-free H_2O to make the reaction mixture up to a final volume of 20µl. The reaction mixture was incubated at 37^0C in a water bath for 2 hours then the products of the restriction digest were visualised by electreophoresis on a 2% agarose gel (Figure 2.5). The PCR product for the newly designed IL-2Rα primer pairs were digested using the restriction enzyme Hae III which cut the oligonucleotide into 3 fragments: 112, 173 and 496 bps in length (data not shown).

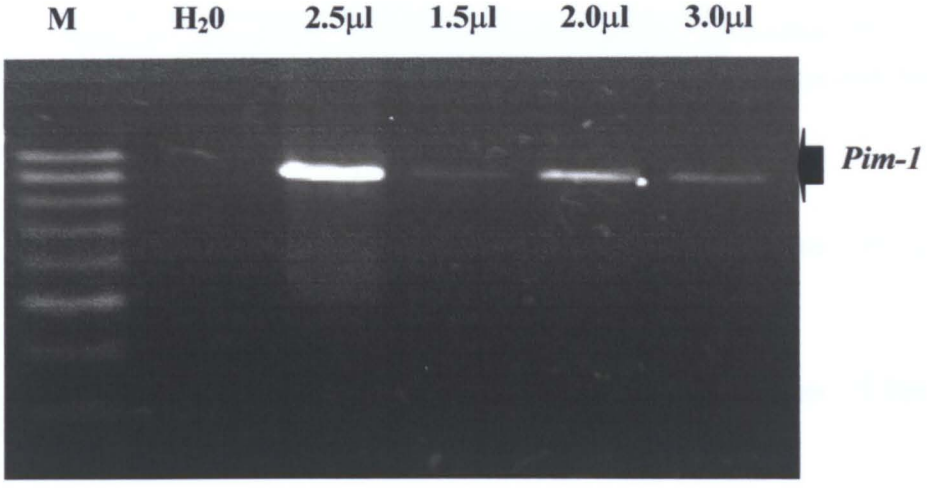


Figure 2.4. The effect of altering the $MgCl_2$ concentration on optimising PCR conditions for the *pim-1* sense and anti-sense primers. **Key:** M-markers, H_2O- PCR negative control, 1.5-3.0µl- volume of 50mM $MgCl_2$ solution per 50µl PCR reaction.

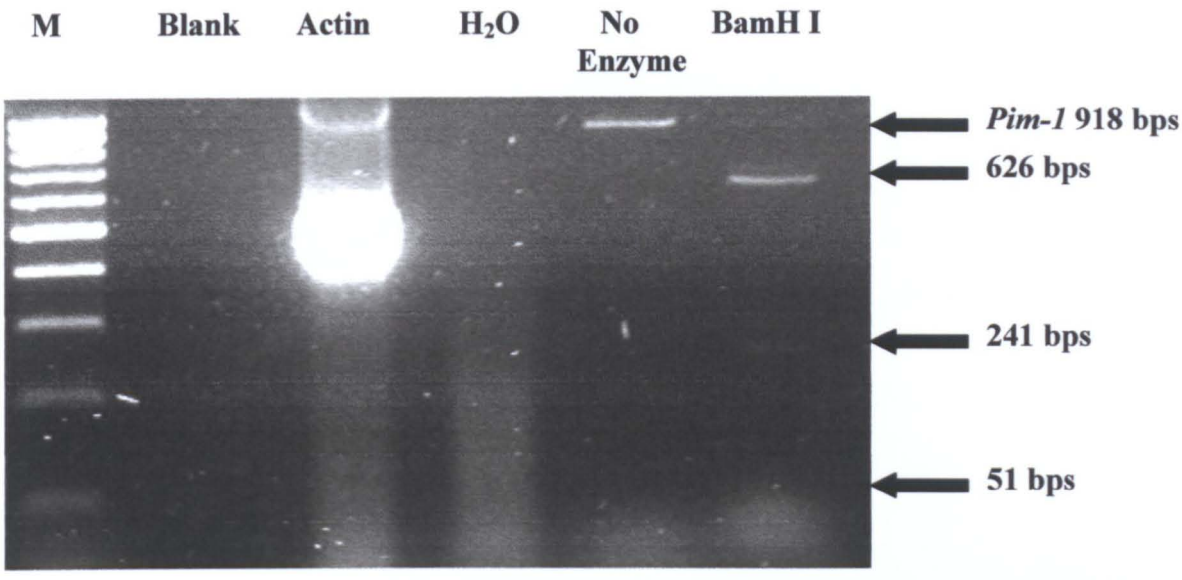


FIGURE 2.5. Restriction digest of gel-purified PCR products derived from *pim-1* sense and anti-sense primers. cDNA derived from activated PBL was amplified using oligonucleotide primers which identified a sequence of the *pim-1* oncogene and after purification, the PCR products were digested by BamH I restriction enzyme resulting in 3 fragments: 51, 241 and 626 bps in length. The 51 bp fragment being small is difficult to identify from the primer dimer band and the 241 bp band is faint. Hae III enzyme alone was used as a control.

PCR AMPLIFICATION AND PRODUCT VISUALIZATION

PCR amplification from cDNA was performed according to the previously published methodology (Innis & Gelfand, 1990). 5μg of mRNA was used in each cDNA synthesis and 2 μl of cDNA reaction mixture (equivalent to 0.5 μg of mRNA) was used in each PCR amplification. Negative control samples were included in which cDNA solution was replaced by RNase-free H_2O. PCR was carried out in 50 μl reaction volumes as follows:

5 μl 10X PCR buffer (Hybaid,UK)
2 μl 50mM $MgCl_2$ (Hybaid,UK)
2 μl cDNA
1 μl 25 μM Sense primer
1 μl 25 μM Anti-sense primer
1 μl 10mM dNTP (Life technologies Ltd)
0.4 μl (2U) AGS Gold Taq polymerase (Hybaid,UK)
37.6μ1 μl dH_2O

Samples were amplified using a PCR Express thermal cycler (Hybaid,UK) and the annealing temperature for the amplification reactions was 60^0C. The reaction mixtures were heated to 95^0C for 5 minutes to dissociate all double stranded species. The amplification consisted of 30 cycles (unless otherwise stated) as follows: 95^0C for 1 minute (denaturation); 60^0C for 1 minute (primer annealing); 72^0C for 1 minute (extension). A final incubation at 72^0C for 5 minutes was carried out to ensure complete extension of the PCR products and following amplification, products were visualised by gel electrophoresis.

The products were analysed by electrophoresis on a 2% agarose gel: 1.4g of electrophoresis grade agarose was dissolved in 70 mls of x1 TBE running buffer by heating in a 650W microwave for 60 seconds. This was allowed to cool to hand-hot temperature and 1 μl of ethidium bromide (10 mg/ml) was added. The gel was poured into the mould of a BioRad Electrophoresis Gel tank (BioRad, UK) and once set, the tank was filled with x1 TBE running buffer until the gel was completely covered. 10 μl of gel loading buffer (Life Technologies) was added to each PCR mixture and 25 μl of PCR mixture/loading buffer were loaded into each well. 10 μl of 1 Kb DNA ladder

(Hybaid), which contained suitably sized fragments to confirm PCR product identification by size. 1 µl of ethidium bromide (10 mg/ml) was added to the TBE running buffer and the gel was electrophoresed at 100V for 1-2 hours and the gels images illuminated by UV light were captured using UViphoto imaging software.

GEL EXTRACTION

The DNA from PCR reactions was purified from amplification reactions visualised on 2% agarose gel, using the QIAquick Gel Extraction Kit (Qiagen,UK) following the manufacturers instructions. The protocol is designed to extract single or double stranded DNA of 100-10,000 bp from TBE agarose gels. Under ultraviolet light, the PCR product of interest was excised from the gel using a sterilised scalpel blade. The gel slice was weighed in a Falcon tube (usually 300 mg ≡ 300 µl of melted gel). To this, 3 volumes (900 µl) of Buffer QX1 was added and incubated at 50^0C for 10 minutes in a water bath. The tubes were mixed gently at regular intervals and once the gel had dissolved, 1 gel volume of isopropanol was added to the mixture and gently agitated. The pH was checked with a pH strip as maximum absorbance of DNA occurs at pH<7.5. If the pH was found to be >7.5, then 10 µl of sodium acetate (pH 5.0) was added and the pH was rechecked. A QIAquick spin column was placed in a 2ml collection tube and the sample was loaded to a maximum of 800 µl/load, with multiple loading required for larger samples. The QIAquick column was then spun at 10,000g for 1 minute and the flow-through was discarded. The column was replaced in the 2 ml collection tube and 500 µl of Buffer QX1 was added and the column was spun at 10,000g for 1 minute. The flow-through was discarded and 750 µl of Buffer PE was added and centrifuged again for 10,000g for 1 minute. The flow-through was discarded and the column spun again at 10,000g for 1 minute, after which the column was placed in a sterile 1.5 ml Eppendorf tube and 50 µl of H_2O was added to the centre of the column. The column was left standing at room temperature for 1 minute before being spun at 10,000g for 1 minute. The purified PCR products were then used in downstream applications.

2.5 STATISTICAL ANALYSIS

Where data represents more than 3 observations, data are presented as a mean± standard error of the mean (SEM). For statistical analysis of the experimental data, Microsoft

Excel Analysis ToolPak software (Microsoft Office2000 Small Business) was used. Direct comparisons of data sets were performed using a two-sample student *t*-test (heteroscedastic *t*-test). When analysis of how changes within one data set compare to changes within another data set, the correlation between the two data sets was calculated and variability between two data sets was analysed by linear regression analysis (the software utilises the "least squares" methodology). The statistical significance of the difference between two data sets is expressed as a probability or *p* value. Statistical significance is attached to differences between data sets if the $p<0.05$, with the greater significance being attached to the lesser *p* value.

CHAPTER 3

Soluble factors from Multiple Myeloma cells inhibit the activation of T-lymphocytes

3.1 INTRODUCTION

Multiple myeloma is a clonal malignant disorder of terminally differentiated B cells which in addition to the main disease characteristics of bone marrow failure, paraproteinaemia and osteolytic bone disease is associated with abnormalities of the immune system, both cellular and humoral (Lauria et al, 1984). A number of phenotypic and functional alterations are representative of a host/tumour interaction in peripheral blood T lymphocytes (PBL) of patients with this disease have been described. Amongst these are the clonal expansion of $CD4^+$ and $CD8^+$ T cells (Janson et al, 1991; Halapi et al, 1997) which produce high levels of IL-2 and Interferon-γ with restricted usage of Vα and Vβ segments (Dianzani et al, 1988; Massaia et al, 1991). These features are suggestive of the potential for T cell responses against myeloma cells as surveillance by the immune system. T cell responses could explain disease stability, even without therapy, seen during plateau phase. Indeed, it has been shown that high serum IL-2 levels in patients with MM correlated with other prognostic indices and were associated with improved survival (Cimino et al, 1990). However, despite these attempts at host immune regulation of the tumour clone, ultimately all patients progress to relapse and this suggests that the T cell responses which develop are unable to keep the disease in check *in vivo*.

Induction of immune responses requires a two signal system for efficient activation (Schwartz, 1989). Following the interaction of cellular adhesion molecules and their ligands, the initial signal is generated by T cell recognition of antigenic peptides (Ag) presented with major histocompatibility complex molecules (MHC) via T cell receptors (Bjorkman et al, 1987). Following this Ag/MHC-restricted signal, a second signal (non-MHC-restricted) is generated via co-stimulatory pathways which determines whether the TCR complex engagement results in functional activation or clonal anergy (Weaver et al, 1990). Of the several co-stimulatory pathways that exist the CD80/CD28 & CD86/CTLA-4 system is thought to particularly important (June et al, 1989). With TCR engagement and secondary co-stimulation, the T cells enter into two consecutive phases of response: activation and proliferation (Crabtree, 1989; Schwartz, 1992).

Tumour cells, in particular myeloma cells, circumvent attempts by the host immune system at regulation of tumour growth and survival either by evading detection or actively suppressing any attempt by the host at immune regulation. In the case of

myeloma tumour cells, there are a number of possible mechanisms which may enable evasion of immune regulation (reviewed Cook & Campbell, 1999). There is a need for a second co-stimulatory signal by the APC to allow T cell activation and prevent anergy. However, myeloma cells, despite expressing an array of adhesion molecules (Cook et al, 1997), generally lack the co-stimulatory molecules CD80 and CD86 on their surface (Schmiltze et al, 1996). It is of interest to note that malignant plasma cells express CD28, the ligand for CD80, and CD86 which is constitutively expressed on the majority of CD4 T cells and approximately half of CD8 T cells, on their surface (Pellat-Deceunynck et al, 1994; Robillard et al, 1998). The surface expression density and intracellular signalling capabilities of CD28 on myeloma cells is similar that of normal T cells, including binding to its ligand, CD80 (Zhang et al, 1998). Blocking experiments have shown that myeloma cells do not require CD28 for growth and differentiation though whether it has a role in auto-stimulation of the tumour clone or contributes to a down-regulation of the immune control of the malignant plasma cells *in vivo* remains to be established.

In addition to evading detection, myeloma cells may influence the function of the immune effector cells directly, suppressing their capabilities to respond to the growing malignant clone. Evidence from patients with this disease indicate that the T cell compartment is compromised in a number of aspects. T cells from patients with MM have increased surface expression of Fas (APO-1/CD95), and lower *Bcl-2* expression and this may imply an increased susceptibility to apoptosis (Massaia et al, 1995). Myeloma tumour cells have been shown to constitutively express FasL on their surface which has been proposed by the authors to be a mechanism by which the tumour cells can disarm the host immune system (Villunger et al, 1997). This "counter attack" or immune privilege mediated by Fas/FasL interactions may not be the sole evasive tactic. The under-glycosylated surface molecule, muc-1, has been shown to efficiently mediate apoptosis of T cells (Agrawal et al, 1998) and myeloma cells have been shown to express muc-1, which can be up-regulated by dexamethasone, and produce a soluble variant which is capable of suppressing an alloantigen T cell response (Treon et al, 1999).

In addition to the cell surface mechanisms indicated, modulation of T cell responses by soluble factors is commonly employed by malignant cells (Kolenko et al, 1997). The production of imunosuppressive cytokines and molecules such IL-10, TGFβ, PGE2,

VEGF, soluble CD27 and soluble muc1 and FasL may also play an important role in the suppression of an efficient host anti-tumour response. Evidence to implicate these soluble cytokines in the evasion of the host immune system is lacking and it is likely that MM tumour cells could adopt various defence mechanisms against rejection, allowing continued growth and dominance in a potentially hostile microenvironment. As yet, there is no clear evidence to indicate what, if any, direct effect the myeloma tumour cells have on the behaviour of T cells, especially in the process of activation.

Experimental Design

The aim of the experiments presented in this chapter is to investigate the effect, if any, that human myeloma cell lines have on the activation process of T lymphocytes from normal donors, stimulated with alloantigen and mitogen. Specifically, do MM tumour cells suppress T cell responses or induce programmed cell death? The experimental data and observations presented in this chapter will form the rationale for the investigations set out in subsequent chapters.

3.2 MATERIALS AND METHODS

All peripheral blood mononuclear cells (PBMNC) used in these experiments were prepared from normal donors as described in chapter 2. The human myeloma cell lines (HMCL) used in these experiments were maintained in complete medium.

3.2.1 MIXED LYMPHOCYTE REACTIONS (MLR).

PBMNC were re-suspended in sterile complete culture medium (CM) at a concentration of $4x10^6$/ml and used as responders in the MLR. 50µl of cell suspension was decanted into each well of a 96 well microtitre plate (Nalge Nunc, Denmark) resulting in a final concentration $1x10^6$/ml ($2x10^5$cells/well). Allogeneic PBMNC were incubated with mitomycin C at a concentration of 25µg/ml (Sigma,UK) at 37^0C for 30 minutes. The cells were washed 3 times in CM and re-suspended at a concentration of $4x10^6$/ml for use as stimulators in one-way MLR. The HMCLs used both as stimulators and to suppress MLR reactions in a 3rd party manner were cultured as described in chapter 2. Cells were incubated with mitomycin C at a concentration of 50µg/ml at 37^0C for 30 minutes. The cells were washed 3 times in CM and re-suspended at a concentration of $4x10^6$/ml. Mitomycin C-treated HMCL and allogeneic PBMNC were used in the experiments in serial dilutions to give PBMNC:stimulator ratios of 1:5 - 1:160. 50µl of the appropriate cell suspension was added to each well, in quadruplicate, of 96 well microtitre plates and CM was added to obtain a final reaction volume of 200µl. Responder and stimulator populations cultured alone served to quantitate the background proliferation. Reactions were cultured for 5 days in 5% CO_2 at 37^0C. Proliferation was assessed by the addition of 0.5µCi of tritiated thymidine ([^{3}H]-dThd) in the last 18 hours of incubation. Incorporated radiolabel was assessed by harvesting the 96 well microtitre plate onto nitrocellulose filters (Packard Instruments, The Netherlands) using a Micromaster 196 cell harvester (Packard Instruments) and analysed using a Packard Matrix 96 counter.

3.2.2 MITOGEN STIMULATION ASSAYS.

PBMNC prepared as described in chapter 2 were re-suspended in sterile complete culture medium (CM) at a concentration of $4x10^6$/ml and 50µl of cell suspension was decanted into each well of a 96 well microtitre plate. Lyophilised Concanavalin A type

V-S (Con A, Sigma UK) was re-suspended in 1 ml of sterile PBS, as per the manufacturers instructions. This was diluted with CM to give a concentration of 200μg/ml, aliquoted in 1 ml quantities and kept at -20^0C until used in stimulation assays. Aliquots were recovered from frozen and diluted 1:20 to a concentration of 10μg/ml with CM. 50 μl of Con A solution was decanted into each reaction well thus with a reaction volume of 200μl, the final Con A concentration was 2.5μg/ml. For experiments using ConA 5μg/ml, aliquots recovered from frozen were diluted 1:10 prior to use. Cultures were incubated for up to 5 days as indicated in the results. Samples for proliferation assays were cultured in quadruplicate in 96 well microtitre plates, and proliferation assessed by the addition of 0.5μCi [^{3}H]dThd (Amersham Pharmacia Biotech, UK) in the last 18 hours of incubation. Incorporated radiolabel was assessed by a Packard Matrix 96 counter. Otherwise, cells were cultured in 24 well plates and harvested as indicated below.

In the stimulation assays where PBMNC were separated from HMCLs by a 0.2μm pore membrane (Life Technologies, Paisley, UK), cultures were initiated in 24 well plates (Nalge Nunc, Denmark) at the same final concentrations of responder PBMNC, HMCLs and ConA indicated in the results. The cells were harvested from the 24 well plates by pipetting and transferred to 96 well plates for the last 6 hours of a 72 hour stimulation assay. 0.5μCi [^{3}H]dThd was added to each well as above and proliferation assessed by incorporated radiolabel using a Packard Matrix 96 counter.

3.2.3 FLOW CYTOMETRY.

Stimulation assays performed to examine surface marker expression, apoptosis and cell cycle were cultured in 24 well (2ml/well) plates at the same final concentrations of responder PBMNC, HMCLs and ConA as per corresponding proliferation assay. Cells were harvested by pipetting and washed x3 in PBS. Cells were re-suspended in an appropriate volume of PBS to give a cell concentration of $1x10^6$/ml and 100μl of cell suspension was decanted into 5ml Falcon BB tubes. The monoclonal antibodies (MoAb) used are listed in Table 3.1. The principles of flow cytometric analysis of cell suspensions including laser alignment, compensation, gating, setting quadrants/markers using isotype negative controls, acquisition and analysis are described in chapter 2. In brief, an appropriate quantity of the directly conjugated test antibody (as per the manufacturer's instructions), which ranged from 5-10μl, was added to the tubes with

Clone	Specificity	Manufacturer	Isotype	Species
S5.2	CD2(FITC)	Becton Dickinson, UK	IgG1	MAH
UCHT 1	CD3(FITC)	PharMingen, Europe	IgG1	MAH
SK3	CD4(PE)	Becton Dickinson, UK	IgG1	MAH
SK1	CD8(PE)	Becton Dickinson, UK	IgG1	MAH
2A3	CD25(FITC)	Becton Dickinson, UK	IgG1	MAH
M-A251	CD25(PE)	PharMingen, Europe	IgG1	MAH
TIC-1	CD122 (PE)	Immunotech, UK	IgG1	MAH
L78	CD69(PE)	Becton Dickinson, UK	IgG1	MAH
FN50	CD69(FITC)	PharMingen, Europe	IgG1	MAH
WT31	TCRαβ(FITC)	Becton Dickinson, UK	IgG1	MAH
MY31	CD56(PE)	Immunotech, UK	IgG1	MAH
L48	CD45RA(FITC)	Becton Dickinson, UK	IgG1	MAH
UCHL1	CD45RO(PE)	PharMingen, Europe	IgG2a	MAH

Table 3.1. Monoclonal antibodies used in cell surface marker identification using flow cytometry. **Key:** FITC- fluorescence isothiocynate, PE- phycoerythrin, MAH- mouse anti-human.

appropriate isotype controls being used as negatives to set gates and markers. For 2-colour flow cytometry, compensation was set using a single positive conjugated test antibody with the opposite conjugated isotype control and the compensation controls set to limit spectral overlap, as described in chapter 2. Data was acquired and analysed using "Cell Quest" software (Becton Dickinson). For blastogenesis studies, the relative number of blasting cells in the different cultures were assessed by gating on the FSC v SSC parameters of the cells. The total lymphocyte population was gated and designated region 1 (R1). Using the resting cells (cultured in CM only), a second region was delineated (R2) within R1, which excluded resting lymphocytes, thereby acquiring blasting cells. Therefore, for quantification of blastogenesis, numbers of cells gated in R2 were expressed as a percentage of the total cell number of R1 (based on 10,000 events measured).

The MoAbs used in these experiments identify T cells in mononuclear preparations (CD3, TCRαβ), T cell subsets (CD4 versus CD8, CD45RA versus CD45RO) and activation markers (CD25 & CD69). CD3 consists of 5 separate non-covalently associated integral membrane proteins (γ,δ,ε,ζ&η) and with the αβ subunits of the T cell receptor (TCR), mediates antigen-specific signaling pathways (Weiss et al, 1986). Antibodies specific for CD3 recognise the ε-component of the TCR/CD3 complex on 100% of primary T lymphocytes in the peripheral blood. Using the MoAb which recognises CD3, after 72 hours of culture in medium alone (resting) 50-60% of PBMNC were $CD3^+$ where as 60-75% were $CD3^+$ after culture with Con A for the same period. The 95^{th} centile range for the percentage of total lymphocytes which are $CD3^+$ is 55-82% (Kotylo et al, 1992). The surface antigens CD4 and CD8, which are co-expressed on thymocytes during development, delineate distinct cellular groups on a functional level (cytokine production and cytotoxicity) and the ratio of these two groups of T cells varies throughout life from a CD4/CD8 ratio of 2.74:1 (whole blood) at birth to 1.4:1 in adult life (Kotylo et al, 1992). The ratio of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells in the studies presented in this thesis are based on PBMNC isolated after density centrifugation and as such, are liable to subtle differences when compared to whole blood. The CD4:CD8 ratio was found to be 1.7:1 when cultured in CM only for 72 hours and 1.2:1 after 72 hours stimulation with Con A.

T cells in humans have been characterized as memory or naïve in phenotype based on their surface expression of differing spliced variants of the surface antigen CD45

(Thomas, 1989). The status of "memory" versus "naïve" is conferred pending the surface staining with antibodies which recognize the CD45RA splice variant which indicates these cells are of a "naïve" phenotype whereas cells which do not stain with anti-CD45RA antibodies but stain with CD45RO antibodies are considered to be of a "memory" phenotype (Sprent & Tough, 1994). This phenotypic division can be seen in both CD4 and CD8 lymphocytes and the ratio of these two "sets" of lymphocytes varies greatly in adult peripheral blood depending on the state of activation of the immune system. Analysis of the PBL in these studies revealed that when cultured in medium alone, 50-64% of $CD3^+$ cells were CD45RA and when cultured in ConA for 72 hours this fell to 35-42% $CD45RA^+/CD3^+$ cells. It is worth noting that fluctuations in absolute values or ratios of T lymphocyte surface marker positive cells may in part be explained by *in vitro* apoptosis/necrosis of other constituent cellular components of PBMNC which alter the balance of cell types analysed after culture.

3.2.4 APOPTOSIS ASSAYS.

Apoptosis is a form of programmed cell death that is characterised by a variety of morphological features, including loss of membrane asymmetry (in particular the distribution of phospholipids between the inner and outer leaflets of the plasma membrane) cell shrinkage, chromatin condensation and chromosomal DNA fragmentation (Castedo et al, 1996). The membrane phospholipid asymmetry is not only true for the choline-containing phospholipids (phosphatidylcholine and sphingomyelin), which are predominantly exterior in location but also the aminophospholipids (phosphadtidylenthanolamin and phosphatidylserine (PS)) which are exclusively found on the internal leaflet of the membrane (Bretscher, 1972). During the early phases of apoptosis, governed by the balance of *bax* and *bcl2* gene families, PS is translocated to the external membrane leaflet, thereby exposing PS to the exterior milieu (Martin et al, 1995). Changes in the plasma membrane phospholipid constitution is recognised as one of the earliest events, where nuclear chromatin condensation is one of the later features of this cellular death pathway.

Based on the physiological events that result in cell death, two assays were adopted to determine if apoptosis is an important feature of T cell/HMCL interactions: DNA fragmentation assay and Annexin-V affinity assay.

DNA Fragmentation Assay.

Culture reactions were carried out in 24 well plates for the time intervals described in the appropriate experiments. The cells were harvested from each reaction well by vigorous pipetting and decanted into autoclaved 1.5 ml tubes (Greiner). The cells were then washed 3 times in cold PBS and then re-suspended in 20μl of DNA Isolate solution (10 mM EDTA, 50 mM TrisHCL (pH 8.0), 0.5% SDS & 0.5 mg/ml proteinase K(Boheringer Manheim, Germany)). The reaction mixture was then incubated for 105 minutes at 50^0C. RNAse (0.5 mg/ml) was heated at 95^0C for 5 minutes and 10μl of heat-treated RNAse solution was added to each reaction mixture and incubated for 60 minutes at 50^0C. A 2% agarose electrophoresis gel was prepared, as described in chapter 2, and the gel was kept dry until samples were loaded. Loading buffer (10 mM EDTA, 1% w/v low melting point agarose, 0.25% w/v Bromophenol blue, 40% w/v sucrose) was heated to 70^0C and 10μl of loading buffer was added to each reaction tube and loaded immediately into the dry wells. Once the loaded samples had set, the running buffer was added and electrophoresis was run over 90 minutes at 75 volts. The gel was then visualised as per chapter 2 and the ethidium-stained DNA was identified. Where apoptosis was present, the fragmented DNA produced a "ladder" effect, resulting from the different sized fragments running at variable speeds. To determine reliability of this assay, IDBL (chapter 2) were cultured in the presence and absence of IL-2 20U/ml for 24 and 48 hours and DNA prepared as outlined.

Annex-V Affinity Assay

As indicated, one of the earliest events in the programmed cell death pathways is the externalisation of the aminophospholipds, in particular phosphatidylserine (PS) (Bretscher, 1972; Martin et al, 1995). Annexin V is a 35-36 kD $Ca2^+$-dependent phospholipid binding protein (Binding Kd of 5 $x10^{-10}$M) which under strict NaCl and Ca^{2+} concentrations can be used as a probe (directly conjugated to the fluorochromes, FITC or PE, PharMingen, UK) to detect PS externalisation early in apoptosis (Engeland et al, 1998). As PS externalisation occurs during necrosis, Annexin V-FITC is used in conjunction with a vital dye, propidium iodide (PI) thus identifying three populations of cells: live cells (An^-/PI^-), early apoptotic cells (An^+/PI^-) and late apoptotic/dead cells (An^+/PI^+). The An^+/PI^+ population contains not only cells that have died from necrosis but also cells in late apoptosis when they have lost their cellular membrane integrity.

For the detection of apoptosis in these experiments using the Annexin V-affinity assay, cells were stained according to the manufacturer's protocol (R&D Systems, UK). Cells were harvested as per the DNA fragmentation assay and washed in cold PBS and re-suspended at a concentration of $1x10^6$/ml in binding buffer (10mM buffered HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5mM $CaCl_2$). 100µl of cell suspension was incubated with 10µl of FITC conjugated Annexin V (AnV) and 10µl of 50µg/ml PI (Sigma,UK) solution for 15 minutes at 20^0C. This was followed by the addition of 400µl of binding buffer without washing. Cells were analysed within an hour by flow cytometry. Using the physical parameters of size (FSC) and granularity/vacuolation (SSC) on a linear dot plot, the PBL were gated and analysed for AnV and PI staining, thereby excluding the HMCL (Figure 3.1). Using unstained cells, a log FL1 and log FL2 dot plot was generated on the PBL gated cells and the detectors were adjusted so that >98% of events were centrally distributed within the lower left quadrant bordered by the first log decade scale on both x and y axis, thus adjusting for auto-fluorescence. The AnV only stained population was run on the log FL1 and log FL2 dot plot and spectral overlap was removed using the compensation controls (FL2-FL1, typically 15-22%). Next the PI only stained cells were run on the log FL1 and log FL2 dot plot and the compensation controls adjusted to remove spectral overlap (FL1-FL2, typically 1-5%). The quadrants were set using a "resting" population of cells stained with both An V and PI. The vertical cursor was set 0.1-0.2 log units beyond the edge of the An V^- population and the horizontal cursor was set 0.1-0.3 log units above the edge of the double negative cluster. The cells of interest were acquired and 10,000 events recorded and analysed using Cellquest software (Becton Dickinson, UK). To determine reliability of this assay, IDBL (chapter 2) were culture in the presence and absence of IL-2 20U/ml for 24 and 48 hours and DNA prepared as outlined.

3.2.5 Cell cycle assays.

Cell proliferation is characterised by a process of DNA synthesis and division. The repetitive growth and division of cells is called cell cycle and can be divided into 5 distinct phases: G_1(growth), S (DNA synthesis), G_2 (growth following DNA synthesis), M (cell division) and G_0 (quiescence). The number of cells in S phase represents the "proliferation potential" of any given population (Shankey et al, 1993). Using PI which stains DNA, a DNA histogram can be generated based on the DNA content of cells at

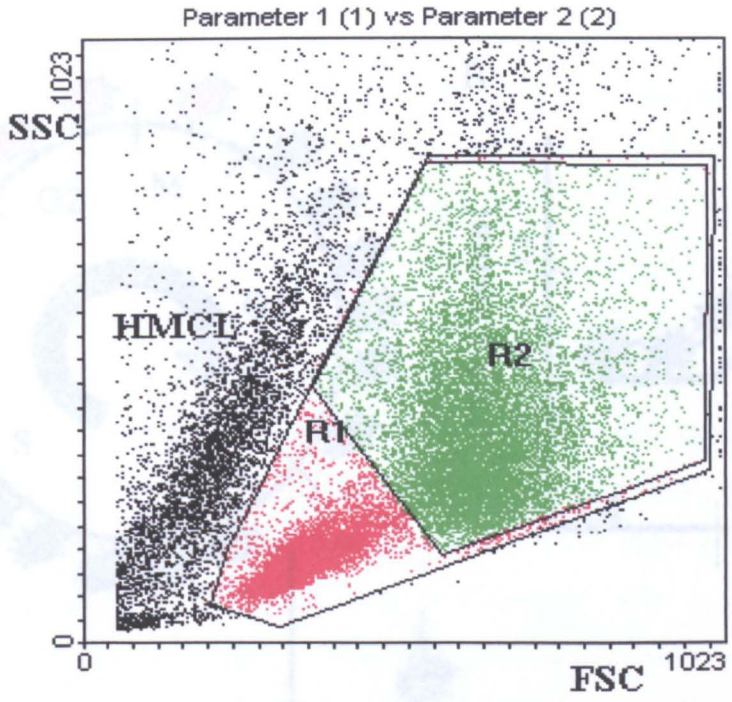


Figure 3.1. R1 was set to capture all PBL with R2 being set to capture the larger, blasting PBL. Using the physical parameters (FSC versus SSC) on a linear scale, PBL (R1+R2) can be differentiated from the HMCL cells and gates set to exclude the latter from analysis. 10,000 events in R1 were acquired for analysis. **Key:** FSC – forward light scatter, SSC – side light scatter.

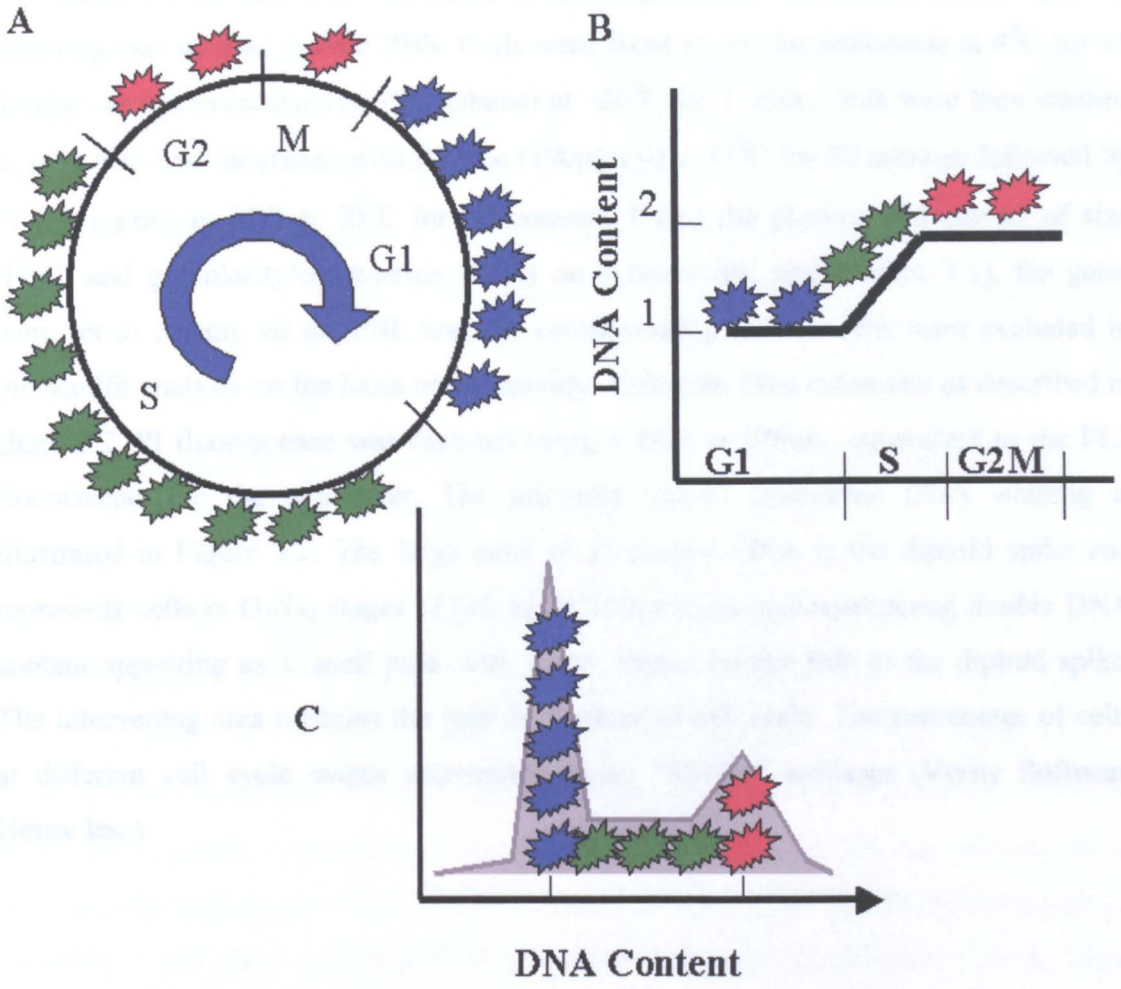


Figure 3.2. A schematic representation of the principle of using cellular DNA content to determine the phase of cell cycle using flow cytometry. The cell cycle (A) is divided in to five phases: G_1, G_2, S, M, and G_0 (not shown). For analysis of DNA content, 3 main areas of interest are generated on a histogram plot: The diploid spike, which represents G_1 (and G_0) cells and the bulk of the cells in any given population which are coloured blue in (A)-(C), the mitotic peak, representing double the DNA content of the diploid spike, coloured in red in (A)-(C) and the cells in the area between representing those actively synthesising DNA prior to proliferation (S phase) coloured green in (A)-(C).

differing stages of cell cycle (Figure 3.2). For cell cycle analysis, cells cultured in 24 well plates for the time intervals stated in each experiment, were harvested by vigorous pipetting and washed in cold PBS. Cells were fixed in 2% formaldehyde at 4^0C for 15 minutes and permeablised in 70% ethanol at -20^0C for 1 hour. Cells were then washed in cold PBS and incubated with RNAse (100μg/ml) at 37^0C for 30 minutes followed by PI (50 μg/ml) in PBS at 20^0C for 30 minutes. Using the physical parameters of size (FSC) and granularity/vacuolation (SSC) on a linear dot plot (Figure 3.1), the gates were set to capture all the PBL and any contaminating tumour cells were excluded in the Modfit analysis on the basis of aneuploidy. Using the flow cytometer as described in chapter 2, PI fluorescence was detected using a filter at 570nm, equivalent to the FL2 fluorescence on the cytometer. The principle behind cytometric DNA staining is illustrated in Figure 3.2. The large peak of PI-stained DNA is the diploid spike and represents cells in G_1/G_0 stages of cell cycle with mitotic cells containing double DNA content appearing as a small peak with x-axis values double that of the diploid spike. The intervening area contains the cells in S phase of cell cycle. The percentage of cells at different cell cycle stages determined using "Modfit" software (Verity Software House Inc.).

3.3 RESULTS

3.3.1 MIXED LYMPHOCYTE REACTIONS

The mixed lymphocyte reaction (MLR) is the proliferative response generated by lymphocytes, which encounter foreign major histocompatibility antigens presented by allogeneic lymphocytes, resulting in the expansion of alloreactive T cell clones. Such reactions employ stimulators that have been rendered incapable of proliferating by pre-treating them with γ-irradiation or mitomycin-C (Chanarin, 1989). The ability of human Myeloma cell lines (HMCL) to stimulate a lymphocyte proliferative response was examined using the MLR.

PBMNC were separated from the peripheral blood of normal, healthy volunteers (n=3), as described in chapter 2, and were co-cultured with mitomycin C-treated allogeneic PBMNC. This resulted in a brisk proliferative response at day 5 of culture, as determined by ^{3}H-Thymidine up-take (Figure 3.3). When the same PBMNC responders were co-cultured with 2 HMCLs (U266 and JIM1) as stimulators, no proliferative responses were detected after 5 days of culture (Figure 3.3). This was tested further when normal, allogeneic PBMNC (n=5) were co-cultured with the HMCL U266, in increasing responder:stimulator ratios, proliferative responses in a 5 day one-way MLR were greatly suppressed. These PBMNC demonstrated normal proliferative responses (54,600$\pm$11,164 cpm – mean $\pm$SEM) when cultured with the mitogen, Con A, alone demonstrating satisfactory functional activity (Figure 3.4).

To examine whether this poor stimulatory function of HMCLs in a one-way MLR was the result of active immunosuppression rather than defective antigenicity, MLR reactions using normal allogeneic PBMNC as responders to mitomycin C-treated allogeneic PBMNC, were set up and HMCL were co-cultured in these reactions in reducing concentrations. The addition of the HMCLs in this 3rd party co-culture reaction greatly reduced the normal MLR proliferative responses suppressed (100% inhibition of response at highest HMCL concentration, 1HMCL:5PBMNC), as determined by the ^{3}H-Thymidine up-take. This suppressive capability of the HMCLs demonstrated a dose-dependent relationship, with full MLR responses only being regained at the lowest HMCL concentrations (1 HMCL: 80 PBMNC), as illustrated in Figure 3.5.

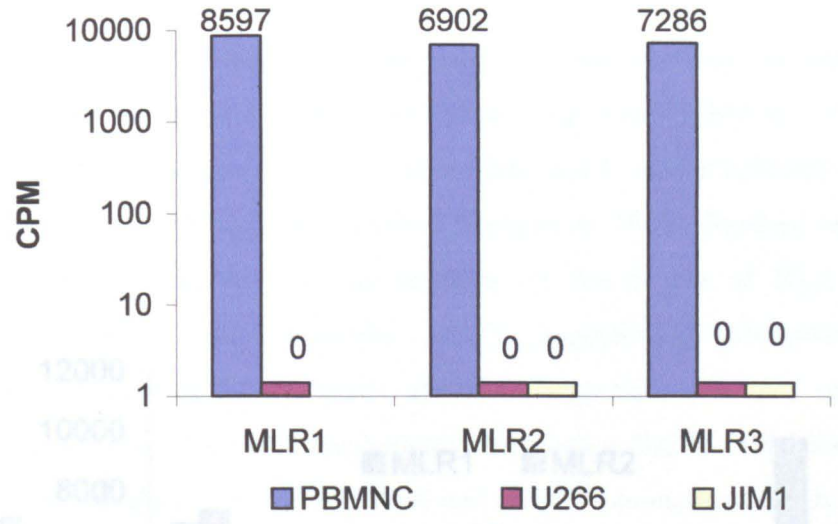


Figure 3.3. Net proliferation (counts.min^{-1}) from 3 normal responder PBMNC in one way mixed lymphocyte reactions using mitomycin C treated allogeneic PBMNC (MLR1-3) or 2 HMCLs (U266 and JIM1) as stimulators in a 5 day culture. Results represent ^{3}H-Thymidine uptake, expressed as counts per minute (CPM).

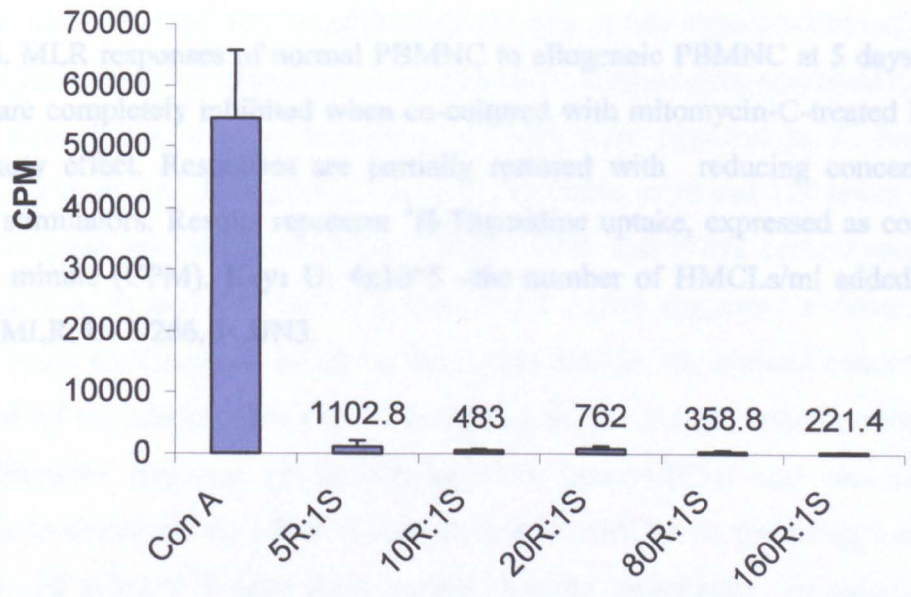


Figure 3.4. MLR responses of normal, allogeneic PBMNC (n=5) to the mitomycin C-treated U266 cells at 5 days. The responsiveness of these normal allogeneic PBMNC to mitogenic stimulation alone (Con A) was satisfactory. Results represent ^{3}H-Thymidine uptake, expressed as mean ±SEM counts per minute (CPM). **Key:** Con A: concanavalin A, 5R:1S : number of responder PBMNC to stimulator HMCL (U266).

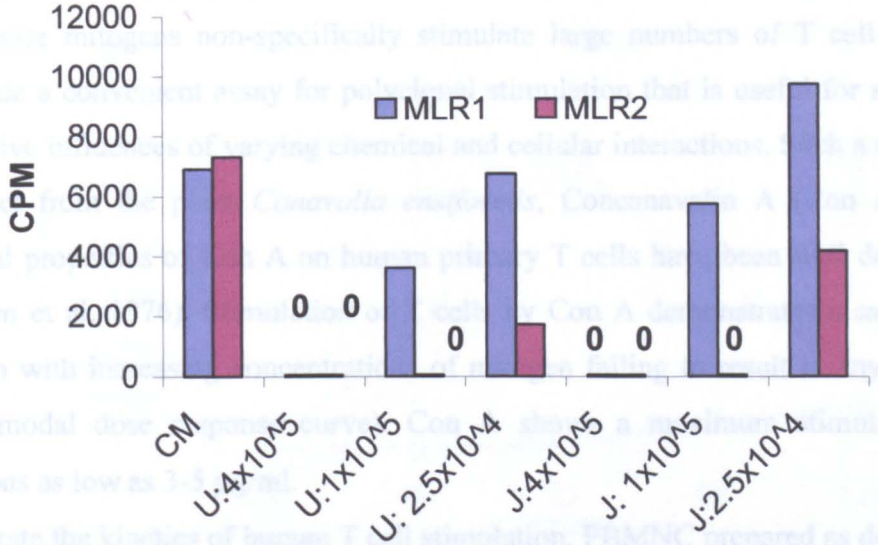


Figure 3.5. MLR responses of normal PBMNC to allogeneic PBMNC at 5 days. MLR responses are completely inhibited when co-cultured with mitomycin-C-treated HMCL in a 3rd party effect. Responses are partially restored with reducing concentration HMCL as stimulators. Results represent ^{3}H-Thymidine uptake, expressed as corrected counts per minute (CPM). **Key:** U: 4x10^5 –the number of HMCLs/ml added to the allogeneic MLR, U- U266, J- JJN3.

3.3.2 MITOGEN STIMULATION

MLR reactions vary in their magnitude depending on the frequency of allo-reactive precursors, which in the normal healthy individual range from 1:544 to 1:47,619 for cytotoxic T lymphocyte precursor (CTLp) frequencies and 1:385 to 1:50,000 for helper T lymphocyte precursors (HTLp) frequencies (Wang et al, 1998). Further, variation in the strength of an alloreactive response depends on the degree of HLA disparity between the stimulator and responder MNC populations. Alternatively, the macromolecular mitogens non-specifically stimulate large numbers of T cell clones. These provide a convenient assay for polyclonal stimulation that is useful for studying the suppressive influences of varying chemical and cellular interactions. Such a mitogen is the extract from the plant *Canavalia ensiformis*, Concanavalin A (Con A). The physiological properties of Con A on human primary T cells have been well described (Cunningham et al, 1976). Stimulation of T cells by Con A demonstrates a saturation phenomenon with increasing concentrations of mitogen failing to result in any further effects (unimodal dose response curve). Con A shows a maximum stimulation at concentrations as low as 3-5 μg/ml.

To demonstrate the kinetics of human T cell stimulation, PBMNC prepared as described in chapter 2, were cultured with complete medium only or two concentrations of Con A (2.5 and 5μg/ml , final concentration) and proliferation kinetics were examined daily for 5 days of culture. Proliferative responses to ConA were maximal at 72 hours, as illustrated in Figure 3.6. Proliferation was still detectable at 96 and 120 hours though demonstrated great inter-donor variability (SEM– 7946 and 17892 cpm, respectively. The responses at 96 and 120 hours to Con A 5.0 μg/ml suggested a decay in the mitogen-induced proliferation. Based on these experiments, the optimal concentration and duration of stimulation with Con A was 2.5μg/ml for 3 days, which resulted in a mean proliferative response of 58,792cpm±4123 (mean±SEM) and was used in experiments to determine the effect of co-culture with HMCLs on the mitogen-induced proliferation of primary T cells form normal, healthy individuals. To calculate the percentage inhibition of proliferative responses by the HMCLs, the following formula was used:

$$\textbf{\%Inhibition} = \frac{\text{(Mean net cpm with ConA) - (Mean net cpm with ConA and HMCL)}}{\text{(Mean net cpm with ConA)}} \times 100(\%)$$

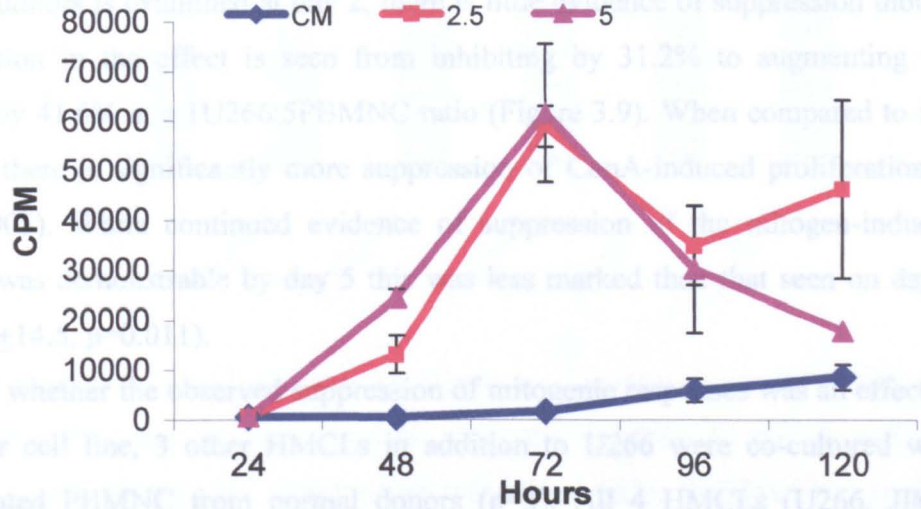


Figure 3.6. The proliferation of normal PBMNC (n=5) to the mitogen ConA assessed ^{3}H-Thymidine uptake at the time points indicated. ConA-induced proliferation peaks at 72 hours with both 2.5 μg/ml and 5.0 μg/ml. **Key:** CM: complete culture medium, 2.5: ConA 2.5 μg/ml, 5: ConA 5.0 μg/ml.

When PBMNC from normal donors (n=6) were activated with Con A for 72 hours, in the presence of the HMCL U266, a strong suppression of the proliferative response was seen compared to stimulation with Con A alone (p=0.02, Figure 3.7). Co-culture of Con A-activated PBMNC with U266 HMCL demonstrated a mean inhibition of 60%±5.9 at the highest HMCL concentration and this suppression was demonstrable even at the lowest concentrations with a mean inhibition of 32%±12.1 at 1U266:160PBMNC (Figure 3.8). When the effect of co-culture of HMCL with Con A-activated PBMNC from normal donors is examined at day 2, there is little evidence of suppression though a wide variation in the effect is seen from inhibiting by 31.2% to augmenting the proliferation by 41.6% at a 1U266:5PBMNC ratio (Figure 3.9). When compared to day 2 of culture, there is significantly more suppression of ConA-induced proliferation at day 3 ($p<0.005$). While continued evidence of suppression of the mitogen-induced proliferation was demonstrable by day 5 this was less marked than that seen on day 3 (mean 37.7%±14.5, p=0.011).

To determine whether the observed suppression of mitogenic responses was an effect of this particular cell line, 3 other HMCLs in addition to U266 were co-cultured with ConA-stimulated PBMNC from normal donors (n=3). All 4 HMCLs (U266, JIM1, JJN3, RPMI8226) demonstrated suppression of Con A-induced proliferation of normal PBMNC with a mean inhibition of 71.9%±4.6 by U266, 93.3%±3.3 by JIM1, 68.4%±11.8 by JJN3 and 78.2%±19.5 by RPMI8226 at a responder: stimulator ratio of 5:1 (Figure 3.10).

The inhibition of mitogen-induced proliferation was seen when HMCLs were co-cultured with the normal PBMNC. To examine whether the suppression resulted from direct cell-cell contact or from the production of a soluble factor or factors, the mitogen-activation experiments were repeated with the HMCLs being physically separated from the PBMNC (n=3) by 0.2μm pore membranes during stimulation. This experiment demonstrated that the suppression of proliferation induced by HMCLs is not reversed by physical separation (Figure 3.11). The mean percentage inhibition of proliferation with contact was 30.0% for U266 and 28.8% for JJN3. When the HMCLs and PBMNC were separated by the 0.2μm pore membranes, there was evidence of further inhibition of ConA-induced proliferation, as demonstrated by the mean percentage inhibition of proliferation of 53.8% for U266 and 71.8% for JJN3. This is represented in Figure 3.11

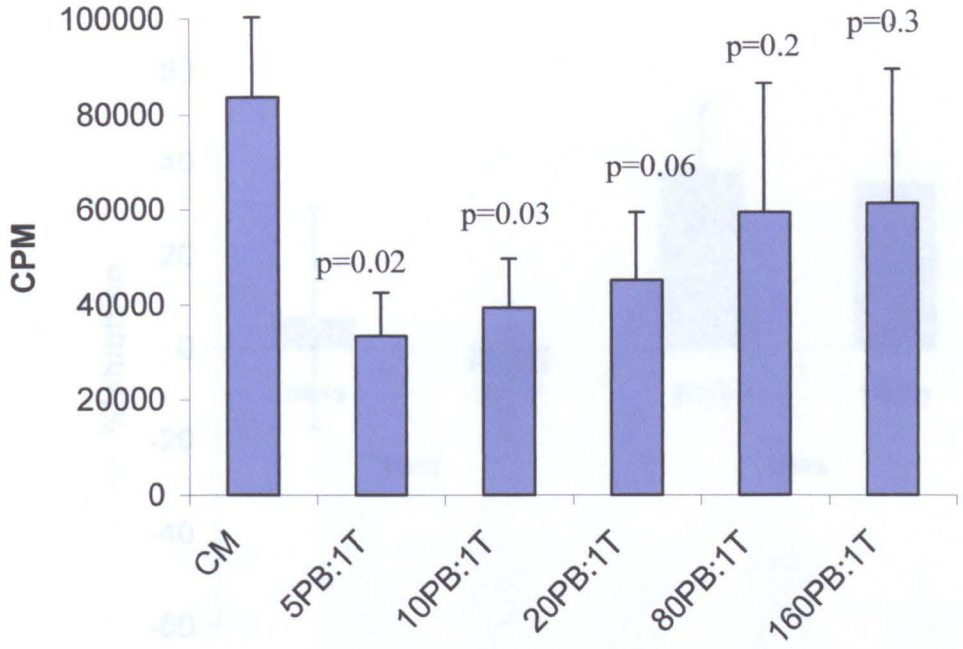


Figure 3.7. Inhibition of ConA–induced proliferation of normal donor PBMNC (n=6, p=0.059) after 72 hours by co-culturing with the HMCL, U266 at different concentrations (5PB:1T, p=0.02). Proliferation was measured by ^{3}H-Thymidine uptake, **Key:** CA- ConA only, 5PB:1T - number of PBMNC to HMCL (U266).

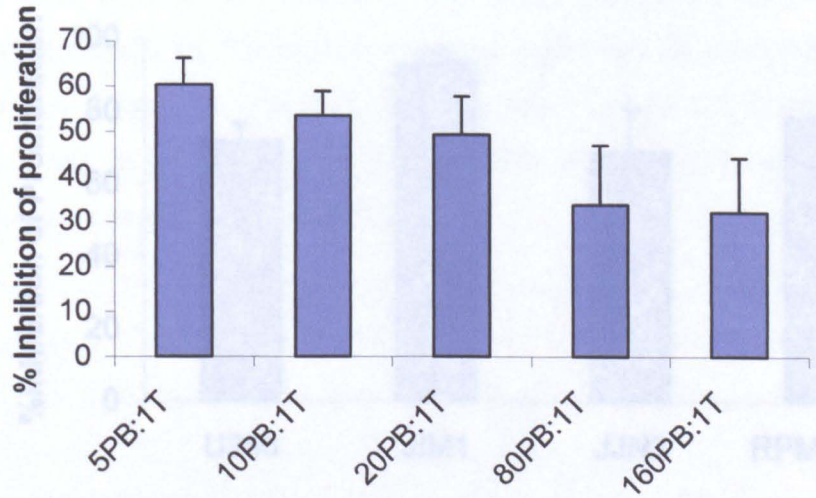


Figure 3.8. Inhibition of ConA induced proliferation in normal PBMNC at 72 hours (n=6) by the HMCL U266, expressed as percentage inhibition. (Key as per Figure 3.7 legend).

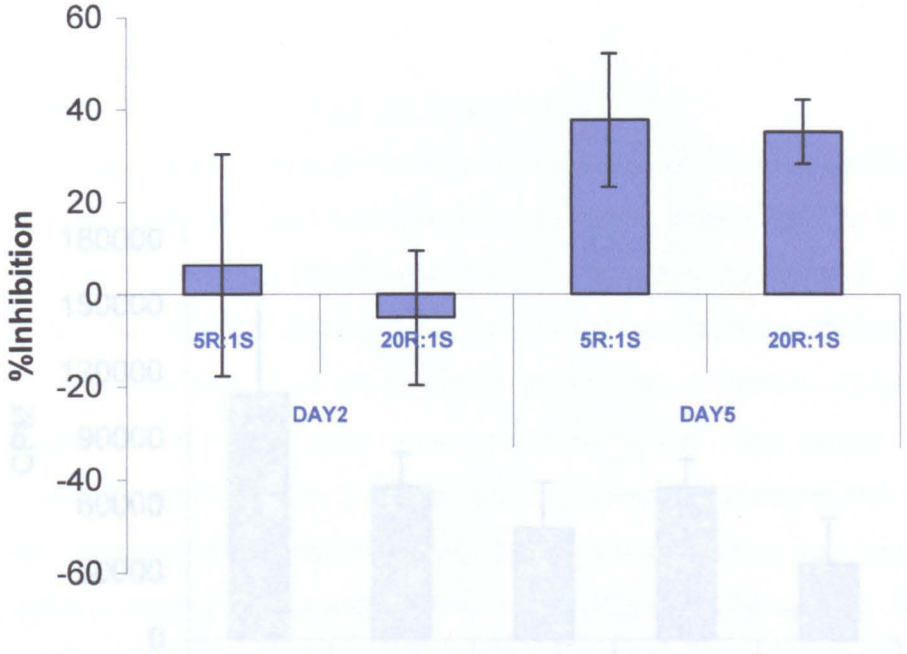


Figure 3.9. Inhibition of ConA-induced proliferation by U266 HMCL at 48 and 120 hours (Day 2 vs Day 5 p=0.011) stimulation, as measured by ^{3}H-Thymidine uptake. **Key:** 5R:1S - number of responder PBMNC to stimulator HMCL (U266).

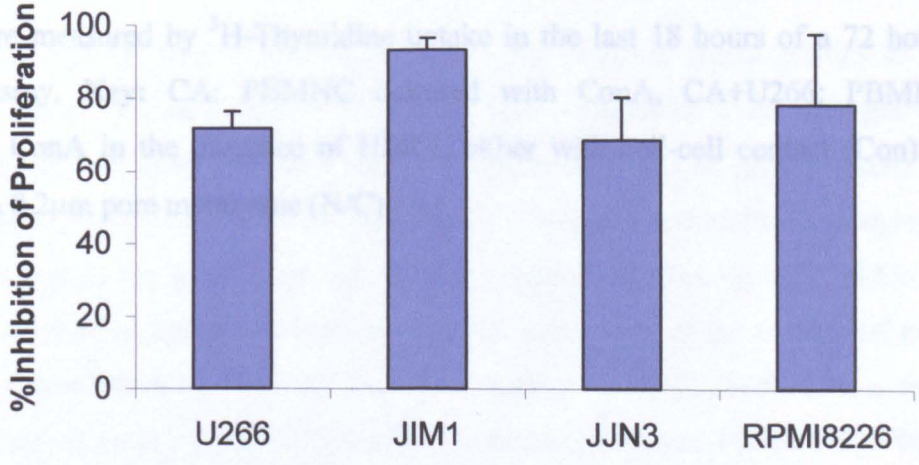


Figure 3.10. Inhibition of ConA-induced proliferation of normal PBMNC from 3 different donors by 4 HMCLs, U266, JIM1, JJN3 and RPMI8226 (5PBMNC:1HMCL), at 72 hours as measured by ^{3}H-Thymidine uptake.

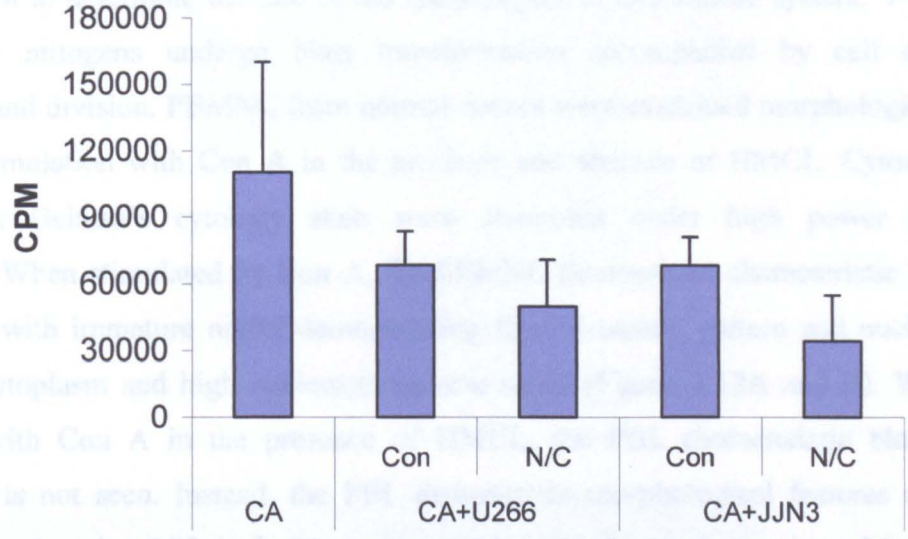


Figure 3.11. The suppression of ConA-induced proliferation of PBMNC from normal donors (n=3) by HMCLs is not reversed by preventing physical contact. Proliferative responses were measured by ^{3}H-Thymidine uptake in the last 18 hours of a 72 hours stimulation assay. **Key:** CA: PBMNC cultured with ConA, CA+U266: PBMNC cultured with ConA in the presence of HMCL either with cell-cell contact (Con) or separated by a 0.2μm pore membrane (N/C).

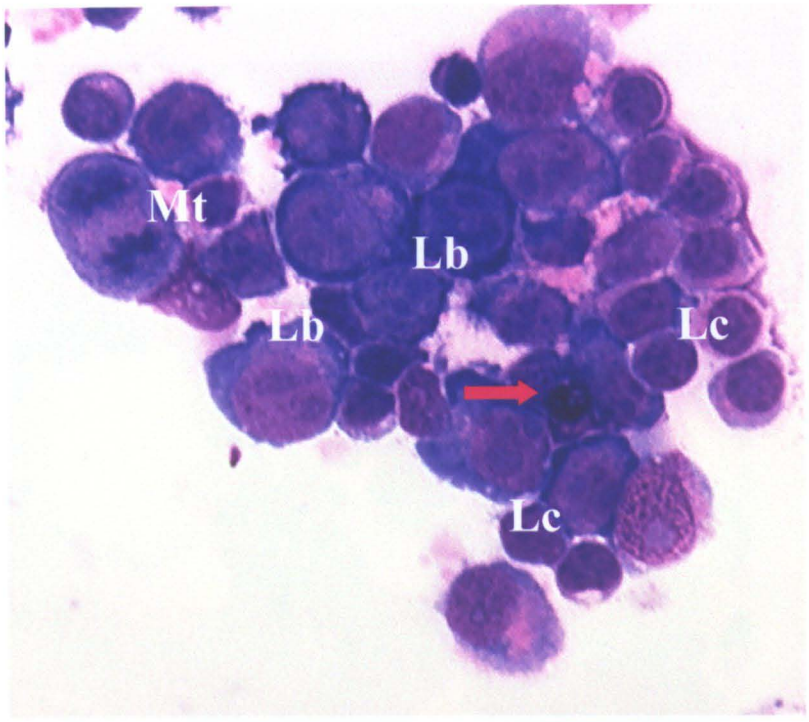
as absolute net counts per minute. This indicates that the inhibition of Con A induced proliferation is mediated by soluble factor (s).

3.3.3 BLASTOGENESIS AND T CELL ACTIVATION

To investigate the suppression of mitogen-induced proliferation of PBMNC by HMCL, it is important to determine the fate of the lymphocytes in this culture system. T cells activated by mitogens undergo blast transformation accompanied by cell cycle progression and division. PBMNC from normal donors were examined morphologically following stimulation with Con A in the presence and absence of HMCL. Cytospins stained with Geimsa's cytology stain were examined under high power light microscopy. When stimulated by Con A, the PBMNC demonstrate characteristic blast morphology with immature nuclei demonstrating fine chromatin pattern and nucleoli, basophilic cytoplasm and high nucleus:cytoplasm ratios (Figure 3.12A and B). When stimulated with Con A in the presence of HMCL, the PBL characteristic blasting morphology is not seen. Instead, the PBL demonstrate morphological features more characteristic of resting PBL including mature nuclei with dense chromatin and lack of cytoplasmic basophilia; the HMCL are easily identified by their large diameter, immature nuclei, basophilic cytoplasm and prominent Golgi apparatus (Figure 3.13A and B).

PBL were analysed by flow cytometry to detect the physical characteristics of the PBMNC stimulated by Con A using FSC versus SCC parameters. Cells were gated at acquisition to exclude dead cells and the HMCLs on the basis of physical size and cellular granularity/vacuolation. As indicated in the materials and methods (chapter 2) 10,000 events within the gates were acquired and number of blasting cells within the cell population could be quantified and expressed as percentage of the number of gated cells. Con A stimulation of PBMNC from normal donors (n=7) resulted in a mean number of blasting cells of 56.5%$\pm$5.8 after 72 hours of culture (Table 3.2). When PBMNC were stimulated with ConA in the presence of the HMCLs, U266 and JJN3 (1 HMCL:5PBMNC, final concentration), the number of blasting cells were significantly reduced to a mean of 28.2%$\pm$5.1% ($p<0.005$, n=7) and 20.4%$\pm$2.3% ($p<0.002$, n=6), respectively. A representative example of the suppression of blastogenesis by HMCL, as detected by flow cytometry, is illustrated in Figure 3.14 (A and B) with the summarised results of all normal donors tested (C).

3.12 A



3.12 B

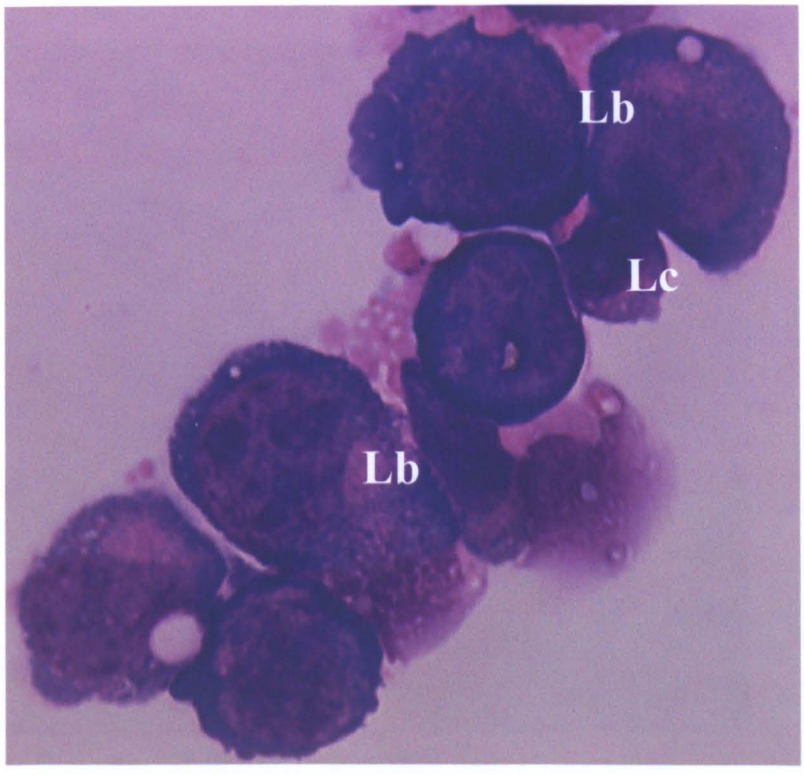
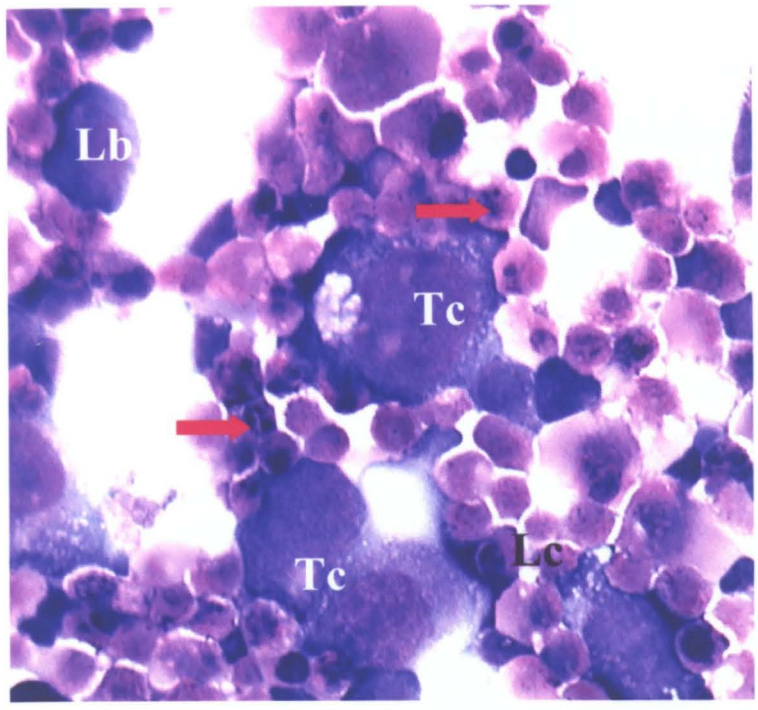


Figure 3.12 Cytospins of PBMNC activated with ConA in the absence of HMCL (A, X400; B, X1000, Geimsa). Lymphocytes (Lc) are seen with mitogen-activated lymphoblast (Lb). Apoptotic cells (arrow) are occasionally seen and mitotic figures (Mt) are frequent.

3.13 A



3.13 B

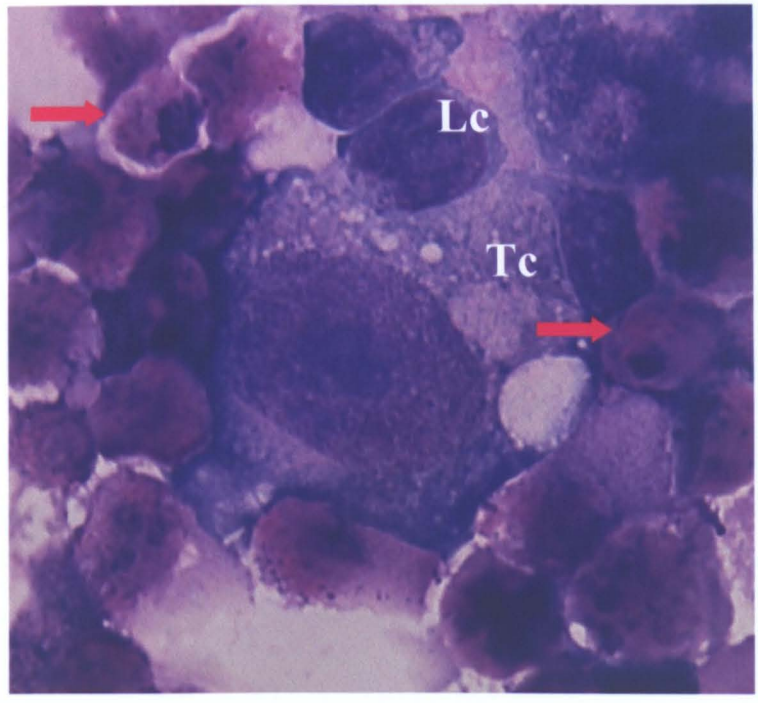


Figure 3.13 Cytospins of PBMNC activated with ConA in the presence of the HMCL, U266 (A, X400; B, X1000, Geimsa). Lymphocytes (Lc) are seen with only occasional mitogen-activated lymphoblast (Lb) when co-culture with myeloma cell lines (Tc). Apoptotic cells (arrows) are occasionally seen when PBL are mitogen-activated in the presence of HMCL. Mitotic figures were infrequent.

A

DONOR	BLASTING (%)		$CD25^+$ (%)		$CD69^-/CD25^+$ (%)	
	CA	CA +U266	CA	CA +U266	CA	CA +U266
1	86.39	14.8	60	24	43	6
2	43	25.32	70	49	69	20
3	67.2	31.22	89	31	53.5	6
4	71	43	82	53	53	28
5	43	23	46	25.6	31	24
6	46	23	53	41.1	30	21
7	41	37	69	58	40	27
Mean±SEM	56.5±5.8	28.2±5.1	67±5.8	40.2±5.1	45.9±5.3	18.9±3.5
p	<0.005		<0.005		<0.001	

B

DONOR	BLASTING (%)		$CD25^+$ (%)		$CD69^-/CD25^+$ (%)	
	CA	CA +JJN3	CA	CA +JJN3	CA	CA +JJN3
1	86.39	31.8	60	6	43	18
2	43	25	70	20	69	12
3	67.2	15.74	89	6	53.5	4.5
4	71	14	82	28	53	8.5
5	43	16.5	46	24	31	9.7
6	46	19.3	53	21	30	-
Mean±SEM	59.4±7.4	20.3±2.3	66.7±6.8	18.9±3.5	46.6±6.1	10.5±2.2
p	<0.002		<0.001		<0.001	

Table 3.2. Physical characteristics and surface expression of activation markers by normal PBMNC activated by ConA in the presence and absence of the HMCLs, U266 and JJN3, after 72 of culture. Results represent identified cells expressed as a percentage of gated cells (10,000 events). **Key:** SEM- standard error of the mean, CA-ConA activated PBMNC.

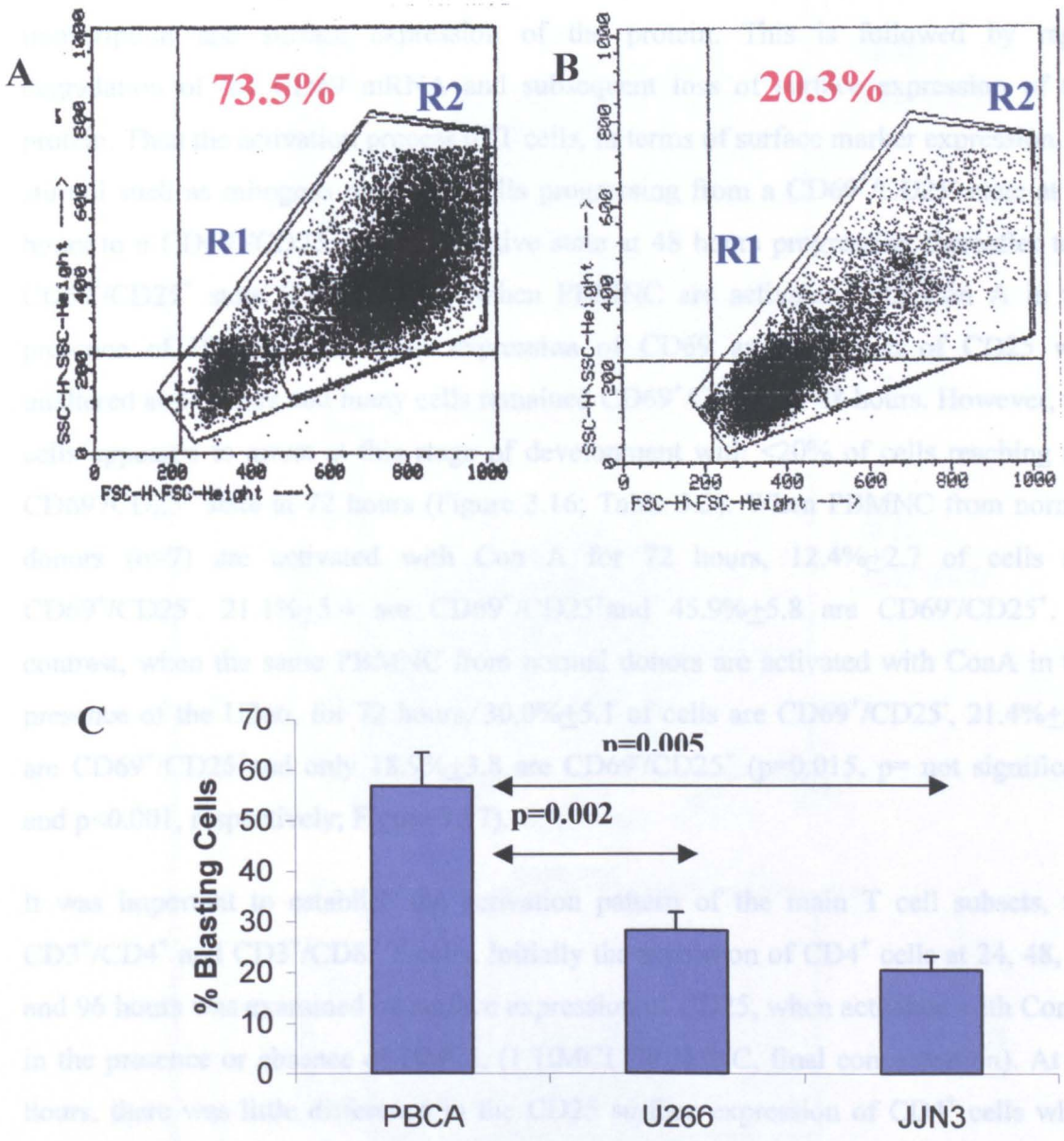


Figure 3.14 Representative dot plots of PBMNC activated by ConA in the absence (A) and presence (B) of the HMCL, U266. The results of suppression of ConA-induced blastogenesis by HMCL U266 and JJN3 are summarised in (C), where n=7 for U266 and n=6 for JJN3. Region 1(R1) was set to gate all (both resting and activated) PBL and region 2 (R2) was set within R1 to gate on blasting cells, indicated by increase in size and granulation/vacuolation. Values indicate the percentage of R1 cells in R2.

The activation of PBMNC was examined by flow cytometry. When PBL are activated *in vitro* they rapidly up-regulate surface expression of CD69 (within 24 hours) which is closely followed by activation of interleukin-2 receptor α (IL-2Rα/CD25) gene transcription and surface expression of the protein. This is followed by rapid degradation of the CD69 mRNA and subsequent loss of surface expression of the protein. Thus the activation process of T cells, in terms of surface marker expression, by stimuli such as mitogens results in cells progressing from a $CD69^+/CD25^-$ state at 24 hours to a $CD69^+/CD25^+$ double positive state at 48 hours progressing thereafter to a $CD69^-/CD25^+$ state (Figure 3.15). When PBMNC are activated with Con A in the presence of HMCLs, the rapid expression of CD69 and initiation of CD25 was unaltered at 24 hours and many cells remained $CD69^+/CD25^+$ at 48 hours. However, the cells appeared to arrest at this stage of development with <20% of cells reaching the $CD69^-/CD25^+$ state at 72 hours (Figure 3.16; Table 3.2). When PBMNC from normal donors (n=7) are activated with Con A for 72 hours, 12.4%±2.7 of cells are $CD69^+/CD25^-$, 21.1%±5.4 are $CD69^+/CD25^+$and 45.9%±5.8 are $CD69^-/CD25^+$. In contrast, when the same PBMNC from normal donors are activated with ConA in the presence of the U266, for 72 hours, 30.0%±5.1 of cells are $CD69^+/CD25^-$, 21.4%±5.8 are $CD69^+/CD25^+$and only 18.9%±3.8 are $CD69^-/CD25^+$ ($p=0.015$, p= not significant and $p<0.001$, respectively; Figure 3.17).

It was important to establish the activation pattern of the main T cell subsets, the $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells. Initially the activation of $CD4^+$ cells at 24, 48, 72 and 96 hours was examined by surface expression of CD25, when activated with Con A in the presence or absence of HMCL (1 HMCL:5PBMNC, final concentration). At 24 hours, there was little difference in the CD25 surface expression of $CD4^+$ cells when activated in the presence or absence of U266 (43.8% *cf* 49.4%, respectively) as illustrated in Figure 3.18. However at 72 and 96 hours there was a significant difference in the CD25 surface expression when the T cells were activated in the presence of U266 (72 hours: 33.1% *cf* 69%, 96 hours: 29.6% *cf* 97%). To determine if this was a specific, differential effect on $CD4^+$ T cells, both $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells from normal donors (n=3) were examined after 72 hours stimulation with Con A in the presence or absence of U266 (1 HMCL:5PBMNC, final concentration). As illustrated, the CD25 surface expression was significantly suppressed ($CD3^+/CD4^+$cells, $p<0.001$and $CD3^+/CD8^+$ cells, $p<0.006$) when the cells were stimulated with Con A in

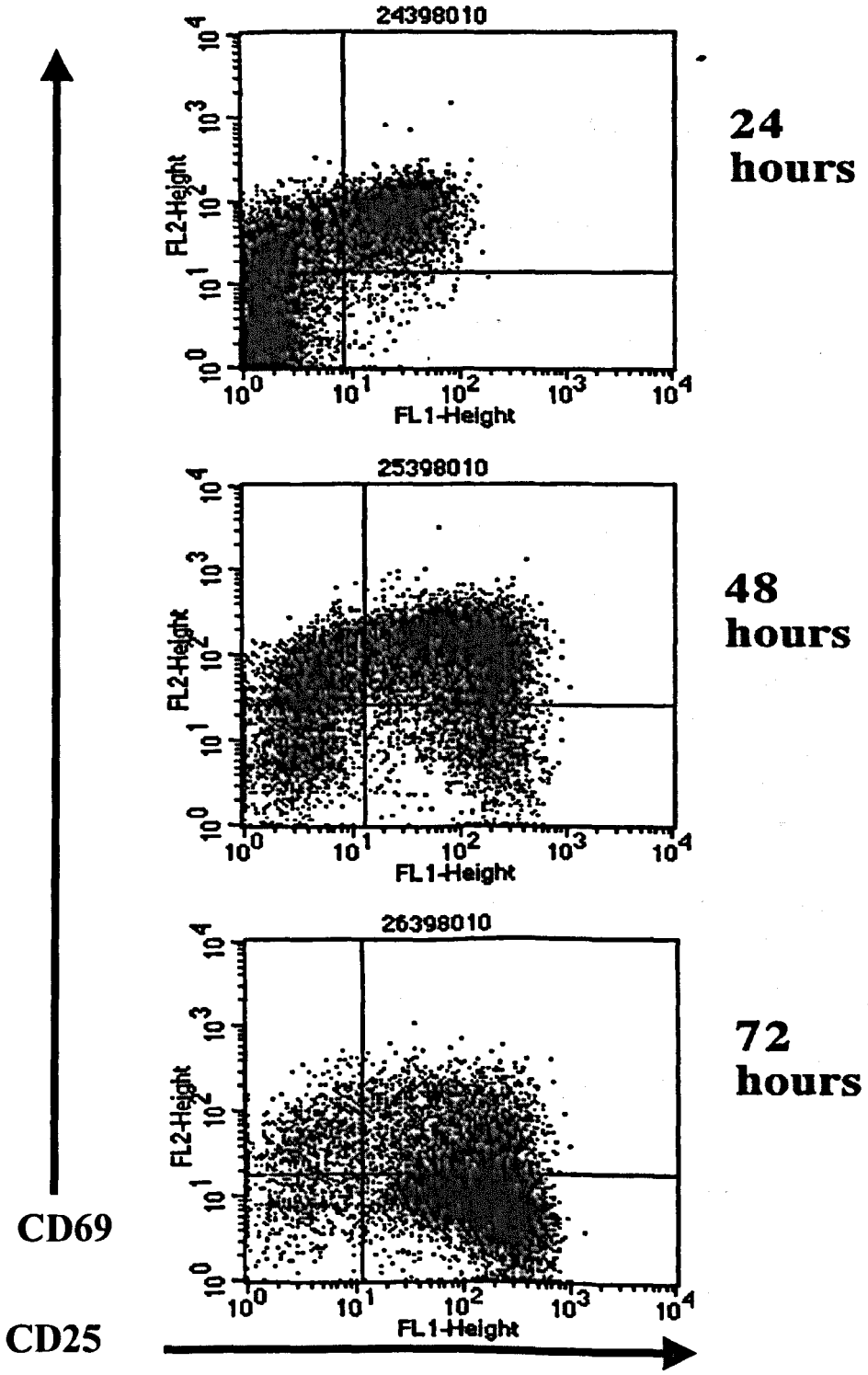


Figure 3.15. Representative dot-plots of CD69 and CD25 surface expression in response to ConA at the time periods. Surface expression of activation markers is analysed on cells gated within the Region 1 , Fig 3.12A.

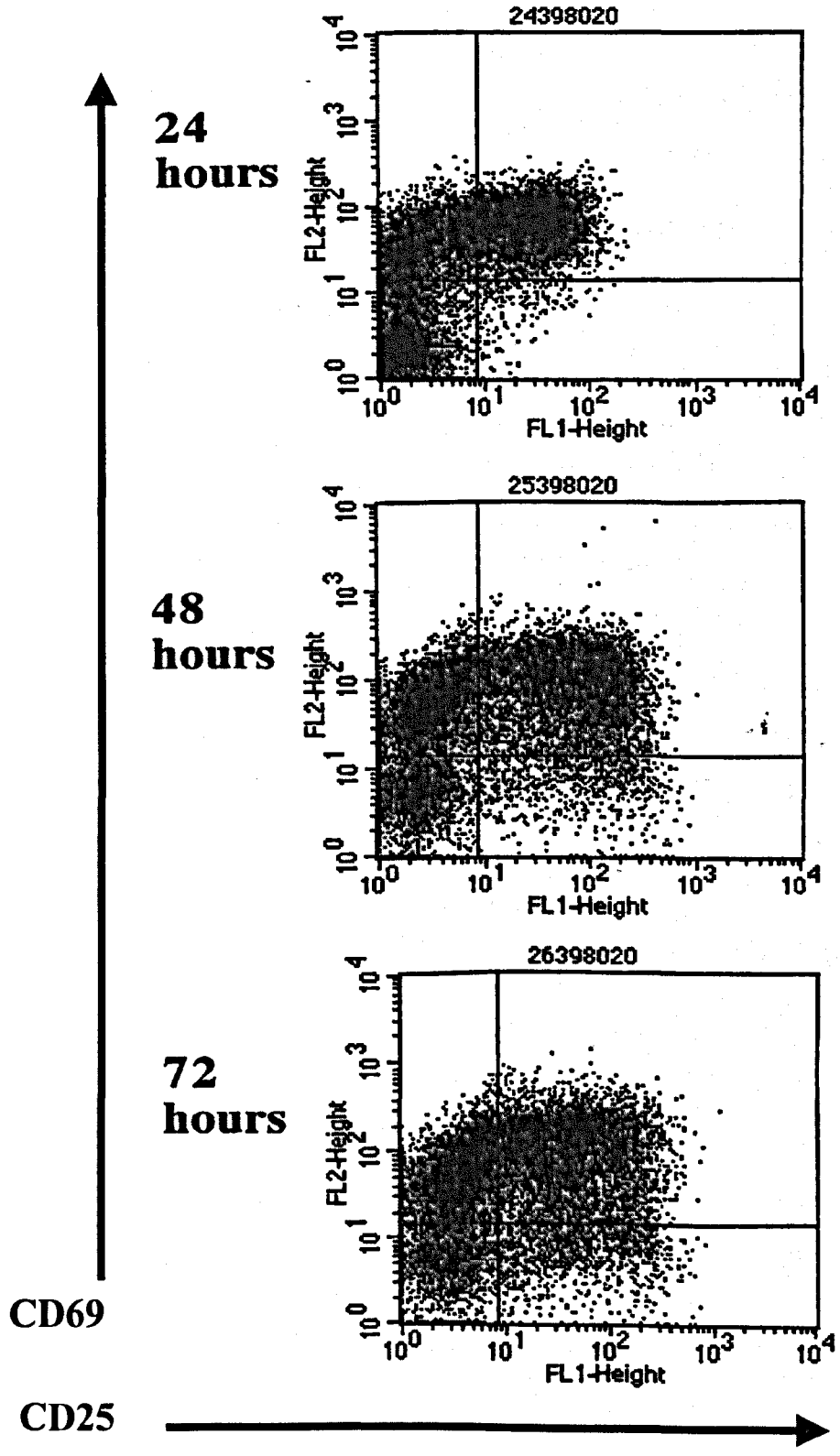


Figure 3.16. Representative dot-plots of CD69 and CD25 surface expression in response to ConA at the time periods indicated in the presence of U266 cell line. Surface expression of activation markers is analysed on cells gated within the Region 1, Fig 3.14A.

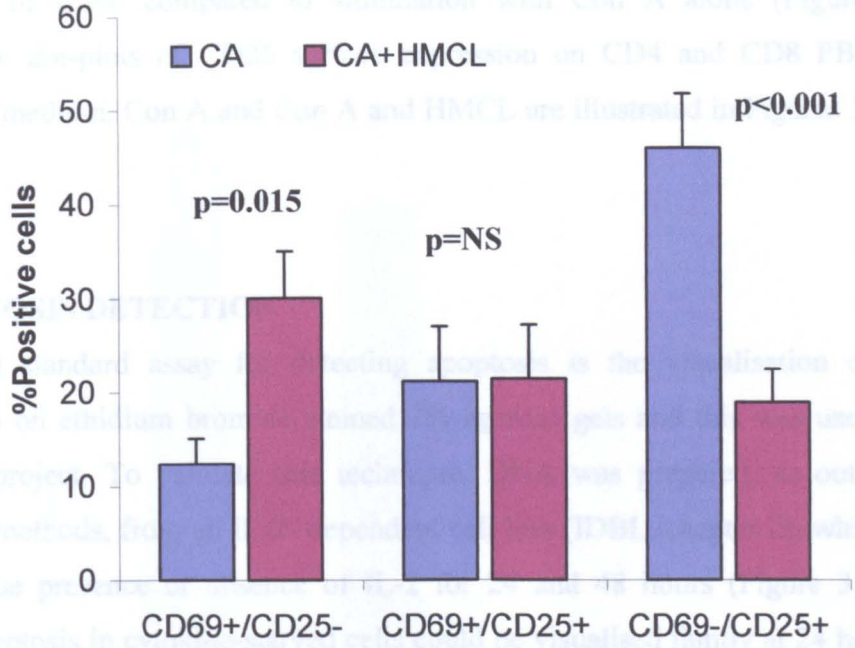


Figure 3.17. The surface expression of activation markers CD69 and CD25 when PBMNC from normal donors (n=7) are activated with ConA in the absence (CA) or presence (CA+HMCL) of U266 in 72 hour cultures. The natural progression from $CD69^+/CD25^-$ to $CD69^-/CD25^+$ is arrested when PBMNC are co-cultured with HMCL.

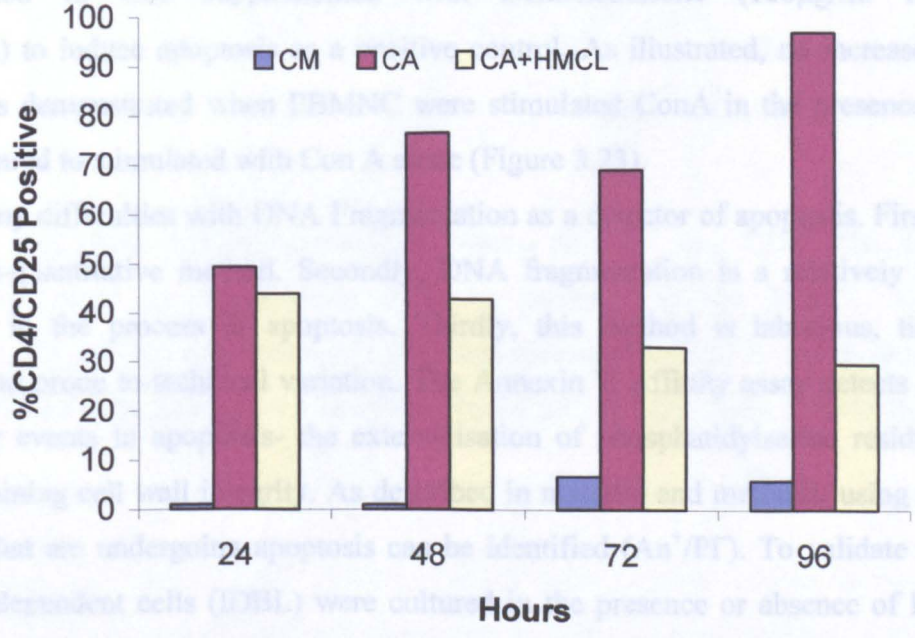


Figure 3.18. The activation of $CD4^+$ T cells, as represented by surface expression of CD25, following culture with complete medium (CM), ConA (CA) or ConA and the HMCL, U266 (CA+HMCL) at 24 hour time intervals.

the presence of U266 compared to stimulation with Con A alone (Figure 3.19). Representative dot-plots of CD25 surface expression on CD4 and CD8 PBL when cultured with medium, Con A and Con A and HMCL are illustrated in Figures 3.20 and 3.21.

3.3.4 APOPTOSIS DETECTION

A recognised standard assay for detecting apoptosis is the visualisation of DNA fragmentation on ethidium bromide stained 2% agarose gels and this was used at the start of the project. To validate this technique, DNA was prepared, as outlined in material and methods, from an IL-2- dependent cell line (IDBL, chapter 2), which were cultured in the presence or absence of IL-2 for 24 and 48 hours (Figure 3.22). As indicated, apoptosis in cytokine-starved cells could be visualised faintly at 24 hours and more clearly at 48 hours. Using this assay, I examined whether increased apoptosis could explain the noted HMCL-induced suppression of proliferative response to Con A of normal PBMNC. PBMNC from a normal donor were activated with Con A in the absence or presence of the U266 and JJN3 (1 HMCL:5PBMNC, final concentration), for 72 hours and DNA was prepared and loaded onto a 2% agarose gel. U266 cells were also incubated in CM supplemented with Dexamethasone (100μg/ml final concentration) to induce apoptosis as a positive control. As illustrated, no increase in apoptosis was demonstrated when PBMNC were stimulated ConA in the presence of HMCL compared to stimulated with Con A alone (Figure 3.23).

There are many difficulties with DNA Fragmentation as a detector of apoptosis. Firstly, this is a non-quantitative method. Secondly, DNA fragmentation is a relatively late phenomenon in the process of apoptosis. Thirdly, this method is laborious, time-consuming and prone to technical variation. The Annexin V Affinity assay detects one of the earlier events in apoptosis- the externalisation of phosphatidylserine residues, whilst maintaining cell wall integrity. As described in material and methods, using this assay, cells that are undergoing apoptosis can be identified (An^+/PI^-). To validate this assay, IL-2- dependent cells (IDBL) were cultured in the presence or absence of IL-2 for 24 hours and prepared as outlined in material and methods (Figure 3.24 A and B). PBMNC from normal donors (n=8) were stimulated with ConA for 72 hours in the absence or presence of the HMCLs, U266 and JJN3 (1 HMCL:5PBMNC, final concentration). No

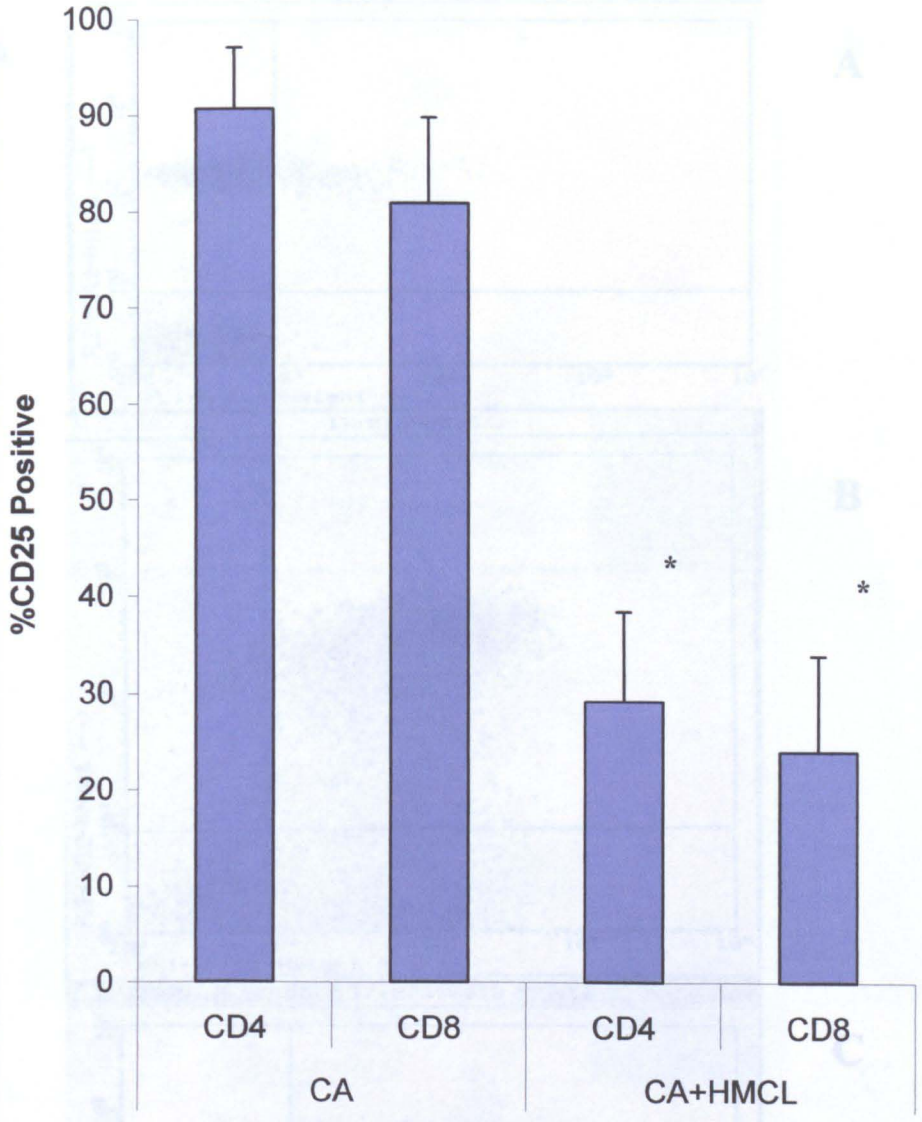


Figure 3.19. The effect of ConA-induced activation on $CD4^+$ and $CD8^+$ PBL from normal donors (n=3) in the absence (CA) or presence of the HMCL, U266 (CA+HMCL). The surface expression of CD25 was measured by FACS and results are expressed as $CD25^+$cells as a percentage of the gated $CD4^+$ or $CD8^+$ cells. (*-$p<0.005$).

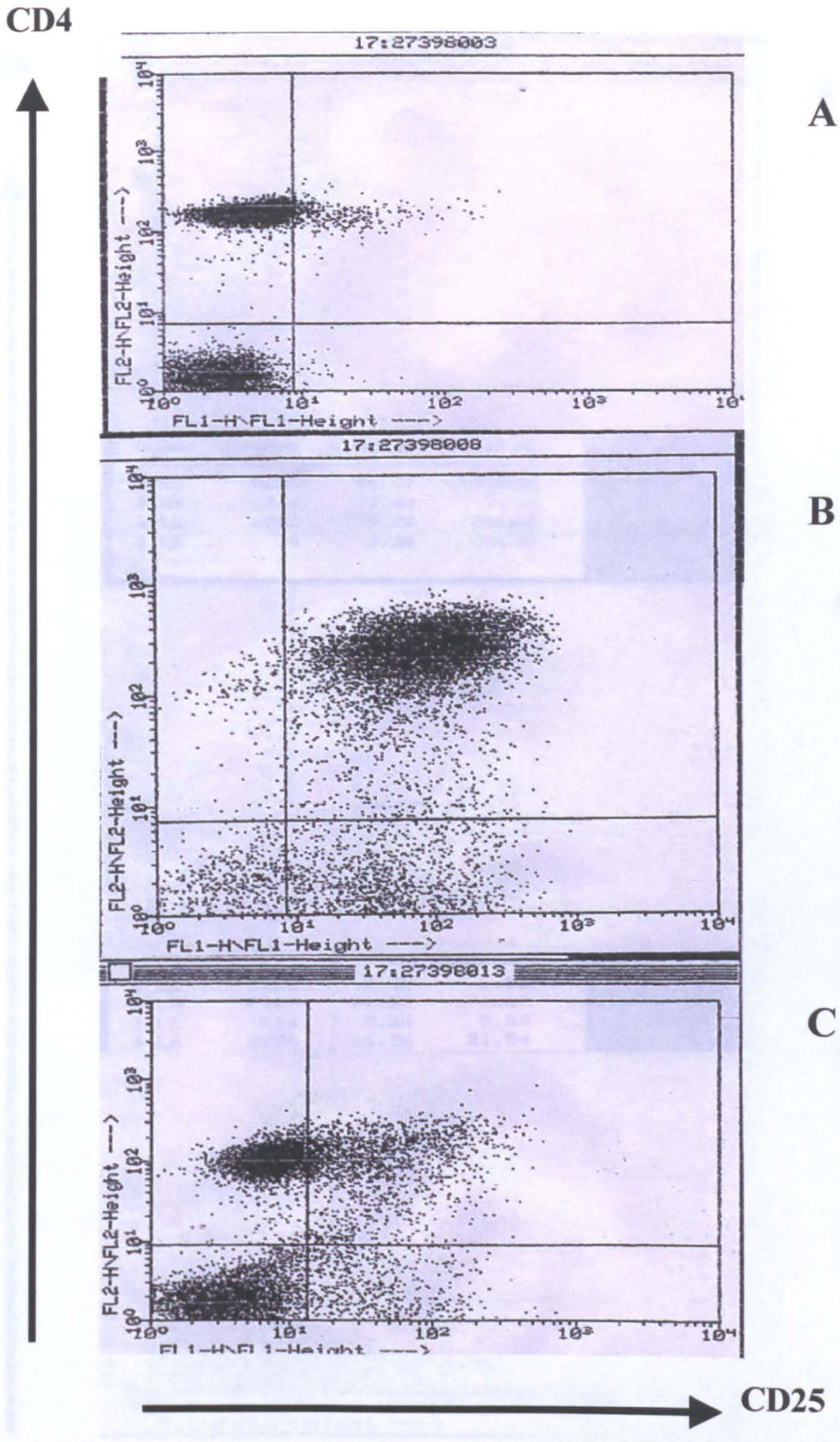


FIGURE 3.20. Expression of CD25 on $CD4^+$ PBL when cultured in medium alone (A), cultured with ConA (B) and culture with ConA and the HMCL, U266 (C) for 72 hours, as determined by flow cytometry.

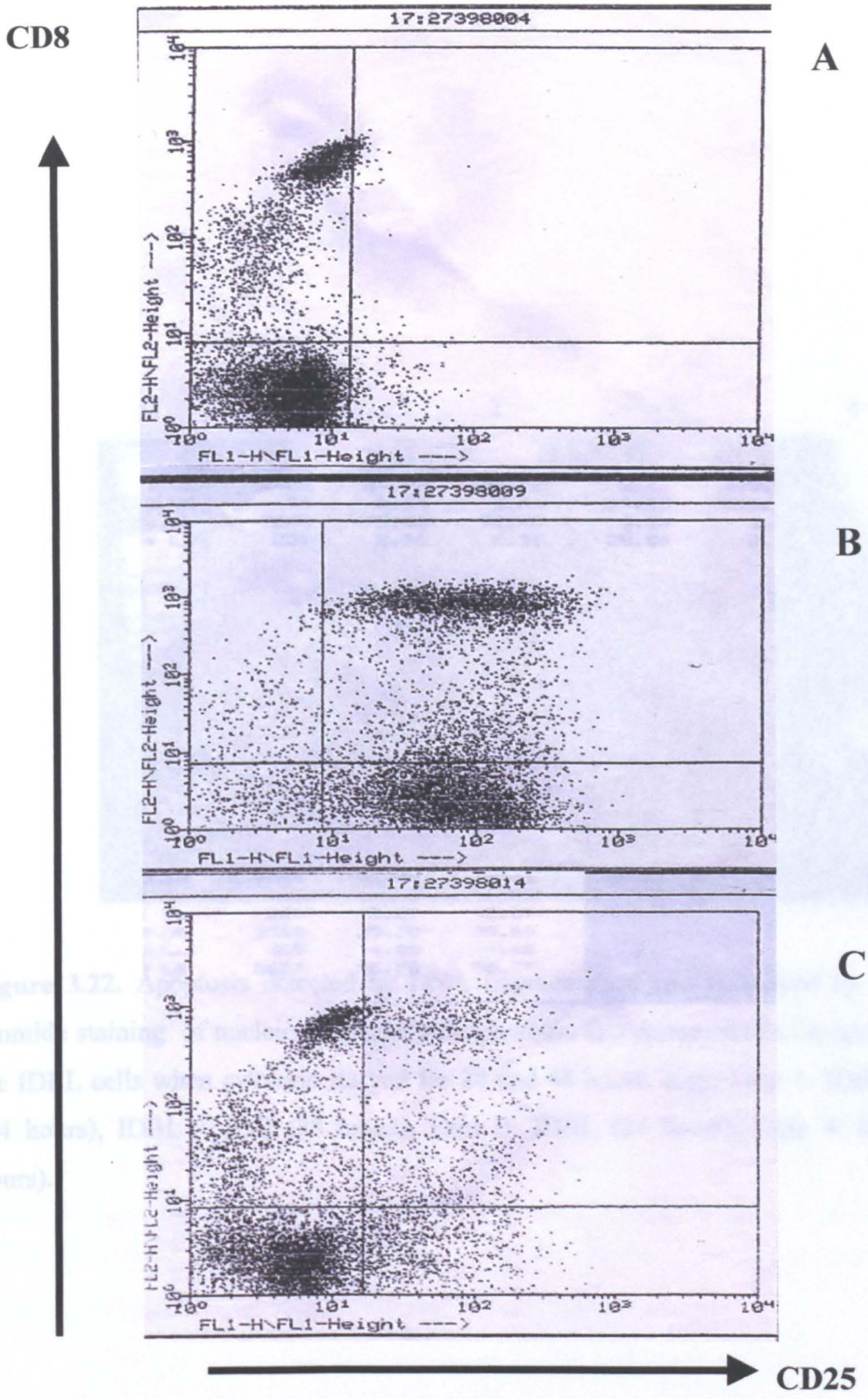


FIGURE 3.21. Expression of CD25 on $CD8^+$ PBL when cultured in medium alone (A), cultured with ConA (B) and culture with ConA and the HMCL, U266 (C) for 72 hours, as determined by flow cytometry.

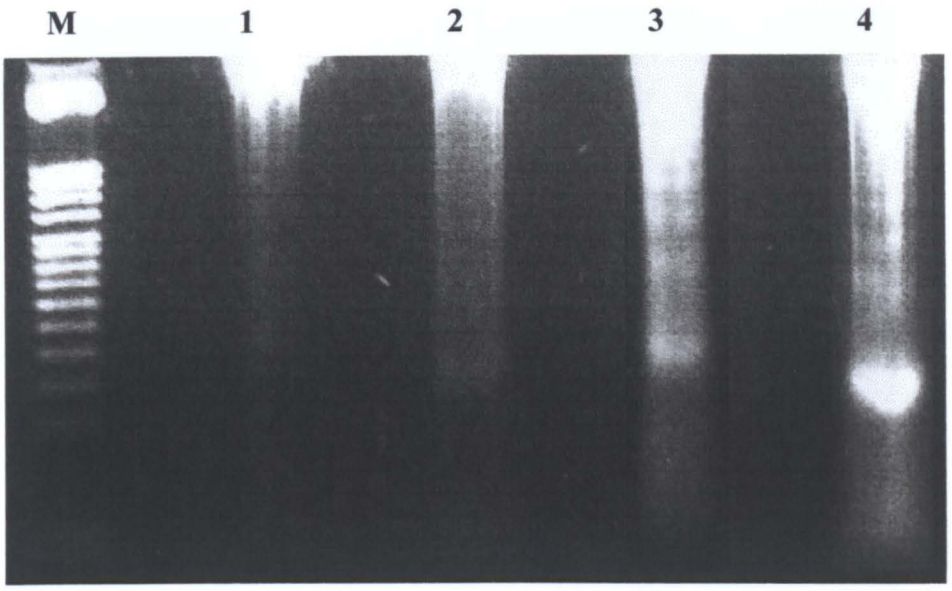


Figure 3.22. Apoptosis detected by DNA fragmentation and visualised by ethidium bromide staining of nucleic acid gel electrophoresis. Gel demonstrates the apoptosis of the IDBL cells when cytokine starved for 24 and 48 hours. **Key:** Lane 1: IDBL + IL-2 (24 hours), IDBL + IL-2 (48 hours), Lane 3: IDBL (24 hours), Lane 4: IDBL (48 hours).

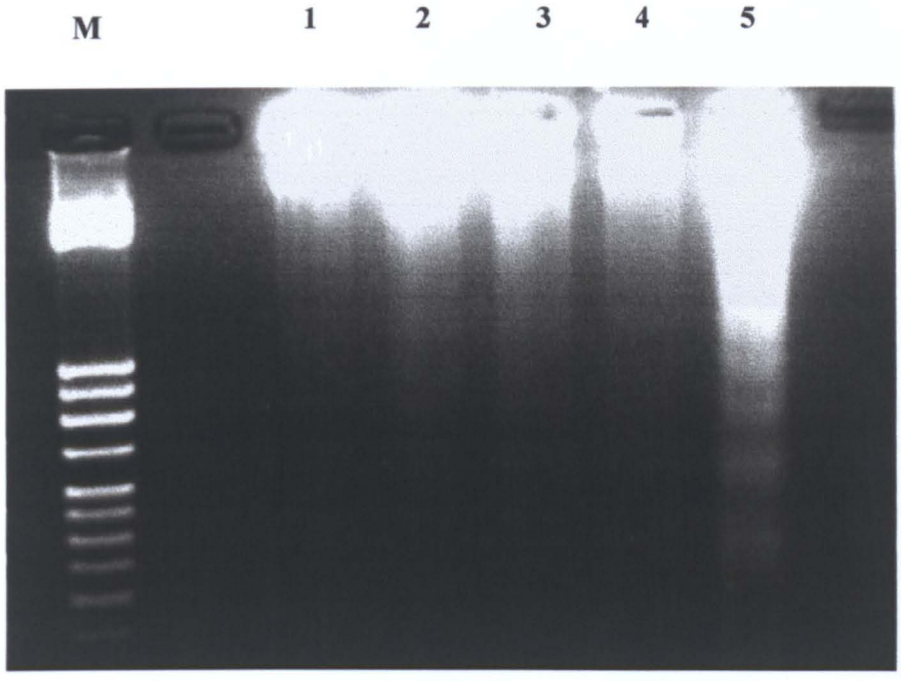
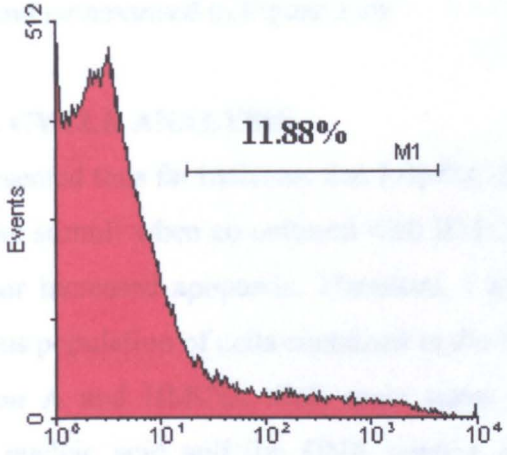


Figure 3.23. Apoptosis detected by DNA fragmentation and visualised by ethidium bromide staining of nucleic acid gel electrophoresis. The gel demonstrates that no obvious increase in apoptosis is seen when PBMNC from normal donors are stimulated with ConA in the presence of HMCL (U266) compared with stimulation with ConA alone. Dexamethasone-treated U266 included as a positive control. **Key:** Lane 1: PBMNC + CM, Lane 2: PBMNC + ConA, Lane 3: PBMNC + ConA + U266, Lane 4: PBMNC + ConA + JJN3, Lane 5: U266 + Dexamethasone.

A



B

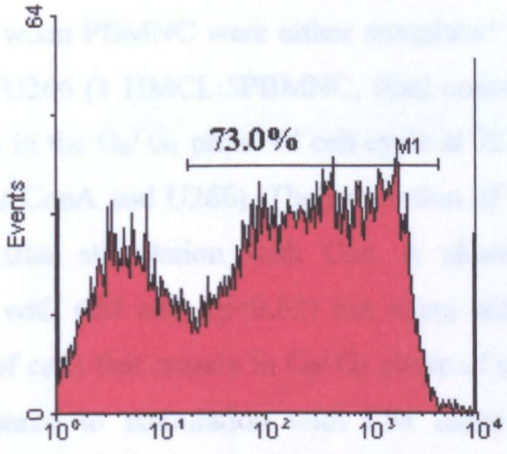


Figure 3.24. Apoptosis as detected by the Annexin V affinity assay, when IDBL cells are cultured in presence (A) or absence of IL-2 (B) for 24 hours.

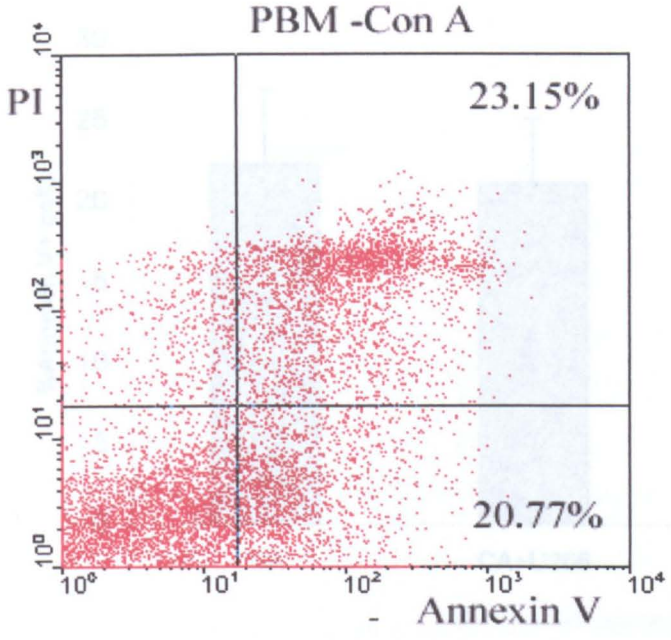
difference in the percentage of PBMNC undergoing apoptosis (An^+ cells) was seen when cultured with Con A and U266 (21.06%±3.4), with Con A and JJN3 (20.45%±3.9) or Con A alone (22.4%±4.6), as illustrated by representative examples in figure 3.25 and summarised in Figure 3.26.

3.3.5 CELL CYCLE ANALYSIS

The data presented thus far indicates that PBMNC from normal donors fail to proliferate to appropriate stimuli when co-cultured with HMCLs, which is neither due to failure of activation nor increased apoptosis. Therefore, I examined the cell cycle status of the heterogeneous population of cells contained in the PBMNC fraction cultured with ConA alone or Con A and HMCLs. Cell cycle status was analysed by propidium iodide staining of nucleic acid and the DNA content of the cells was measured by flow cytometry and analysed using Modfit software.

When the cell cycle status of PBMNC activated with Con A (n=5) was examined at 72 hours, a mean of 48%±8.2 cells were determined to be in the G_0/G_1 phase of cell cycle. In contrast, when PBMNC were either stimulated with complete medium only or with Con A and U266 (1 HMCL:5PBMNC, final concentration), the greatest proportion of cells remain in the G_0/G_1 phase of cell cycle at 72 hours (mean 82%±7.55 for CM and 77%±8.2 for ConA and U266). The proportion of cells that remain in G_0/G_1 phase of cell cycle after stimulation with Con A alone is significantly lower than after stimulation with CM only ($p<0.02$) but when stimulated with Con A and U266, the proportion of cells that remain in G_0/G_1 phase of cell cycle are not significantly altered when compared to stimulation with CM alone (Table 3.3). Representative DNA histograms of PBMNC stimulated with Con A and Con A with U266 are illustrated Figure 3.27.

A



B

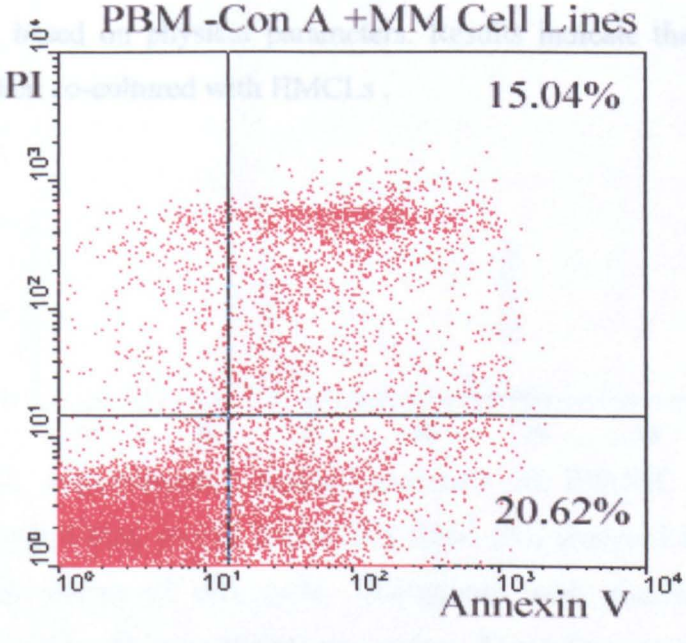


Figure 3.25. Representative examples of apoptosis of PBMNC, as detected by Annexin V^+ surface staining on gated cells, when stimulated in the absence (A) or presence of U266 (B). Figures displayed as dot plots to demonstrate the apoptotic (An^+/PI^-) and necrotic (An^+/PI^+) cell populations in each set of culture conditions.

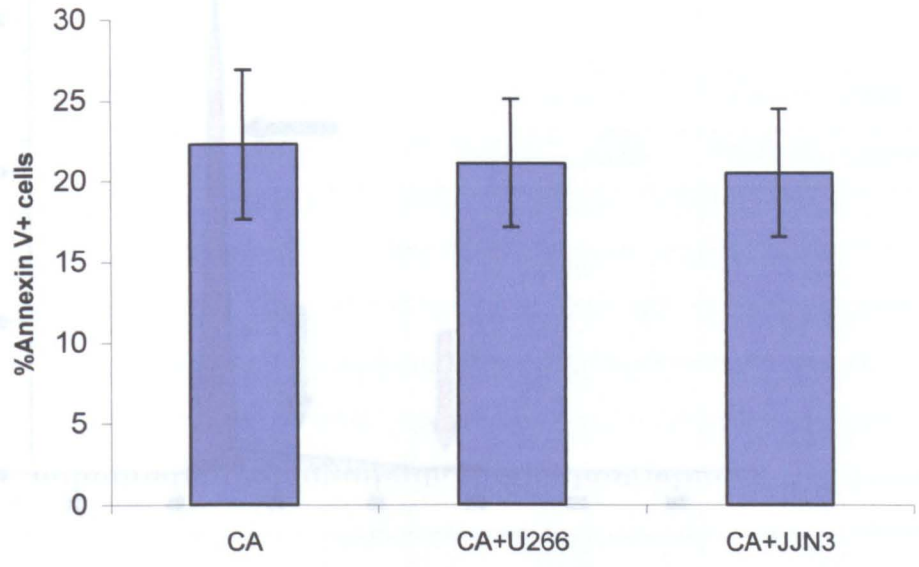
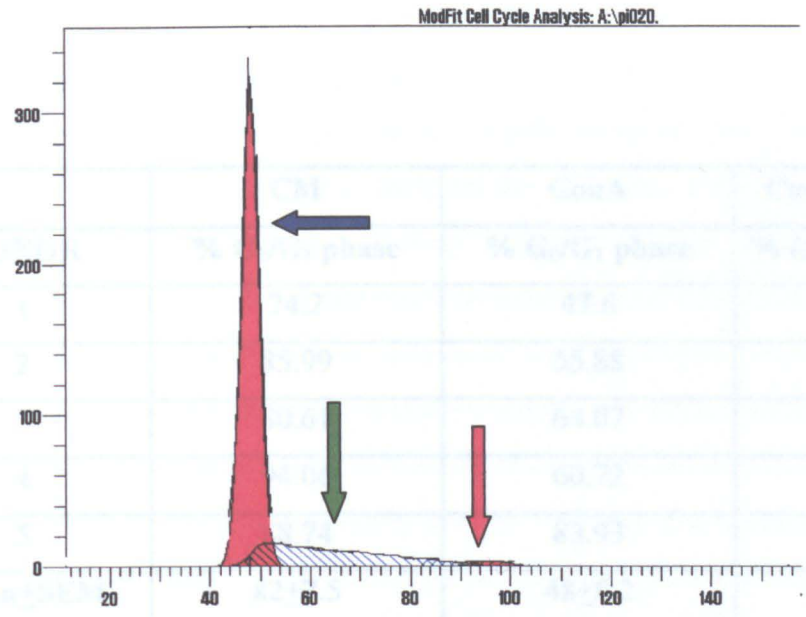


Figure 3.26. Apoptosis of PBMNC from normal donors (n=8) when cultured with ConA alone or with ConA and the HMCLs, U266 and JJN3. Gates were set to exclude the HMCL, based on physical parameters. Results indicate that the apoptosis rate is unaltered when co-cultured with HMCLs .

A



B

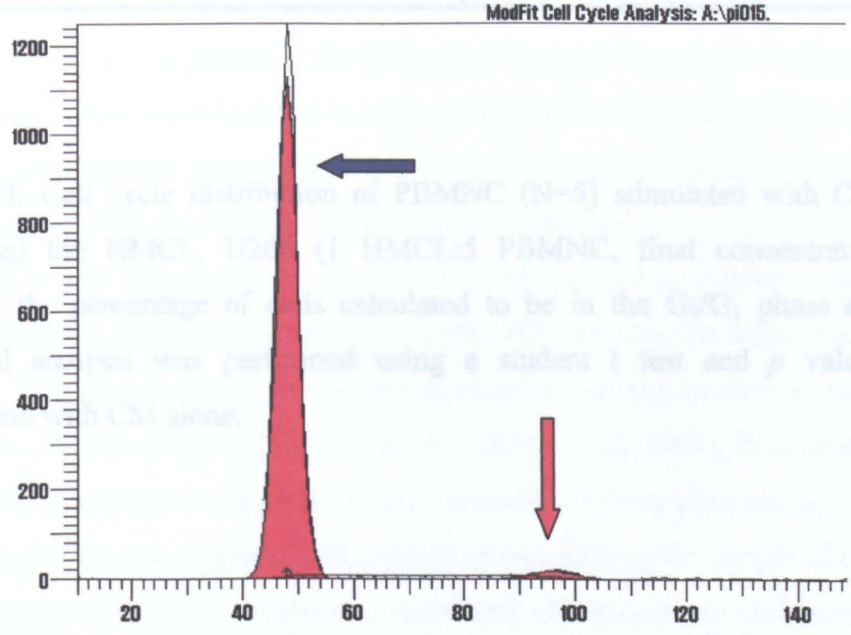


Figure 3.27. Representative DNA histograms of PBMNC from normal donors stimulated with ConA (A) or ConA and U266 (B), analysed by ModFit software to delineate the phases of cell cycle. Histograms were generated from gated cells, excluding tumour cells on physical parameters. The cells contained within the Diploid peak represent cells in the G_0/ G_1 phase (blue arrows) of cell cycle, with cells to the right of the diploid peak representing cells that have progressed into the S (green arrows) and G_2/M phase (red arrows) of cell cycle.

	CM	ConA	ConA+ U266
DONOR	**% G_0/G_1 phase**	**% G_0/G_1 phase**	**% G_0/G_1 phase**
1	74.2	47.6	76.7
2	85.99	55.88	72.91
3	80.61	64.07	85.85
4	94.06	60.72	77.7
5	88.74	83.93	84.96
Mean±SEM	82±7.5	48±8.2	77±8.2
p	-	<0.02	>0.5

Table 3.3. Cell cycle distribution of PBMNC (N=5) stimulated with CM, ConA or ConA and the HMCL, U266 (1 HMCL:5 PBMNC, final concentration). Results represent the percentage of cells calculated to be in the G_0/G_1 phase of cell cycle. Statistical analysis was performed using a student t test and *p* values represent comparison with CM alone.

3.4 DISCUSSION

Myeloma tumour cells suppress activating T cells.

Although there is little doubt that tumour-specific antigens exist for most human cancers, it is still unclear why these antigens do not cause rejection in the tumour bearing host. A successful immune response against a tumour is dependent on the detection of that tumour and the ability of the host immune system to mount an effective response. In multiple myeloma many defects in the host immune system have been identified including phenotypic and functional changes in T-cells, B-cells, macrophages and NK/LAK cells (Lauria et al, 1984; Massaia et al, 1988; Mellstedt et al, 1982; Wen et al, 1990). Several investigators have studied different mechanisms which could explain the abnormalities of the peripheral blood immune cells including evasive tactics by the tumour cells and apoptosis induction of the immune cells by the tumour (Janson et al, 1991; Massaia et al, 1995). However, to date, no evidence has been presented to demonstrate the exact nature of the effect that myeloma tumour cells have on primary immune cells. The data presented in this chapter demonstrates that the myeloma tumour cells can directly affect primary immune cells from normal, healthy volunteers resulting in reduced responses to alloantigen and mitogen stimulation.

The HMCLs did not stimulate primary MLR when used in similar concentrations to non-transformed allogeneic stimulators (PBMNC), despite the high alloreactive CD8 precursor frequencies seen in the peripheral blood of normal, healthy individuals (CTLp frequencies in the range of 1:544 -1:47619; Wang et al, 1998). This raises two broad issues: firstly, are myeloma tumour cell incapable of presenting antigen to effector T cell and secondly, are myeloma cell capable of suppressing the proliferative response to appropriate stimulation? As previously indicated, effective T cell activation requires the presentation of a suitable antigen with co-stimulation. It has been shown that myeloma tumour cells demonstrate suitable levels of HLA class I and II molecules on their surface but no expression of the co-stimulatory molecules, CD80 and CD86, could be detected either at the protein level by FACS or mRNA level by RT-PCR (Cook & Campbell, 1999). However, other investigators have shown that myeloma tumour cells can act as antigen presenting cells which suggested that the tumour cells possessed competent antigen processing and presentation capabilities, perhaps utilising a supplementary co-stimulatory system (Yi et al, 1997). Therefore, myeloma cells may be able to present antigen in a suitable form to activate T cells and, as such, the second

issue of active suppression of the T cells becomes a more dominant explanation of the experimental data. The suppression of one-way allogeneic MLR by HMCL in a 3rd party/bystander manner supports the theory that HMCL can suppress alloreactive T cell responses. Once again this phenomenon demonstrates a dose effect with reducing concentrations of HMCL being associated with a reduction in the suppression of the MLR proliferative responses.

However, in these experiments, the MLR responses were varied which may reflect the degree of HLA-disparity amongst the normal donors used in these studies. As such, to gain further understanding of the effect HMCL have on activating T cells, mitogen stimulation was used to obtain a more uniform activation of the normal, primary T cells. Once again, the HMCL demonstrated a potent suppressive effect on the activation of the T cells in response stimulation with mitogen, which also demonstrated a dose-effect. This suppressive effect was not a biological anomaly related to this one HMCL, U266 as similar results were obtained when a total of 4 HMCLs (U266, RPMI 8226, JJN3 & JIM1) were co-cultured with 3 different, healthy donors. This suggests that this immunosuppressive function may be a characteristic of the B cell malignancy that these transformed cell lines represent.

However, as with all research using *in vitro* models based on transformed cell lines, the behaviour of cell lines may represent either a feature of their *in vitro* transformation or an exaggerated response in comparison to their true malignant counter parts. Myeloma cell lines are generated from patients with advanced disease, usually with leukaemic phase or body-cavity infiltration such as peritoneal or pleural deposits. The extramedullary dissemination of myeloma tumour cells is associated with alterations in phenotype and biology compared with malignant plasma cells obtained from the bone marrow cavity of patients (San Miguel et al, 1995). For example, the NK cell associated surface antigen, CD56 (NCAM) is expressed by malignant plasma cells in up to 67% of patients with myeloma but is not detected on normal plasma cells (Harada et al, 1993). This is an adhesion molecule that with other adhesion molecules such as VLA-4 (CD49d), VLA-5 (CD49e), CD44 (HCAM) and CD54 (ICAM), may facilitate "anchorage" to the bone marrow stroma (San Miguel et al, 1995; Cook et al, 1997). However, extramedullary spread is associated with a dramatic down-regulation of both CD56 and VLA-5 (Drew et al, 1996; Cook et al, 1997). These VLA-5$^-$ plasma cells detected in peripheral blood have been shown to have a higher proliferative potential and response to exogenous IL-6 than the VLA-5$^+$ plasma cells though the latter group

secrete higher amounts of paraprotein (Kawano et al, 1993). The use of transformed cells lines permit the initiation of experimental investigations to establish theories of behaviour and function, aiming to extend these studies to true patho-physiological material. These experiments have indicated that myeloma-derived cell lines are capable of suppressing activation of T cells from normal healthy volunteers. Evidence does exist to demonstrate that T cell subsets are disrupted in patients with multiple myeloma, in particular a deficit of $CD4^+/CD45RA^+$ cells, and this is more commonly associated with advancing disease stage, associated with poor prognostic markers such as a high β_2-microglobulin and signs of bone marrow failure (San Miguel et al, 1992). This suggests a link between advancing disease state, alterations in the host immune system and the *in vitro* behaviour of HMCLs presented in this chapter and implies, though does not prove, that the observed effect on the activation of normal T cells is in fact related to the in vivo tumour cells which the cell lines represent. Clearly, the identification of the mediating factor(s) from the HMCLs and its subsequent identification in clinical samples form patients with multiple myeloma is essential. It would be important to demonstrate the effect that serum, both peripheral blood and bone marrow, not only contain the putative mediator of immunosuppression but also do in fact prevent activation of normal T cells, as described here and this is the subject of parallel studies to this project.

The observed suppression of Con A-induced proliferation by HMCLs was maximal at 3 days, though a suppressive effect could still be detected, to a lesser extent, at day 5. The timing of the maximal suppression coincides with the kinetics of Con A induced proliferation, which is maximal at day 3 and starting to fade by day 5. This suggests that the suppressive effect of HMCLs on the mitogen-induced proliferation of normal peripheral blood lymphocytes is dependent on the initiating of the activation sequence. This is supported by the data presented here and by parallel data which demonstrates that, suppression is inversely related to the degree of initiation of the activation process (Campbell et al, unpublished observation).

Perturbation of the TCR/CD3 complex by antigen or mitogen results in the activation of at least two interrelated second messenger systems: a protein tyrosine kinase (PTK) pathway and a membrane polyphosphoinositides hydrolysis pathway mediated by phospholipase CγL (PL CγL) (Chan et al, 1994). The activation of both these pathways and the activation of $p21^{ras}$ pathway mediate tyrosine phosphorylation of multiple

substrates including the IL-2Rβ Rγc subunits, JAK3/JAK1/p56^{lck} kinases and STAT3/STAT5 (Kolenko et al, 1997). These latter events result in up-regulation of gene transcription including IL-2Rα, IL-2 and other cytokines. The fact that the degree of suppression of T cell responses to mitogen and alloantigen are related to the degree of activation implies that this effect is dependent on blocking these mediators of lymphocyte activation. There are many known factors, which are capable of suppressing T cell activation by blocking different stages of the activation and/or proliferation phases of lymphocyte responses. One such example is TGFβ which can inhibit T cell proliferation by down-regulating IL-2 mediated-signals mediated through reduced tyrosine phosphorylation of proteins of 50- 120 kDa range and inhibition of IL-2 mediated phosphorylation of the retinoblastoma susceptibility gene product, which is pivotal in the progression through the cell cycle (Fontana et al, 1989; Ahuja et al, 1993). The experiments using a 0.2μm pore membrane demonstrate that physical cell-cell contact is not required for the mediation of the immunosuppressive effects. FasL, which is a member of the TNFR/NGFR family, can mediate T cell inactivation through inducing apoptosis. This can act via direct cell-cell contact of the membrane forms with its receptor, Fas on activated T cells (0'Connell et al, 1999). It is worthy of note that a soluble form of FasL has been shown to be effective in countering T cell survival mediated by apoptosis (Tanaka et al, 1996). However, it does indicate that a soluble factor or factors are produced by the HMCL that directly suppress the proliferative responses of normal PBL in response to appropriate stimulation. Identification of such factor or factors is the subject of further experiments presented in chapter 5.

Myeloma cells prevent T cell transition from activation to proliferation phases.

To understand the possible effects that myeloma cells may have on normal T cells when appropriately stimulated, it is important to understand that the T cell response to antigen/mitogen occurs in two contingent phases comprising distinct molecular events: an activation phase and a subsequent proliferation phase (Crabtree, 1989). Important activation phase events include the induction of IL-2 genes, its high affinity receptor (IL-2Rαβγc) and CD69 surface expression (Testi et al, 1994). The proliferative phase takes place when IL-2 engages its receptor and initiates cell cycle progression – entry into the IL-2 autocrine growth pathway. CD69, a 27/33 kDa homodimeric surface glycoprotein, is rapidly expressed on the surface of T cells (mRNA detected 30-60

minutes after stimulation, surface protein detected 2-3 hours) following appropriate stimulation with a variety of agents including anti-CD3/TCR, activators of protein kinase C and mitogens (Testi et al, 1994). Whilst the natural ligand for CD69 is not known, cross-linking studies demonstrate a rise in intra-cellular Ca^{2+}, synthesis of cytokines and their receptors (IL-2, IFNγ, TNFα), the expression of *c-myc* and *c-fos* proto-oncogene and proliferation (Carmen et al, 1997). The association of *c-fos* with *c-jun* results in the AP-1 complex, which through the co-operation with NF-AT (nuclear factor of activated T cells) activates gene transcription of IL-2Rα (Kuo & Leiden, 1999). This explains in part, at a molecular level, the normal sequence of surface expression of activation markers. That is, from a $CD69^+/CD25^-$ state to $CD69^+/CD25^+$ upon activation. By 72 hours, however, these cells normally become $CD69^-/CD25^+$ after 72 hours of stimulation owing to the rapid degradation of CD69 mRNA resulting from selective destabilisation associated with several AU-rich sequence motifs within the 3'-untranslated region (Testi et al, 1994; Santis et al, 1995; Castellanos et al, 1997). This transition in the surface activation marker composition reflect the entry into the IL-2 autocrine growth pathway and of cell cycle progression and proliferation.

When T cells are stimulated with mitogen in the presence of HMCL, entry into the activation phase, as represented by up-regulated surface CD69 expression, is seen. However, unlike the normal transition of T cells under stimulation, the T cells co-cultured with HMCL fail to up-regulate IL-2Rα (CD25) and remain, largely, $CD69^+$ after 72 hours of stimulation. This coupled with the data demonstrating poor ^{3}[H]dThd uptake indicating poor S phase entry, strongly suggests that the cells initiate their response pathways, enter the activation phase but fail to progress to the proliferation phase because of failure to enter the autocrine IL-2 growth pathway. The disruption of the IL-2 autocrine pathway is fundamental to the regulation of clonal expansion in response to suitable stimuli, both proliferation and apoptosis associated with activation (both passive "lymphokine-withdrawl" and active "antigen-induced" forms) which aim to co-ordinate the immune response to achieve the most effective clonal expansion (reviewed by Lenardo et al, 1999).

Myeloma cell-derived factors induce cell cycle arrest rather apoptosis.

The data presented here demonstrate that normal primary T cells fail to proliferate in response to allogeneic and mitogenic stimuli as a result of arrest in the G_1/G_0 phase of

cell cycle rather than activation-associated apoptosis. As indicated above, activation-associated apoptosis of T lymphocytes, both active and passive forms, is more pronounced in the proliferation phase when a balance between antigen and cytokine are important in regulating the immune response (Boehme et al, 1993). T cells are sensitive to activation-associated apoptosis when they have at least traversed the cell cycle several times, particular when in the S phase. The T cells in these experiments demonstrate poor ^{3}[H] dThd uptake indicating failure to enter S phase of the cell cycle which is corroborated by the reduced DNA synthesis indicated on the DNA cytometry analysis. The finding that T cells undergo cell cycle arrest rather than apoptosis when stimulated in the presence of HMCL is in contradiction to the published data which suggests that HMCL induce T cell apoptosis mediated by the Fas/FasL system (Massaia et al, 1995).

Myeloma cells have been shown to express FasL on their surface, as detected by flow cytometry. However, as myeloma cells also express Fas, then why do the tumour cells that are Fas+/FasL+ not commit autocrine "suicide" or juxtacrine "fractricide"? Many theories have been suggested to account for this including expression of antagonistic soluble Fas, failure to establish death-inducing signalling complexes (DISCs) and altered regulation and function of the caspases (Nagata, 1996; Villunger et al, 1997; Debarats et al, 1998). One possibility is that the FasL on the surface of myeloma cells may have reduced functional capability. Experiments to date to determine the functionality of myeloma-expressed FasL have relied on cytotoxicity assays using a T cell lymphoma-derived cell line that is exquisitely sensitive to FasL-mediated apoptosis. It is important to note that T cells which are activating, whilst expressing Fas on their surface are not susceptible to Fas-induced apoptosis in these initiation phases however when stimulated repeatedly with antigen and IL-2, the then become susceptible to activation induced cell death (Chappell et al, 1999; Leonardo et al, 1999). In the *in vitro* assays described here, the T cells from normal healthy volunteers are not being exposed to chronic antigenic stimulation in the presence of IL-2 and as such, it is not surprising that Fas/FasL-mediated apoptosis does not appear to play a significant role in mediating the tumour-induced immunosuppression.

Furthermore it has been shown in a melanoma model that it is FasL expressed on the surface of activated CTLs that induce Fas/FasL-mediated apoptosis in T cells rather than tumour derived FasL (Zaks et al, 1999). If the apoptosis-induction in tumour-reactive T cells arises from activated T cells, then interaction between tumour cells and

T cells would induce apoptosis owing to T cell activation as an initial step. The data presented here strongly suggests that T cells are prevented from becoming activated as a primary event when co-cultured in the presence of HMCL, thus negating the T cell-T cell Fas/FasL induced apoptosis. This is supported by the absence of increased apoptosis of T cells in the culture system when HMCL are added. However, *in vivo* the role of the Fas/FasL system remains to be clarified. The T cells in this situation are exposed to chronic antigen stimulation, in the form of the tumour-related idiotype, which may render the T cells from patients more susceptible to Fas mediated apoptosis (Massaia et al, 1995). However, it is likely that the weakened T cell response seen in patients with myeloma represents an orchestration of immunosuppressive strategies employed by the malignant clone.

Therefore, from this data it can be concluded that the T cells under go cell cycle arrest when stimulated in the presence of the HMCLs owing to a soluble factor (s). These observations may be the effect of a single cytokine such as transforming growth factor β (TGFβ) which has been described as promoting T cell survival by inducing a resting state and even synergising with IL-2 to prevent apoptosis, particularly of Th_2 cells (Zhang et al, 1995). It is also possible that the observed effect may be the result of an orchestration of cytokines and antigen such as quiescence-inducing and apoptosis protecting abilities of IL-15 in antigen–experienced T cells without cell cycle progression and proliferation (Dooms et al, 1998). The role of muc-1 in inducing apoptosis in T cells has been shown in an in vitro breast cancer model, however it may also cause T cell anergy and quiescence (Agrawal et al, 1998). The role of other cytokines such as IL-10 acting alone or in synergy with other molecules and cytokines needs further attention.

The data presented here indicating the effect that myeloma cells have on the activation process of T cells are novel. Defects in T cells from patients with other malignancies have been described. For example, T cells from patients with intracranial glioblastoma have been shown to have defective TCR-mediated signalling and renal carcinoma cell supernates are capable of suppressing the mitogen-induced activation of T cells from normal volunteers (Kolenko et al, 1997; Morford et al, 1997). If the tumour cells of myeloma are capable of an array of defence mechanisms which can prevent the generation of an immune response or switch off an established response, then this is important for physicians and scientists who are devising immunotherapy strategies. The

design of immunotherapy regimes will need to overcome the effects of a hostile microenvironment in terms of developing immune responses and this may require a re-evaluation of the vaccination strategy dogma which has been learned from microbiological vaccination protocols and which have been adopted in cancer trials in favour of new approaches which aim to circumvent the tumour-associated hostility towards the hosts immune system if the true potential of immunotherapy is to be maximised.

The data presented in this chapter clearly demonstrate that HMCL, an *in vitro* model of multiple myeloma, prevent T lymphocytes from normal, healthy donors from activating when stimulated with alloantigen and mitogen. This effect results from cell cycle arrest and failure to progress to the proliferation stage of activation, mediated by soluble factor or factors, as yet to be determined. Before investigating the potential factors which could explain the observations, it is necessary to develop an *in vitro* model to mimic the two contingent phases of T cell responses to stimulation, activation and proliferation. The establishment and validation of such a model is the subject of the chapter 4.

CHAPTER 4

An *in vitro* model of T lymphocyte activation for investigating the immunosuppressive effects of myeloma cell lines.

4.1 INTRODUCTION

The data presented in chapter 3 demonstrate that myeloma tumour cells, at least HMCLs, can interrupt the activation process of T cells from healthy donors in response to alloantigen and mitogen. This effect is mediated by a soluble factor(s) and in order to investigate this effect, an *in vitro* model system based on the activation process of peripheral T cells is necessary. To establish such a model system to mimic the activation process of T cells, an understanding of the homeostasis of T cells in terms of regulating activation and apoptosis is required.

The immune response involves an impressive expansion of specific T cell clones with potent effector functions (Pantaleo et al, 1994). The doubling time of mature T cells in response to antigen has been shown to be as little as 4.5 hours with the estimation that after 7 days, one T cell could produce up to 10^{12} progeny though with a single protein having up to 10 different epitopes, as many as 10-100 naïve T cells could be activated in any one immune response (Kurts et al, 1997; Murali-Krishna et al, 1998). A balance of cell production and destruction is needed to ensure that the T cell compartment is maintained and can respond to appropriate stimuli and that such a response is not excessive. It is not known whether like other formed elements of blood, T cells have a predetermined life-span. However, under specific conditions, the T cell growth factor IL-2 and antigen can induce apoptosis and to understand this relationship, it is important to recognise that T cell response to antigen occurs in two contingent phases characterised by distinct molecular events: an activation phase and a subsequent proliferation phase, schematically represented in Figure 4.1 (Crabtree, 1989). Contrary to popular moniker, "activation-induce cell death", there is little or no apoptosis in this phase of stimulating resting T cells, which aims to allow a protective immune response to develop (Radvanyi et al, 1996). The proliferative phase takes place when IL-2 engages its receptor and initiates cell cycle progression – entry into the IL-2 autocrine growth pathway. Once cells have progressed through the cell cycle, perhaps more than once and have entered late G_1 or S phase, they become exquisitely sensitive to apoptosis (Boehme et al,1993). This cell cycle progression and susceptibility to apoptosis both involve the proto-oncogene *c-myc* (Boehme et al, 1996).

This propriocidal or "feed-back response" regulation of T cells determines the fate of cycling T cells in the prevailing conditions of the immune response. In particular, when there is no further antigen the expression of IL-2 and its receptor are down-regulated resulting in "passive" or cytokine-withdrawal apoptosis. In contrast, if cycling cells are

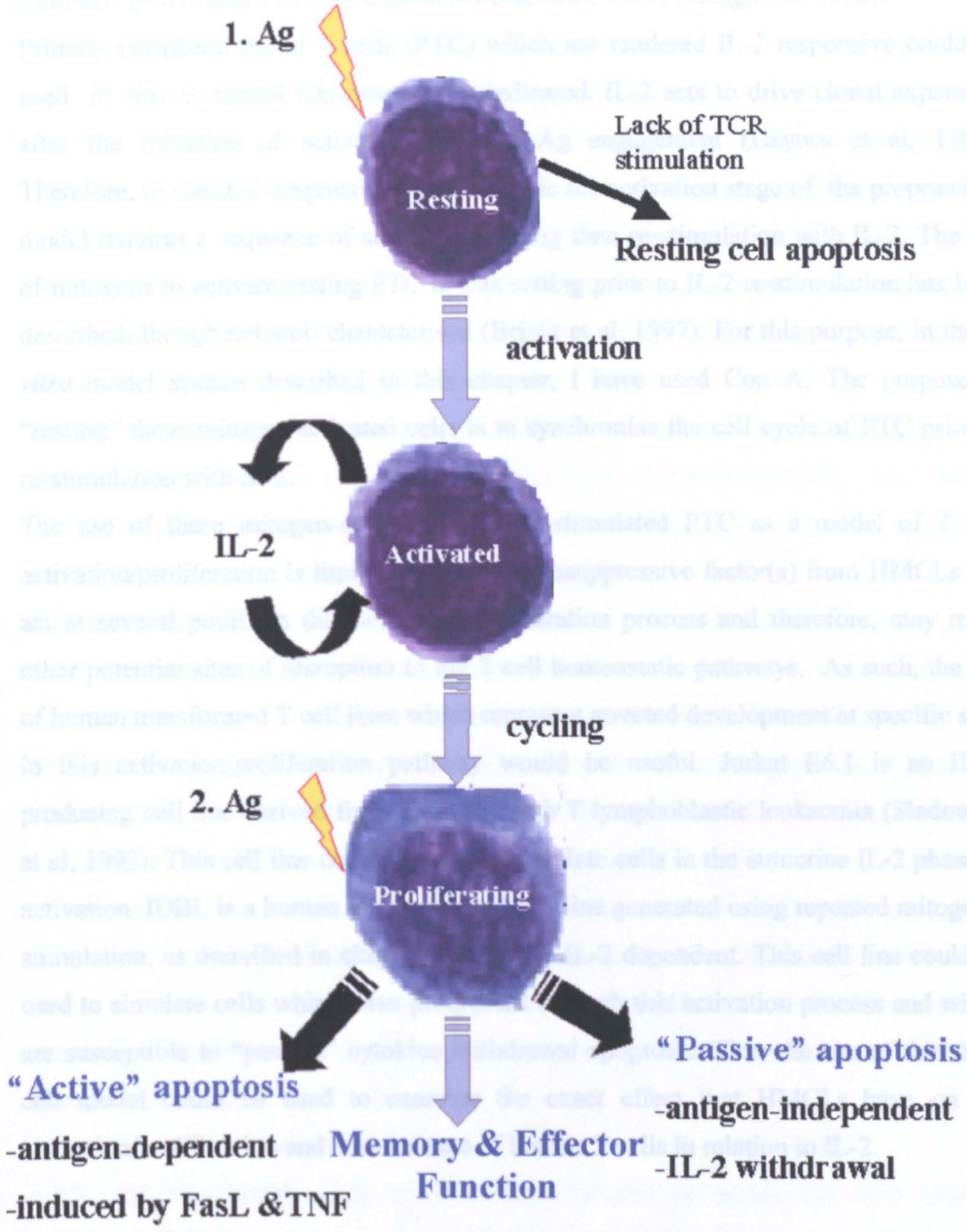


FIGURE 4.1. Schematic representation of the propriocidal paradigm of T lymphocytes. T cell activation occurs in 2 phases: activation leading to entry into the IL-2 autocrine pathway and proliferation resulting from cell cycle progression.

re-exposed to antigen, "active" or antigen-induced apoptosis can occur mediated by Fas/FasL system and TNFα (Alexander-Miller et al, 1996; Zheng et al, 1998).

Primary peripheral blood T cells (PTC) which are rendered IL-2 responsive could be used *in vitro* to model this process. As indicated, IL-2 acts to drive clonal expansion after the initiation of activation by TCR-Ag engagement (Haynes et al, 1999). Therefore, to use IL-2-responsive PTC to mimic the activation stage of the propriocidal model requires a sequence of activation, resting then re-stimulation with IL-2. The use of mitogens to activate resting PTC in this setting prior to IL-2 re-stimulation has been described, though not well characterised (Bright et al, 1997). For this purpose, in the *in vitro* model system described in this chapter, I have used Con A. The purpose of "resting" these mitogen-activated cells is to synchronise the cell cycle of PTC prior to re-stimulation with IL-2.

The use of these mitogen-activated, IL-2 re-stimulated PTC as a model of T cell activation/proliferation is limited if the immunosuppressive factor(s) from HMCLs can act at several points in this activation/proliferation process and therefore, may mask other potential sites of disruption to the T cell homeostatic pathways. As such, the use of human transformed T cell lines which represent arrested development at specific sites in this activation/proliferation pathway would be useful. Jurkat E6.1 is an IL-2-producing cell line derived from a patient with T lymphoblastic leukaemia (Sladowski et al, 1993). This cell line could be used to simulate cells in the autocrine IL-2 phase of activation. IDBL is a human T lymphoblast cell line generated using repeated mitogenic stimulation, as described in chapter 2, which is IL-2 dependent. This cell line could be used to simulate cells which have progressed through this activation process and which are susceptible to "passive" cytokine withdrawal apoptosis. Thus the use of this three cell model could be used to examine the exact effect that HMCLs have on the activation/proliferation and maintenance of human T cells in relation to IL-2.

Experimental Design

The aim of the work presented in this chapter was to establish an *in vitro* model of T cell activation, which will permit detailed investigation of myeloma-derived mediators of immuno-suppression. The development of such a model will enable the delineation of the precise point in the activation process where such mediators act and provide a model to examine the molecular events that are disrupted.

4.2 MATERIALS AND METHODS

4.2.1 GENERATION OF IL-2 RESPONSIVE T CELLS.

PBMNC were prepared from peripheral blood of normal healthy volunteers as described in chapter 2. PBMNC were re-suspended at a concentration of $4x10^6$/ml and stimulated with 5μg/ml of Con A for 5 days in CM. Following this culture period, the cells were washed three times in cold PBS. The cells were then re-suspended in one of the following culture media: CM, RPMI supplemented with 0.5% FCS or X-Vivo15 culture medium (BioWhitaker, UK). The effect that these media had on resting proliferation rates, as determined by [^{3}H]-dThd uptake, and ability to support re-stimulation was tested, as described in the results section. This culture period without stimulation was intended to synchronise the cell cycle staging of the activated PBL. The optimal duration of this cell cycle synchronisation phase was assessed by [^{3}H]-dThd uptake as an indicator of S phase entry and proliferation. Cell cycle analysis was performed by flow cytometry analysis of PI DNA staining, as described in chapter 3.

Following the cell cycle synchronisation period, the viable cells were purified by density-gradient centrifugation using Ficoll-Hypaque (Sigma, UK). Recovered cells were washed twice in PBS before being re-stimulated in CM supplemented by rhIL-2 (Sigma, UK) at the dose and for the duration described in the results section. Proliferation was determined by ^{3}[H] thymidine uptake.

The phenotypic characteristics of the IL-2-responsive primary T cells were analysed by flow cytometry. Cells ($5x10^5$/sample) were stained by direct immunofluoresence for two-colour flow cytometry using isotype-matched controls, as described in chapter 2. Monoclonal antibodies used are listed in chapter 3 and data was analysed using Cell Quest software (Becton Dickinson).

4.2.2 GENERATION AND MAINTENANCE OF HUMAN TRANSFORMED CELL LINES.

The IDBL cell line was generated as described in chapter 2. The cell viability and proliferation were maintained in CM supplemented with hrIL-2 20U/ml. Cells were maintained at a concentration of 10^4-10^5/ml and were sub-cultured twice per week when fresh CM supplemented with rhIL-2 20 U/ml was added. Once a week, dead cells and debris were removed by density-gradient centrifugation using Ficoll-Hypaque. On

recovering the viable cells from the Ficoll-Hypaque, cells were washed three times in PBS before being re-suspended in CM supplemented with IL-2 20U/ml. IDBL cells were used at low passage numbers, i.e. 2-30, and prior to use in the experiments, cells were washed three times in PBS before being re-suspended at the cell concentrations indicated in the result section in CM supplemented with IL-2. Experiments to establish the cell division kinetics were conducted with varying doses and exposure of IL-2, with the proliferative responses assessed by [^{3}H]-dThd uptake and cell cycle analysis performed by flow cytometry analysis of PI DNA staining, as described in chapter 3. The effect of cytokine starvation on the apoptosis of IDBL cells was assessed by the Annexin V affinity assay.

Jurkat cells are cytokine-independent cells, which produce IL-2 and interferon-α. Cells were maintained at a concentration of 10^4-10^5/ml and were sub-cultured twice per week when fresh CM was added. Once a week, dead cells and debris were removed by density-gradient centrifugation using Ficoll-Hypaque. On recovering the viable cells from the Ficoll-Hypaque, cells were washed three times in PBS before being re-suspended in CM. Prior to use in experiments, cells were washed three times in PBS and re-suspended in CM at the cell concentrations indicated in the results section. Cell division kinetics was examined in proliferation experiments where proliferation was assessed by [^{3}H]-dThd uptake. Cell cycle analysis was analysed by PI DNA staining.

The phenotypic characteristics of the transformed human T cell lines were analysed by flow cytometry. Cells ($5x10^5$/sample) were stained by direct immunofluoresence for two-colour flow cytometry using isotype-matched controls, as described in chapter 2. Monoclonal antibodies used are listed in chapter 3 and data was analysed using Cell Quest software (Becton Dickinson).

4.2.3 INTRACELLULAR CYTOKINE STAINING.

Intracellular cytokine staining is a method that allows a snapshot in time of the protein content of a single cell. The aim is to make use of the rapid flow cytometric analysis of cells to provide both surface phenotype and intracellular cytokine content of single cells within a population of cells responding to antigenic or mitogenic stimulation. The technique initially requires fixation of cells in paraformaldehyde, which cross-links proteins and prevents their loss through leakage. Secondly, permeabilization of cellular membranes with a detergent generates pores in the membrane, allowing specific

cytokine antibodies access to the cells' interior. The cytokine specific antibodies are fluorochrome-conjugated thus fluorescent labelled cells are indicative of cytokine production with the intensity of staining being proportional to the amount of cellular protein present. This technique ultimately allows for the investigation of the cytokine secretory pattern of individual cells along with their surface phenotype features. Northern blotting, *in situ* hybridization, RT-PCR, cellular bioassays and sandwich ELISAs, which have been recognised as suitable assays for studying the cytokine profile of activated cells, are time consuming, labour intensive and in some cases only give information that reflects the properties of the entire population of cells being analysed. In contrast, intracellular cytokine staining of cells and subsequent flow cytometric analysis can yield rapid and specific information on the cytokine production properties of individual cells within a mixed population of cells.

Cells to be analysed by intracellular cytokine staining were first washed three times in cold PBS and re-suspended at a concentration of $1x10^6$/ml in Fix and Perm solution (Beckton Dickinson, USA). If cells were to be surface stained, then this antibody was added and the cells washed before re-suspending in Fix and Perm solution. Cells were incubated in this solution for 10 minutes at room temperature. The cells were then washed three times in PBS. 2.5 µl of the anti-cytokine antibody conjugated to a fluorochrome (Anti-IL-2 conjugated with FITC) was added to the cells and incubated for 20 minutes at room temperature in the dark. Appropriate isotype control antibodies were used to determine auto-fluorescence. Cells were then acquired using a FACScan (Beckton Dickinson) and analysed using Cellquest software.

4.2.4 MAGNETIC CELL SORTING OF CELL POPULATIONS.

After 5 days of *in vitro* stimulation with Con A, subsets of PBL were separated on the basis of surface expression of IL-2Rα (CD25) using the MACS magnetic cell sorting system by Miltenyi et al, (1990). This system differs from other magnetic separation techniques in that the magnetic beads conjugated to the secondary antibody are extremely small which allows cells to analysed by flow cytometry immediately post separation as the beads do not have to become detached from the cells and membrane perturbation is kept to a minimum. In brief, the basic principle of MACS cell separation results from cells being labelled with a specific primary monoclonal antibody – in this instance anti-human CD25 – which is PE-conjugated. A secondary antibody directed against PE and conjugated to metal beads is added and the cell suspension is passed

through a column containing plastic coated steel wool in the presence of a powerful magnet. This results in positively labelled cells being retained within the column with non-magnetic cells being flushed out. The magnetic-labelled cells are then recovered by removing the magnetic source and flushing the cells out, as summarised in Figure 4.2 (A-K).

The autoclaved column was assembled with a sterile 3-way-stopcock. A leur-lock syringe filled with ice-cold PBS/1%BSA was attached to the side port and the assembled column was placed into the MACS magnetic separator and filled with the PBS/1%BSA solution from the syringe (Figure 4.2 A & B). The column was allowed to soak in PBS/1%BSA until use allowing protein to coat the column and minimise cellular damage. Immediately prior to use, the syringe was then refilled with ice-cold PBS/1%BSAand several volumes were passed through the column (Figure 4.2 C). To restrict the flow maximising the positive selection of magnetic bead-labelled cells, a hypodermic needle (retained within its sheath) was attached to the down port of the 3-way-stopcock, with the flow indicator in the "no flow" position and the tip of the plastic needle sheath was cut using pliers (Figure 4.2 D). The column was then ready to receive appropriately labelled cell suspensions.

Labelling of PBL was performed using cells that had been washed in PBS/1%BSA three times and kept on ice until required. The cells were counted and the cell suspension was centrifuged at 1,500G for 5 minutes. The supernatant was decanted and 10µl/10^7cells of mouse-anti-human CD25 monoclonal antibody conjugated to PE (PharMingen, Europe) was added. After 30 minutes incubation on ice the cells were washed twice with PBS/1%BSA and re-suspended with anti-PE monoclonal antibody conjugated metal beads (10 µl per 10^7 cells), which was free of sodium azide (removed as per manufacturers instructions). The cells were incubated in the dark at 4^0C (in a refrigerator) for 15 minutes. The cells were then washed in PBS/1%BSA and re-suspended at 10^7/ml and added to the prepared column (Figure 4.2 E).

Once the labelled cells were added to the column, 5 volumes of ice-cold PBS/1%BSA were added from the top of the column allowing the cell suspension to drip through the column and the non-magnetic population (negative fraction: $CD25^{Dim}$) were collected via the down-port (Figure 4.2 F). The column was then removed from the

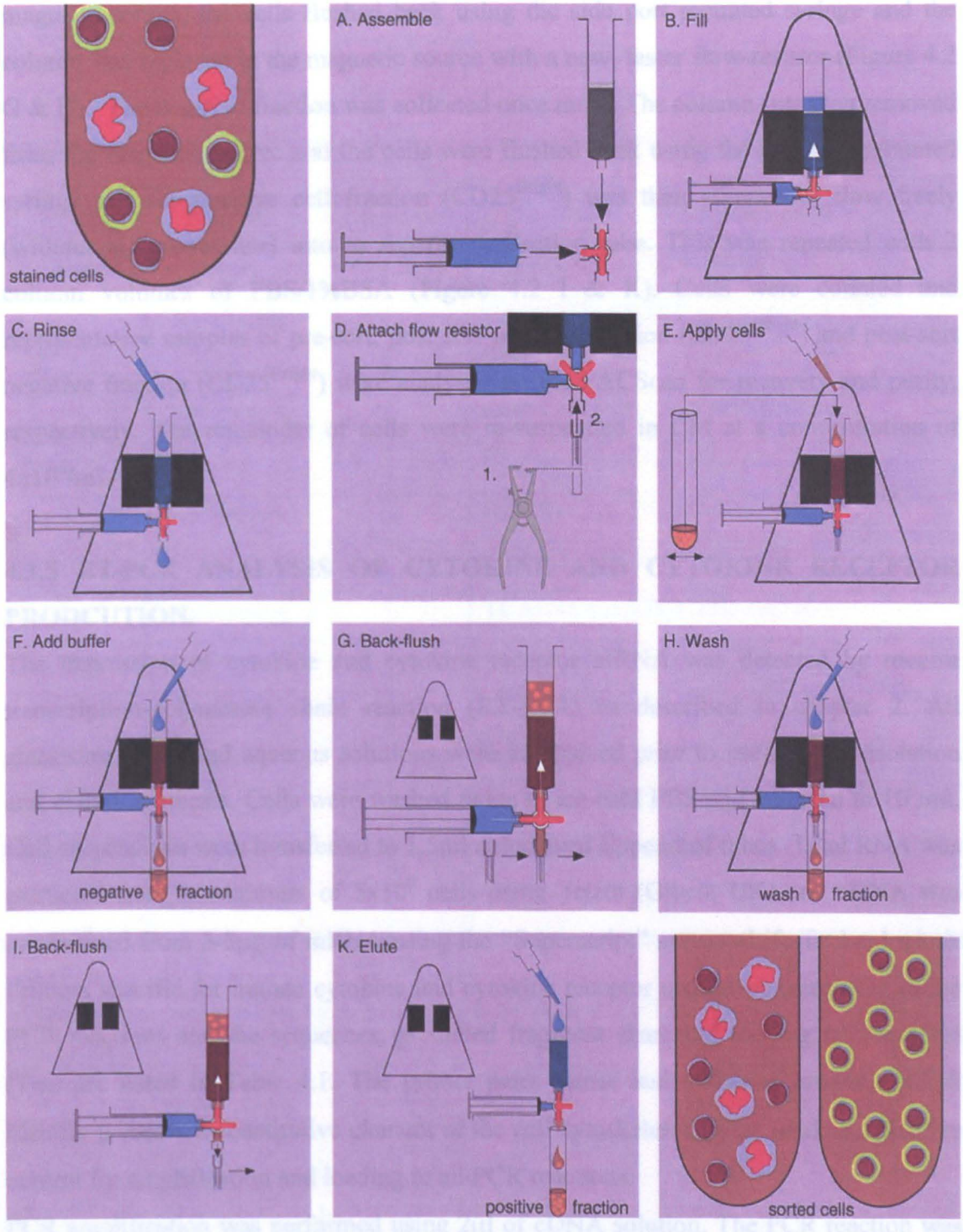


FIGURE 4.2. Diagram summarising the procedure for sorting cells using magnetic beads coated with MoAb directed against FITC or PE conjugated primary MoAB).

magnetic source, the cells flushed back using the side port mounted syringe and the column was replaced in the magnetic source with a new, faster flow-resistor (Figure 4.2 G & H). The negative fraction was collected once more. The column was then removed from the magnetic source and the cells were flushed back using the side-port mounted syringe and the positive cell fraction ($CD25^{Bright}$) was then allowed to flow freely (without a flow-resistor) into to separate collection tube. This was repeated with 2 column volumes of PBS/1%BSA (Figure 4.2 I & K). Cells were counted and representative samples of pre-sort, post-sort positive fraction ($CD25^{Bright}$) and post-sort negative fraction ($CD25^{Bright}$) were analysed on the FACScan for recovery and purity, respectively. The remainder of cells were re-suspended in CM at a concentration of $4x10^6$/ml.

4.2.5 RT-PCR ANALYSIS OF CYTOKINE AND CYTOKINE RECEPTOR PRODCUTION.

The expression of cytokine and cytokine receptor mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) as described in chapter 2. All glassware, tubes and aqueous solutions were autoclaved prior to use in RNA isolation and cDNA synthesis. Cells were washed twice in ice cold PBS and adjusted to 10^7/ml. Cell suspensions were transferred to 1.5ml autoclaved Eppendrof tubes. Total RNA was extracted from a minimum of $5x10^6$ cells using Trizol (Gibco, UK) and cDNA was synthesized from 3-5μg of mRNA using the "Superscript" system (Life Technologies). Primers specific for human cytokine and cytokine receptor sequences were used in the PCR reactions and the sequences, predicted fragment sizes and melting temperatures (Tm) are listed in Table 4.1. The primer pairs (sense and antisense primers) which identify β-Actin, a constitutive element of the cell cytoskeleton, were used as a positive control for amplification and loading in all PCR reactions.

PCR amplification was performed using 2μl of cDNA solution. The PCR reaction was carried out in 50μl volumes as described in chapter 2 and control samples, which contained all reaction constituents except cDNA, were used to exclude environmental contamination. The PCR primers were in-house sequences with known optimal reaction conditions except the sense and anti-sense primer pairs to amplify IL-2Rβ and γ_c chains that have previously been described by Steinberger et al (1997). The samples were heated to 95^0C for 5 minutes to ensure that all the RNA/DNA complexes were

	Strand	AT (^{0}C)	Size (bp)	5'-Seqnuence-3'
β-Actin	Sense	60	548	GTGGGGCGCCCCAGGCACCA
	Anti-sense			CTCCTTAATGTCACGCACGATTTC
IL-2	Sense	55	266	ACTCACCAGGATGCTCACAT
	Anti-sense			AGATAATCCATCTGTTCAGA
IL-2Rα	Sense	60	781	CCTGCTGATGTGGGGACTG
	Anti-sense			GTCTCCGCTGCCAGGTGA
IL-2Rγc	Sense	60	420	CCAGAAGTGCAGCCACTATC
	Anti-sense			TCACTCCAATGCTGAGCACT
IL-2Rβ	Sense	60	531	ACCTCTTGGGCATCTGCACC
	Anti-sense			CGTCTCCAGGCAGATCCATT
IL-4	Sense	60	456	ATGGGTCTCACCTCCCAACTGCT
	Anti-sense			CGAACACTTTGAATATTTCTCTCTCAT
Interferon-γ	Sense	60	494	ATGAAATATACAAGTTATATCTTGGCTTT
	Anti-sense			GATGCTCTTCGACCTCGAAACAGCAT

TABLE 4.1 Oligonucleotide primers used for RT-PCR. **Key:** AT- annealing temperature.

completely denatured and amplification consisted of 30 cycles as follows: 95^0C for 1 minute (denaturation), 60^0C for 1 minute (primer annealing) and 72^0C for 1 minute (primer extension). A final step at 72^0C for 5 minutes was carried out to ensure complete extension of products. Following amplification, samples were visualised by 2% agarose gel electrophoresed. PCR amplification for IL-2R subunits were conducted using the above cycle conditions but 35 cycles were performed. PCR products were visualised by 2% agarose gel electrophoresis at 100V and the image captured using UViphoto software.

4.3 RESULTS

4.3.1 SUPPRESSION OF T CELL ACTIVATION BY HMCL IS NOT RESCUED BY INTERLUEKIN-2

As discussed in chapter 3, PB T cells activated by mitogen in the presence of HMCLs fail to proliferate which is associated with a lack of entry into the IL-2 autocrine pathway. This posed the question whether these cells could be rescued by exogenous IL-2, allowing entry into the IL-2 autocrine pathway, cell cycle progression and proliferation. Therefore, the mitogen-activation of PBMNC from normal healthy donors in the presence and absence of HMCLs, as described in chapter 3, was repeated with the addition of human recombinant IL-2 20 U/ml (rIL-2, Sigma, UK). Proliferation was measured by ^{3}[H] thymidine uptake in a 3 day assay. The suppression of proliferation of normal PBL in response to mitogen induced by the HMCLs, U266 and JJN3 was not reversed by the addition of rIL-2 (Figure 4.3). This demonstrates that HMCLs produce a suppressive factor(s), which controls the T cell activation process including cell cycle progression and proliferation. In order to investigate the mechanisms involved and to identify, in functional assays potential mediators of this immunosuppression, an *in vitro* model of T cell activation/proliferation needed to be established as stated.

4.3.2 GENERATION OF IL-2 RESPONSIVE PRIMARY T CELLS.

Culturing PBMNC with Con A for 5 days as described in the material and methods of this chapter, results in a purified population of activated primary T lymphocytes. At the end of the 5-day stimulation period, the surface phenotype of the viable cells was examined using the MoAb described in Table 3.1 using flow cytometry. This population of cells represented an enrichment of $CD3^+$ T cells (89.7%$\pm$4.1, n=4) with a mean 50.5%$\pm$6.1 $CD3^+/CD4^+$ cells and a mean 33.2%$\pm$3.6 $CD3^+/CD8^+$ cells. The $CD3^-$ cells were identified as NK cells by their surface expression of CD2 and CD56 with a lack of expression of TCR$\alpha\beta$. Most cells (72.6%$\pm$12.4) demonstrated CD45RO surface expression and 63.9%$\pm$2.3 $CD3^+$ cells demonstrated surface expression of the α subunit of the IL-2R. These results are summarised in Table 4.2 and representative dotplots are shown in Figure 4.4.

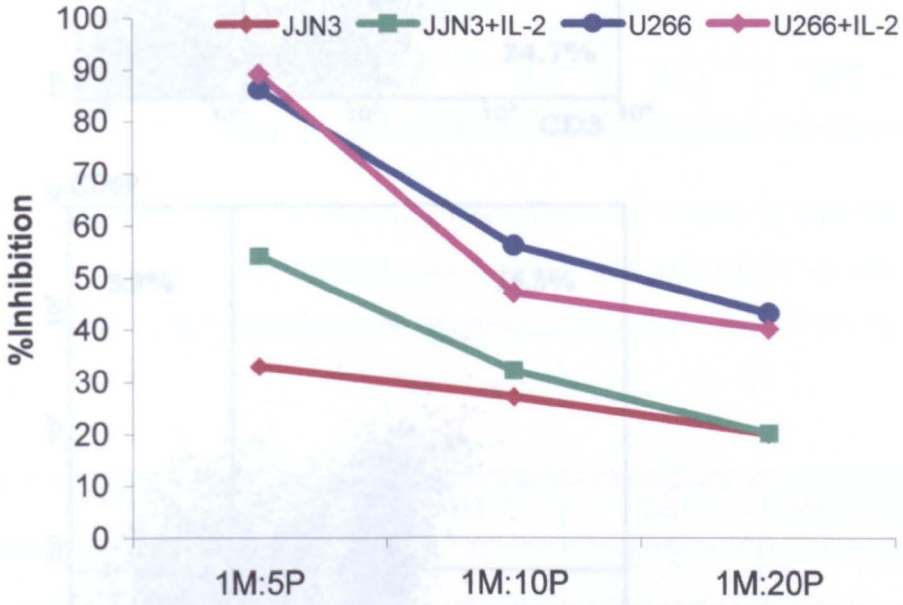


FIGURE 4.3. Inhibition of ConA-induced proliferation of PBMNC by HMCLs, U266 and JJN3, is not reversed by exogenous rhIL-2 in a 72 hour stimulation assay. Key: 1M:5P – indicates the stimulator:responder ratio, filled diamond – JJN3, filled square – JJN3+rhIL-2, filled circle – U266, filled triangle – U266+rhIL-2.

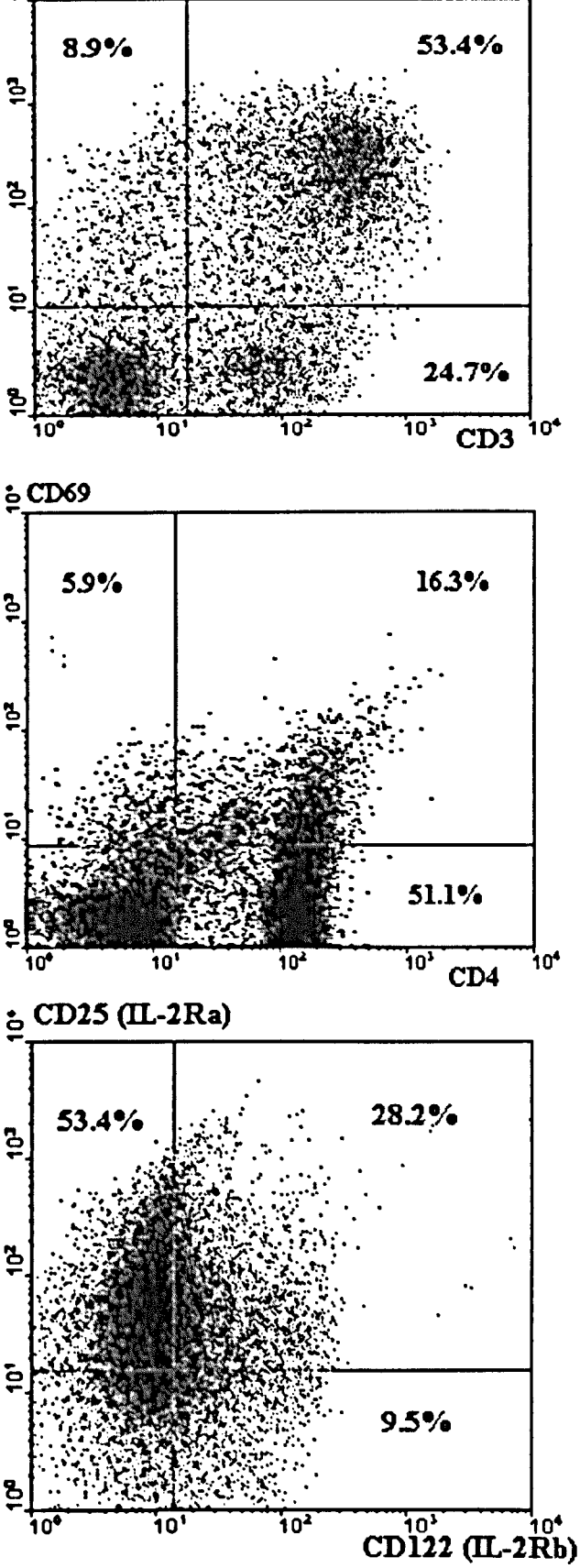


FIGURE 4.4. Representative dotplots of surface phenotype of PBL stimulated with ConA for 5 days. Immuno-fluorescent surface staining analysed by flow cytometry (FACScan, Becton Dickinson).

(%)	D1	D2	D3	D4	MEAN±SEM
$CD3^+$ Cells	98.1	81.3	82.17	97.3	89.7±4.1
$CD3^+/CD4^+$ Cells	30.01	53.6	56.99	61.4	50.5±6.1
$CD3^+/CD8^+$ Cells	42.1	28.8	26.18	35.7	33.2±3.6
$CD3^+/CD25^+$ Cells	63.83	57.44	67.08	67.13	63.9±2.3
$CD3^+/CD122^+$ Cells	46.2	12.6	54.3	37.8	37.7±9.1
$CD3^+/CD45RO^+$ Cells	76.6	94.8	37.4	81.5	72.6±12.4

TABLE 4.2 Surface phenotype of viable cells following 5 day culture of PBMNC from healthy donors (n=4) with ConA. Results are expressed as percentage of total cells expressing the indicated surface antigens as determined by isotype control MoAb, using flow cytometry.

	D1	D2	D3	MEAN±SEM
$CD25^{Bright}$				
$CD25^+$	89.6%	90.6%	87.9%	89.4%±0.8
$CD4^+$	92.2%	88.8%	86.5%	89.4%±1.9
$CD45RA^+$	35.1%	42.2%	39.7%	39.0%±2.1
$CD69^+/CD25^-$	21.1%	18.73%	19.6%	19.81%±0.7
$CD69^+/CD25^+$	53.26%	62.47%	64.8%	60.84%±2.9
$CD25^{Dim}$				
$CD25^+$	33.3%	26.6%	23.3%	27.7%±3.0
$CD4^+$	55.78%	62.1%	57.1%	58.94%±2.6
$CD45RA^+$	64.2%	55.8%	61.3%	60.4%±2.5
$CD69^+/CD25^-$	3.1%	2.53%	4.6%	3.41%±0.6
$CD69^+/CD25^+$	7.53%	8.69%	9.1%	8.44%±0.5

TABLE 4.3. Phenotypic analysis of T cells sorted on surface expression of IL-2Rα (CD25) after culture in 0.5%FCS/RPMI1640. Results are expressed, as the percentage of cells deemed positive for the surface expression of the antigen indicated based on comparison to the isotype control.

Mitogenic stimulation of PBMNC results in polyclonal proliferation of lymphocytes which will have widely differing proliferation and cell cycle kinetics, owing to many factors including whether they have prior exposure to specific antigen (i.e. memory cells). As a result, at the end of a 5-day stimulation culture using Con A, the cells will be at various stages of activation and cell cycle. In order to synchronize these cells in terms of cell cycle distribution, a "rest period" was employed to allow maximisation of the cells to the G_0/G_1stage of the cell cycle. However, the optimal medium to allow synchronization of cycling cells whilst maintaining viability had to be discerned. Using PBMNC from healthy donors (n=4), after a 5 day stimulation with Con A, as described above, cells were washed x3 with cold, sterile PBS. The cells were re-suspended at a concentration of $1x10^6$/ml and split into to three samples with each sample being incubated with one of three test media: CM, RPMI1640+0.5%FCS (with 2mM L-glutamine, 100 u/ml penicillin, 100μg/ml streptomycin) and X-Vivo. The basal proliferation was determined by ^{3}[H]tThd up-take in the last 6 hours of the "rest period". 0.5%FCS-supplemented RPMI1640 resulted in lower mean basal proliferation (691±287 cpm) than CM (2922±1553 cpm, p=0.137) and X-Vivo15 (2488±592 cpm, p=0.072), as illustrated in Figure 4.5.

The effect that resting in 0.5%FCS/RPMI1640 had on cell cycle distribution was examined using PI DNA-staining analysed by flow cytometry before and after the 24 hour resting incubation. The mean number of cells in G_0/G_1stage post Con A-stimulation was 65.49%±13.6, which rose to 85.4%±8.8 following resting in 0.5%FCS/RPMI1640 (Figure 4.6). Representative DNA histograms are illustrated in Figure 4.7.

It was important to determine the appropriate rhIL-2 dose and duration of exposure for the re-stimulation phase. When PBL from normal healthy donors (n=3) were rested in 0.5%FCS/RPMI1640 24 hours and the dead cells and debris were removed by density gradient separation, the PBL were re-stimulated with incremental doses of recombinant human rhIL-2 for 24, 36 and 48 hours with proliferative responses being measured by ^{3}[H] thymidine up-take. No significant difference in proliferative responses were noted between 20 U/ml and 100 U/ml at 24 or 48 hours (p=0.197 and p=0.338, respectively). When the proliferative response to 20U/ml of rhIL-2 was compared at 24, 36 and 48 hours, it was demonstrated that this was significantly higher at 36 hours compared to 24 hours (p=0.014) though no significant difference in proliferative responses were noted

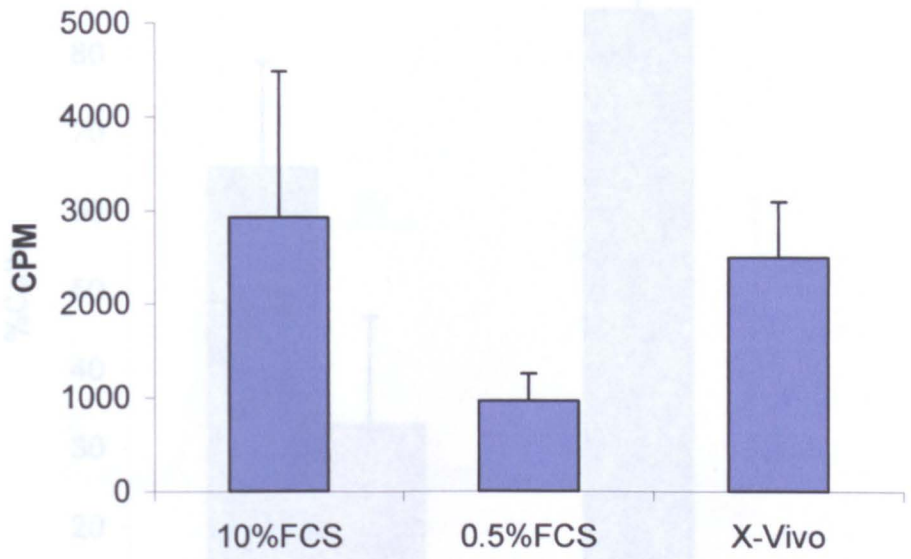


FIGURE 4.5. The effect of three "resting" media on the basal proliferation of mitogen activated PBMNC from normal donors (n=4). 0.5%FCS-supplemented RPMI1640 resulted in a lower mean basal proliferation than 10%FCS-supplemented RPMI1640 (p=0.137) and X-Vivo.

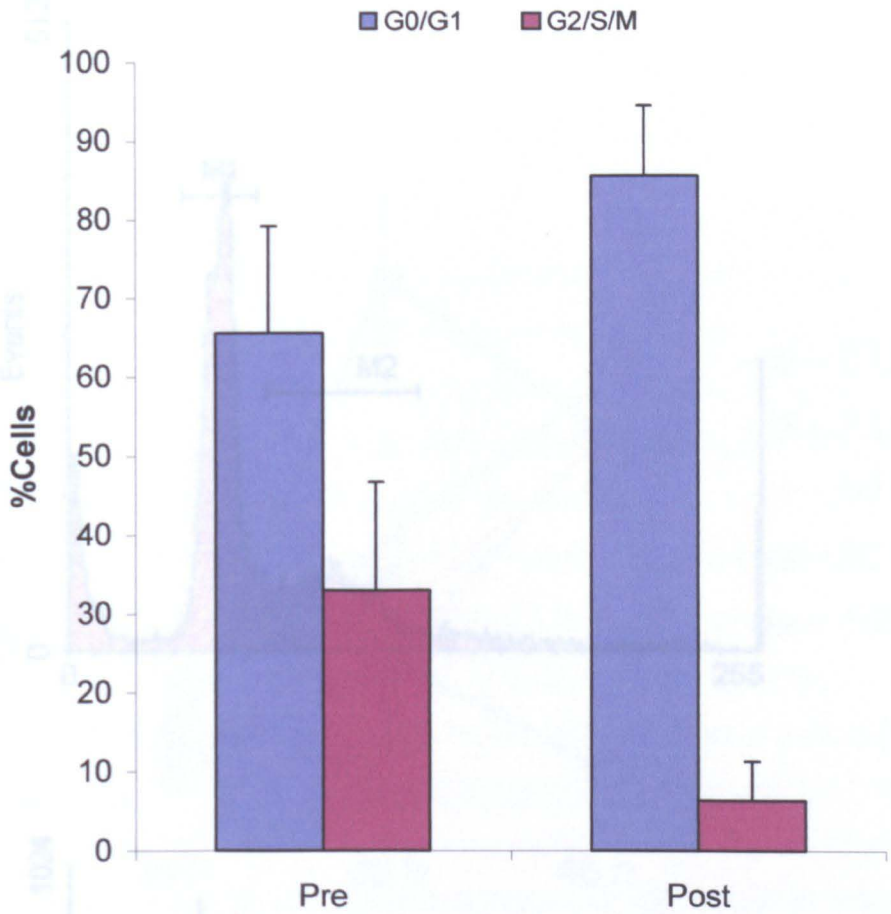
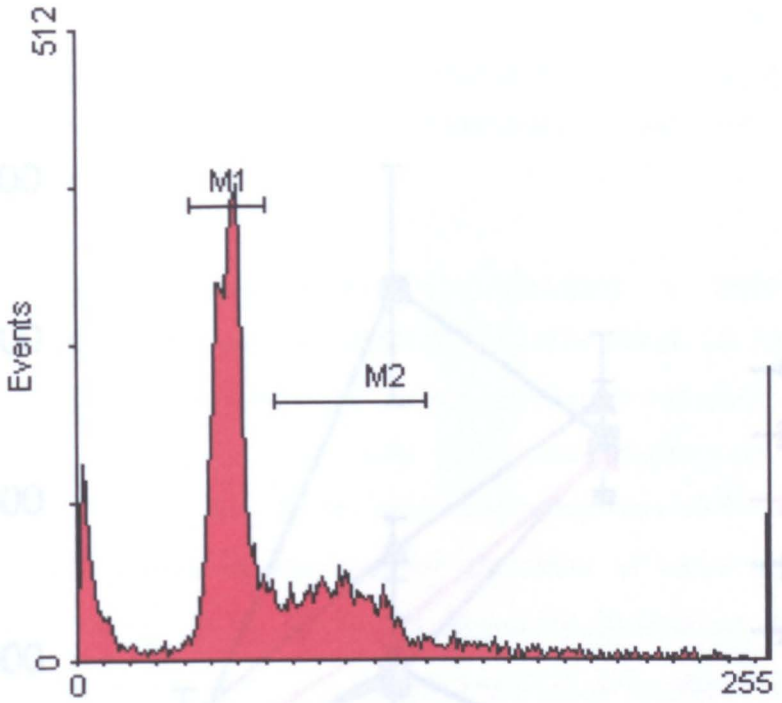


FIGURE 4.6. The effect on cell cycle synchronisation of ConA-activated T cells from normal donors (n=4) by culturing in 0.5% FCS-supplemented RPMI1640. Cells accumulate in G_0G_1 phase of cell cycle following culture for 24 hours (p=0.147).

A



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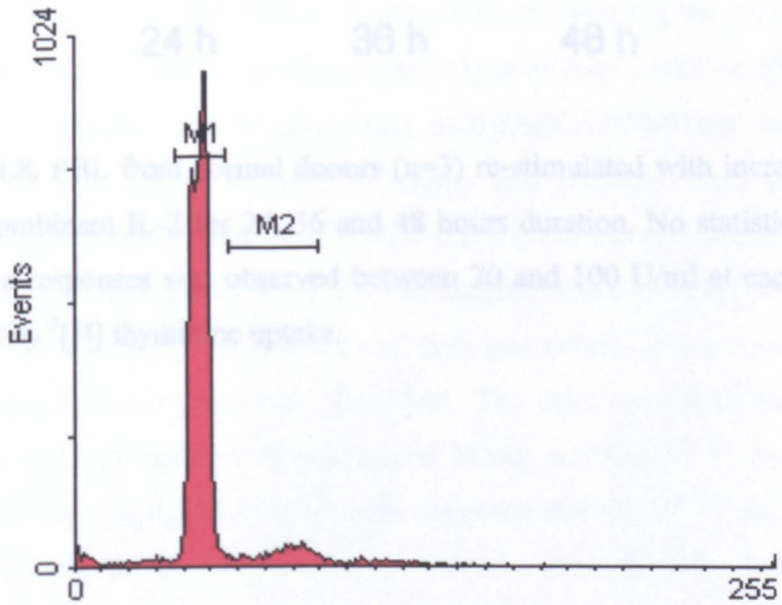


FIGURE 4.7. Representative DNA histograms of cell cycle analysis of primary T cells post-stimulation (A) and post-resting in 0.5%FCS/RPMI1640 (B). Marker M1 represents the diploid peak which contains the cells which are in G0/G1 phase of cell cycle and marker M2 represents those cells which are traversing the cell cycle phases of S, M and G_2.

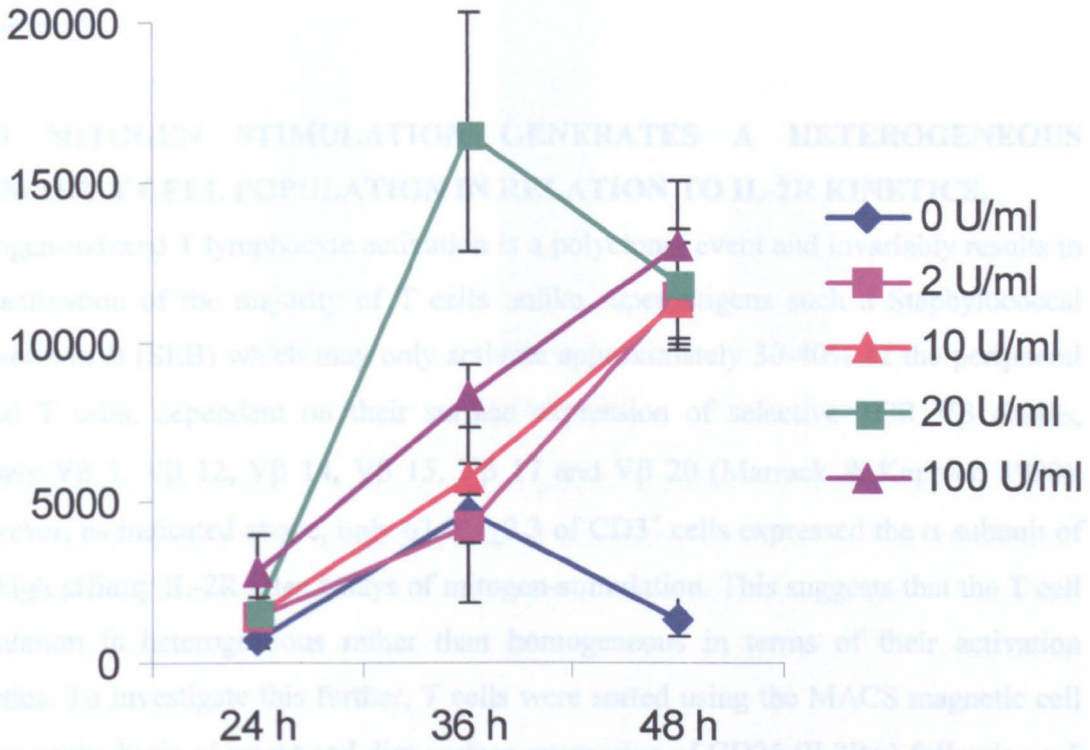


FIGURE 4.8. PBL from normal donors (n=3) re-stimulated with incremental doses of human recombinant IL-2 for 24, 36 and 48 hours duration. No statistical difference in proliferative responses was observed between 20 and 100 U/ml at each time point, as determined by ^{3}[H] thymidine uptake.

between 36 and 48 hours stimulation (p=0.157), as illustrated in Figure 4.8. Therefore, 0.5%FCS-supplemented RPMI1640 was adopted for the resting period followed by rhIL-2 20U/ml for 36 hours for the re-stimulation conditions in all subsequent experiments.

4.3.3 MITOGEN STIMULATION GENERATES A HETEROGENEOUS PRIMARY T CELL POPULATION IN RELATION TO IL-2R KINETICS.

Mitogen-induced T lymphocyte activation is a polyclonal event and invariably results in the activation of the majority of T cells unlike superantigens such a Staphylococcal enterotoxin B (SEB) which may only activate approximately 30-40% of the peripheral blood T cells, dependent on their surface expression of selective TCR Vβ chains, namely Vβ 3, Vβ 12, Vβ 14, Vβ 15, Vβ 17 and Vβ 20 (Marrack & Kappler, 1990). However, as indicated above, only 63.9%±2.3 of $CD3^+$ cells expressed the α subunit of the high affinity IL-2R after 5 days of mitogen-stimulation. This suggests that the T cell population is heterogeneous rather than homogeneous in terms of their activation kinetics. To investigate this further, T cells were sorted using the MACS magnetic cell sorter on the basis of bright and dim surface expression of CD25 (IL2Rα) following a 5 day stimulation with Con A and resting in 0.5%FCS/RPMI1640 for 24 hours. The efficiency of this system in terms of purity and recovery were assessed then the activation kinetics and phenotype of both T cell populations were examined.

PBMNC from normal donors (n=8) were stimulated with Con A for 5 days and rested in 0.5%FCS/RPMI1640 for 24 hours. Dead cells and debris were removed using density gradient separation, as previously described. The cells were then stained for surface expression of CD25 using a PE-conjugated MoAb and $CD25^{Bright}$ were selected using MACS. Prior to sorting, 64.4%±5.5 cells demonstrated $CD25^{Bright}$ surface staining and following sorting, the positive fraction contained a mean 87.9%±1.0 $CD25^{Bright}$ T cells whereas the negative fraction contained a mean 30.2%±3.7 $CD25^{Bright}$ T cells (Figure 4.9, $p<0.001$). Whilst providing a purification of T cells on the basis of surface expression of CD25, the recovery of cells from this system is of equal importance. When the absolute numbers of $CD25^{Bright}$ and $CD25^{Dim}$ T cells were calculated pre-sorting and in the positive and negative fractions respectively, a mean recovery of 34.6%±6.3 $CD25^{Bright}$ cells and 56.1%±7.8 $CD25^{Dim}$ cells was noted (Figure 4.10).

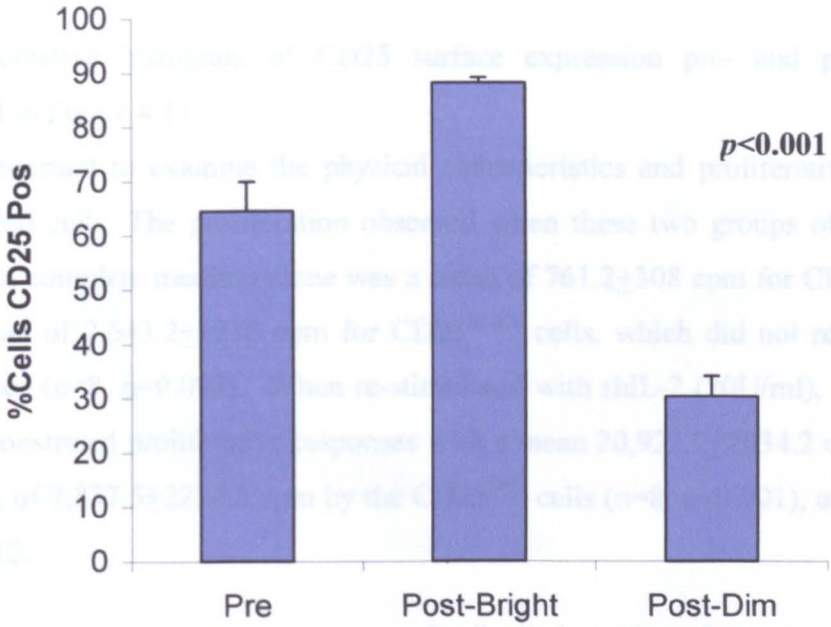


FIGURE 4.9. The surface phenotype of peripheral T cells from normal donors (n=8) stimulated for 5 days with ConA then sorted according to CD25 surface expression. Key: Pre – Percentage $CD25^{Bright}$ T cells prior to sorting, Post-Bright- Percentage $CD25^{Bright}$ T cells in the positive fraction, Post-Dim- Percentage $CD25^{Bright}$ T cells in the negative fraction.

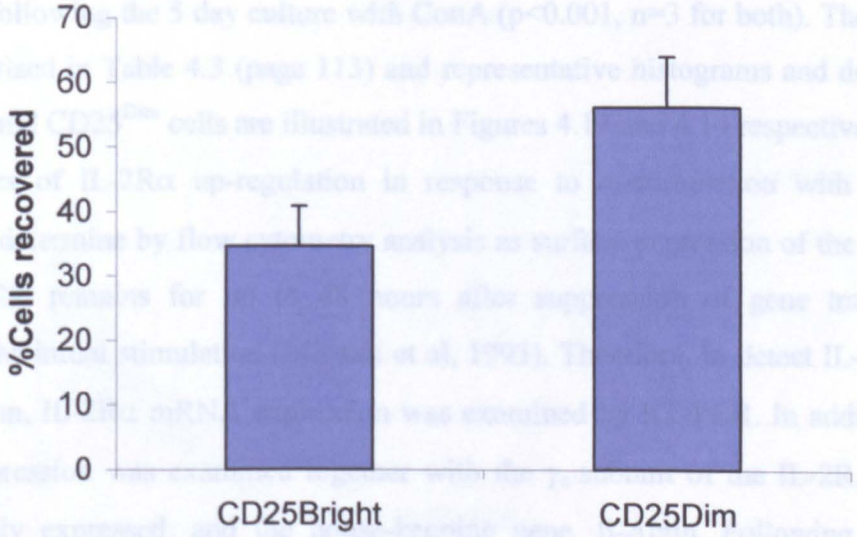


FIGURE 4.10. The recovery of $CD25^{Bright}$ and $CD25^{Dim}$ T cells in the positive and negative fractions respectively.

A representative histogram of CD25 surface expression pre- and post-sorting is illustrated in Figure 4.11.

It was important to examine the physical characteristics and proliferative capacity of these sorted cells. The proliferation observed when these two groups of T cells were cultured in complete medium alone was a mean of 761.2±308 cpm for $CD25^{Dim}$ T cells and a mean of 2,643.2±1218 cpm for $CD25^{Bright}$ cells, which did not reach statistical significance (n=8, p=0.093). When re-stimulated with rhIL-2 (20U/ml), the $CD25^{Bright}$ cells demonstrated proliferative responses with a mean 20,922.2±2034.2 cpm compared to a mean of 7,837.5±2224.3 cpm by the $CD25^{Dim}$ cells (n=8, $p<0.001$), as illustrated in Figure 4.12.

To further characterise these two groups of cells, their surface phenotype was examined by flow cytometry analysis following culture in 0.5%FCS/RPMI1640 for 24 hours. $CD25^{Bright}$ cells contained more $CD4^+$ T cells than the $CD25^{Dim}$ population (89.4%±1.9 vs. 58.94%±2.6, $p<0.001$, n=3) with fewer cells with the naive surface phenotype ($CD45RA^+$: 39.0%±2.1 vs. 60.4%±2.5, p=0.0013, n=3). When the surface activation marker CD69 was examined in conjunction with CD25 surface expression, more $CD25^{Bright}$ cells had progressed to the $CD69^+/CD25^+$ and $CD69^-/CD25^+$ stages of activation following the 5 day culture with ConA ($p<0.001$, n=3 for both). These results are summarised in Table 4.3 (page 113) and representative histograms and dot plots of $CD25^{Bright}$ and $CD25^{Dim}$ cells are illustrated in Figures 4.13 and 4.14 respectively.

The kinetics of IL-2Rα up-regulation in response to re-stimulation with rhIL-2 is difficult to determine by flow cytometry analysis as surface expression of the α subunit of the IL-2R remains for up to 48 hours after suppression of gene transcription following the initial stimulation (Minami et al, 1993). Therefore, to detect IL-2Rα gene up-regulation, IL-2Rα mRNA expression was examined by RT-PCR. In addition, IL-2 mRNA expression was examined together with the γ_c subunit of the IL-2R, which is constitutively expressed, and the house-keeping gene, β-Actin. Following 24 hours incubation with rhIL-2 (20U/ml), IL-2Rα and IL-2 mRNA could be detected in $CD25^{Bright}$ cells along with IL-2Rγ_c and β-Actin whereas $CD25^{Dim}$ cells did not express detectable levels of IL-2Rα or IL-2 mRNA but still expressed IL-2Rγ_c and β-Actin mRNA (Figure 4.15).

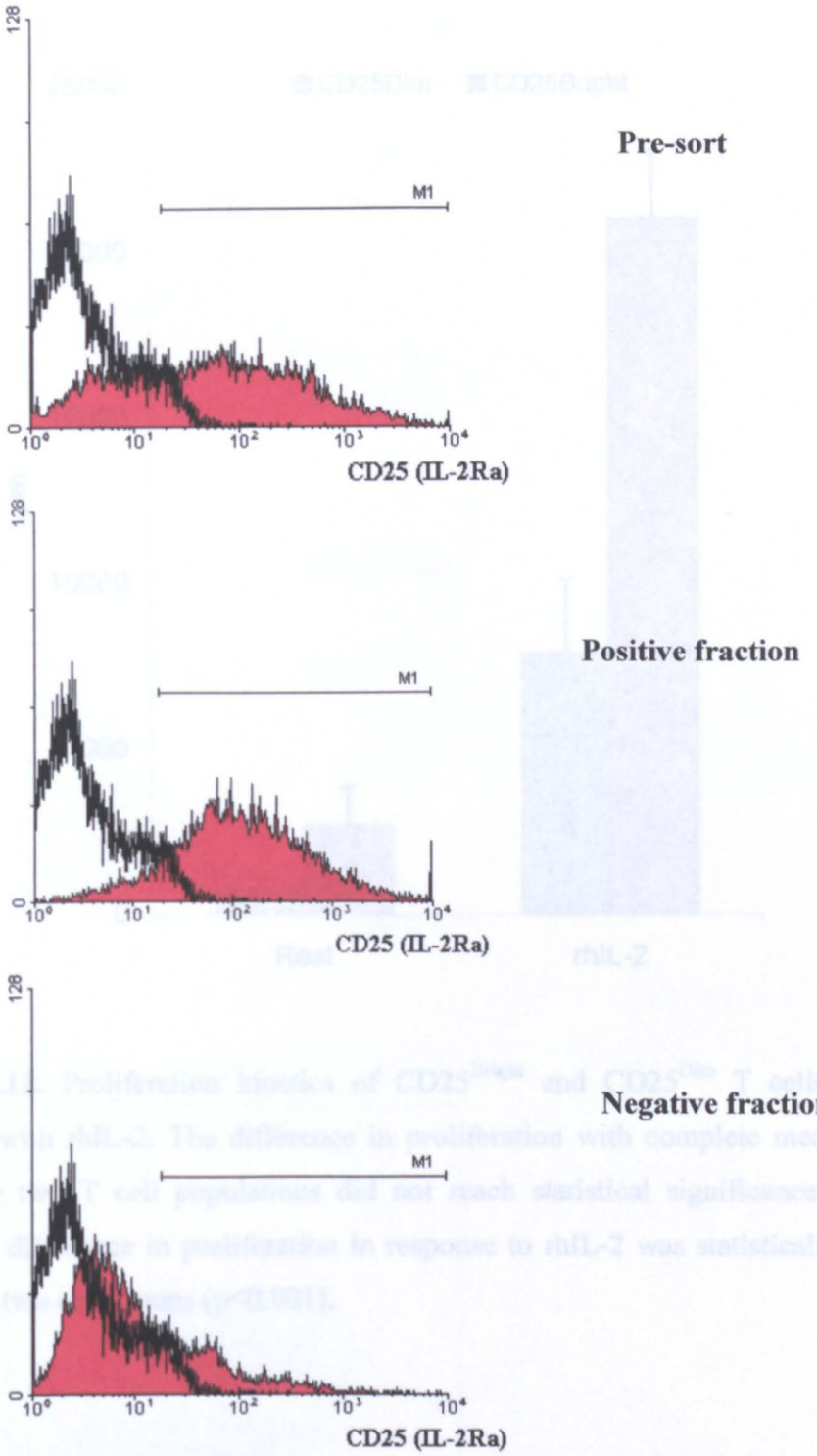


FIGURE 4.11. Representative histograms of CD25 surface expression of peripheral blood T cells stimulated with ConA for 5 days. Histograms indicate CD25 surface expression before sorting and after sorting. Hollow histogram represents isotype control MoAb.

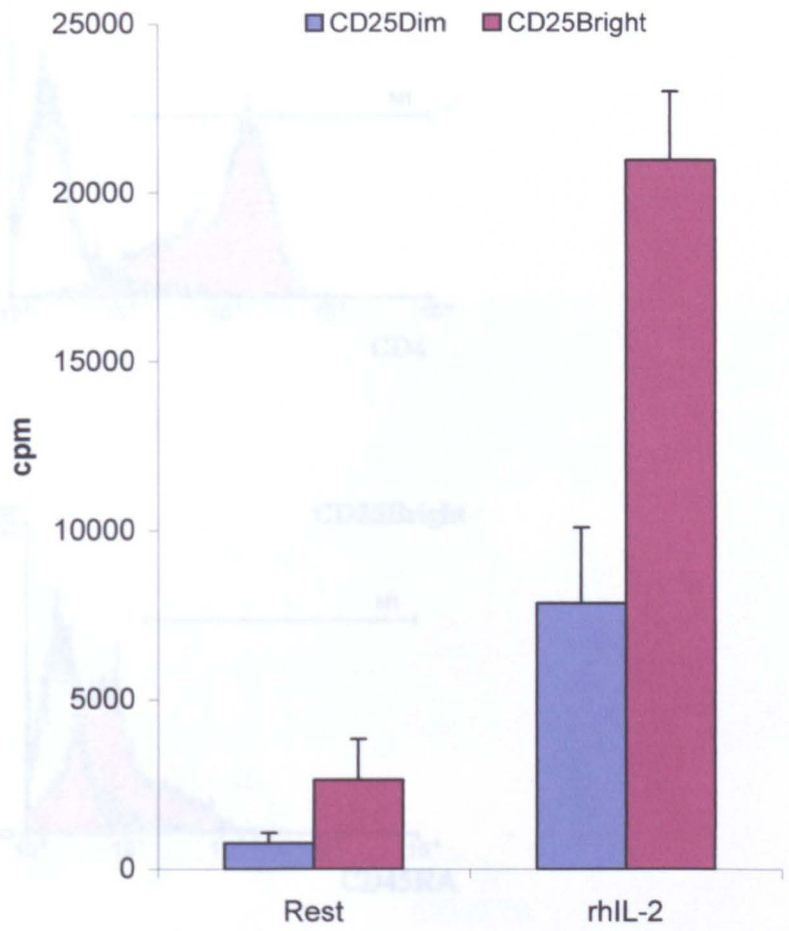
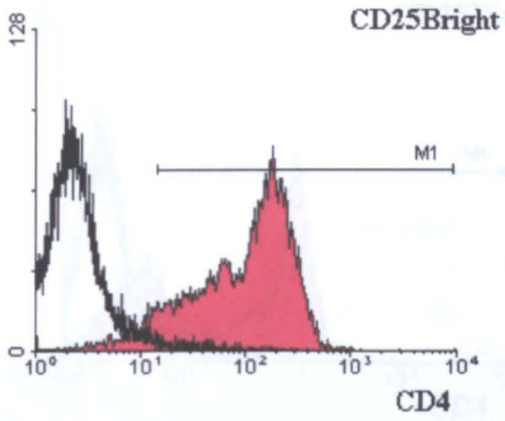


FIGURE 4.12. Proliferation kinetics of $CD25^{Bright}$ and $CD25^{Dim}$ T cells upon re-stimulation with rhIL-2. The difference in proliferation with complete medium alone between the two T cell populations did not reach statistical significance ($p=0.093$) whereas the difference in proliferation in response to rhIL-2 was statistically different between the two cell groups ($p<0.001$).

A



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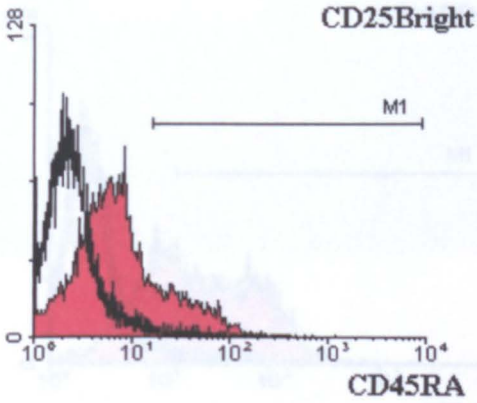

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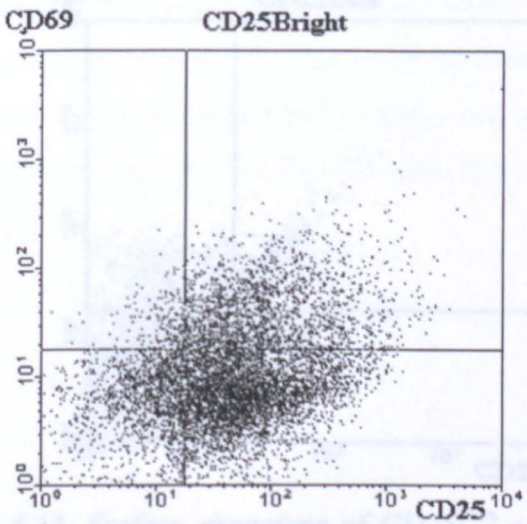
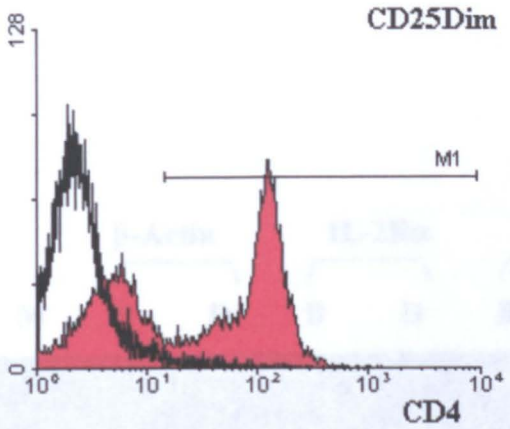


FIGURE 4.13. Surface phenotype of $CD25^{Bright}$ cells cultured in 0.5%FCS/RPMI1640 for 24 hours. (A) CD4 expression, (B) CD45RA expression and (C) CD69 and CD25 expression.

A



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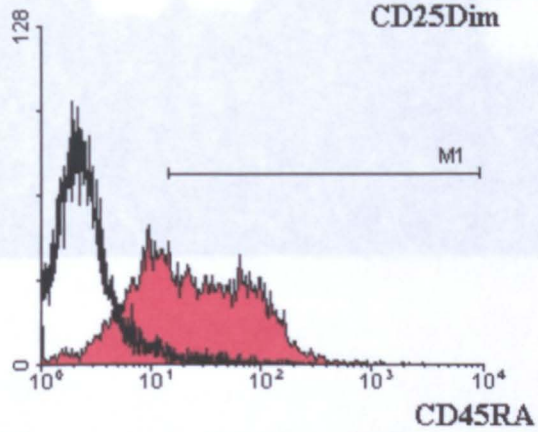

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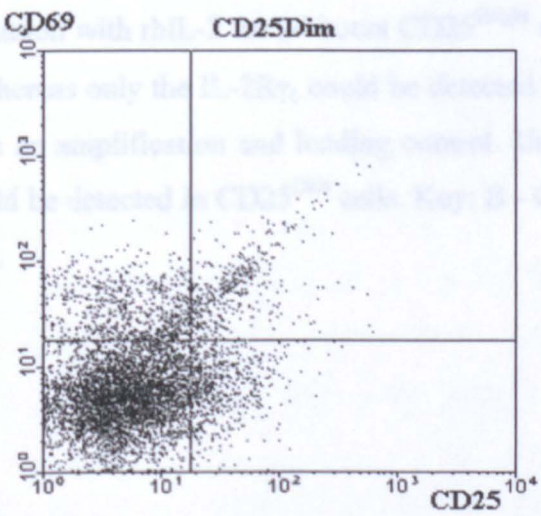


FIGURE 4.14. Surface phenotype of $CD25^{Dim}$ cells cultured in 0.5%FCS/RPMI1640 for 24 hours. (A) CD4 expression, (B) CD45RA expression and (C) CD69 and CD25 expression.

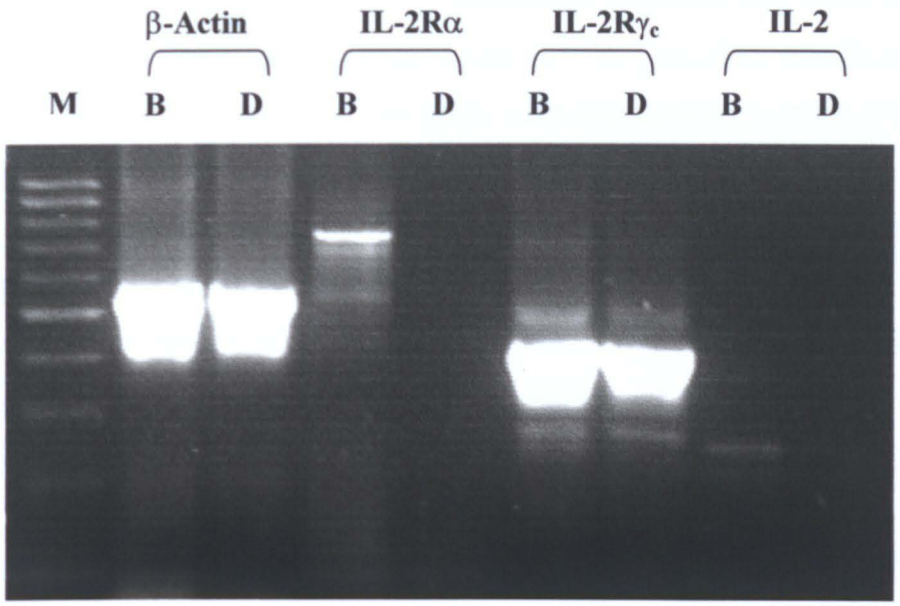


FIGURE 4.15. Expression of IL-2Rα, IL–2Rγc, and IL-2 mRNA detected by RT-PCR. After stimulation with rhIL-2 for 24 hours $CD25^{Bright}$ cells expressed the IL-2R α and γc sub-units whereas only the IL-2Rγc could be detected in $CD25^{Dim}$ cells. β-Actin mRNA was used as an amplification and loading control. Unlike the $CD25^{Bright}$ cells, no IL-2 mRNA could be detected in $CD25^{Dim}$ cells. Key: B - $CD25^{Bright}$ cells, D- $CD25^{Dim}$ cells, M- markers.

4.3.4 CHARACTERISATION OF A HUMAN IL-2-DEPENDENT T CELL LINE.

An IL-2-dependent blast cell line (IDBL) was generated as described in chapter 2. Cells were used in these experiments were maintained in complete medium supplemented with rhIL-2 and used at low passage numbers (2-30). I sought to characterise this cell line in terms of the surface phenotype, expression of cytokines and their ligands and the proliferation kinetics. Phenotypically, IDBL consistently expressed low levels of CD3, IL-2Rβ (CD122) and CD4 but no detectable levels of CD2, or IL-2Rα (CD25) could be demonstrated (Table 4.4, Figure 4.16). The IDBL cells expressed high levels of Fas and the activation marker, CD69, on their surface (Figure 4.16).

By RT-PCR, IDBL did not express the mRNA of the cytokines IL-2, interferon-γ or IL-4 (Figure 4.17). When analysed after 24, 48 and 72 hours of culture with rhIL-2, no expression of IL-2Rα mRNA could be detected by RT-PCR (Figure 4.18). Whilst IL-2Rγ_c mRNA could be detected after 72 hours of culture with rhIL-2, IL-2Rβ mRNA could not be amplified by RT-PCR on bulk-populations despite detection of surface expression, albeit at a low level (Figure 4.19).

The proliferation kinetics of IDBL to cytokine stimulation was assessed by ^{3}[H]dThd uptake. When assessed at differing time intervals, IDBL demonstrated maximal proliferation to rhIL-2 at 72 hours with a mean 265,159±12594 cpm compared with a mean 202,658±13,897 cpm at 48hours ($p=0.02$) and a mean 75,046±9,097 cpm at 24 hours ($p<0.001$) as illustrated in Figure 4.20. It is worthy of note that the proliferation in CM without IL-2 steadily decreased over the time of the experiment. When the proliferative response to increasing doses of rhIL-2 was tested, it was shown, using a linear regression analysis model, that IDBL demonstrated a linear relationship with the concentration of rhIL-2 ($r^2=0.616$, $p=0.007$; Figure 4.21). Therefore the optimal experimental conditions in terms of proliferation for this cell line are 20U/ml of rhIL-2 for 72 hours culture. However, owing to the magnitude of proliferation and the extent of ^{3}[H]thymidine incorporation seen when IDBL cells were cultured at a final concentration of $1x10^6$/ml, then IDBL cells were used at a final concentration of $1x10^5$/ml.

The proliferative response to another T cell stimulatory cytokine, IL-12, was important to establish. IL-12 signals gene transcription through different intra-cellular signals to that associated with IL-2/IL-2R interactions: Jak2/Tyk2/STAT4 *cf.* Jak1/Jak3/STAT3/STAT5 (Leonard & O'Shea, 1998).

MARKER	(%)	(%)	(%)	MEAN±SEM
IDBL Cells				
CD2	0	2.3	3.75	2.0±1.9
CD3	84.89	92.1	87.3	88.2±3.7
CD4	47.95	10.5	27.5	36.0±11.0
CD25	0	2.19	1.12	0.4±0.6
CD122	70.8	74.6	67.9	71.1±3.4
CD69	81	74.6	78.6	78.1±3.2
CD45RA	72	69.6	81.6	74.4±6.3
CD95 (Fas)	100	94.7	97.5	97.4±2.7
Cyto IL-2	1.25	2.80	0.9	1.6±0.8
Cyto IFNγ	3.5	2.7	1.8	2.7±0.9
Jurkat Cells				
CD2	94.07	83.6	99.08	92.3±4.57
CD3	46.17	98.73	98.03	81.0±17.43
CD4	39.55	92.3	92.18	74.7±17.58
CD25	2.46	4.89	0.06	2.5±1.4
CD122	4.91	2.74	5.96	4.5±0.95
CD69	13.44	83.08	70.45	55.7±21.45
CD45RA	98.02	67.6	70.78	78.8±9.66
CD95 (Fas)	60.6	98.46	99.40	86.2±12.79
Cyto IL-2	89.4	94.5	99.82	94.6±3.01
Cyto IFNγ	3.62	2.01	4.41	3.4±0.71

TABLE 4.4. Phenotypic characteristics of two human T cell lines, IDBL and Jurkat E6.1 as determined by direct immuno-fluorescence staining and flow cytometry. Surface phenotyped examined on 3 different occasions.

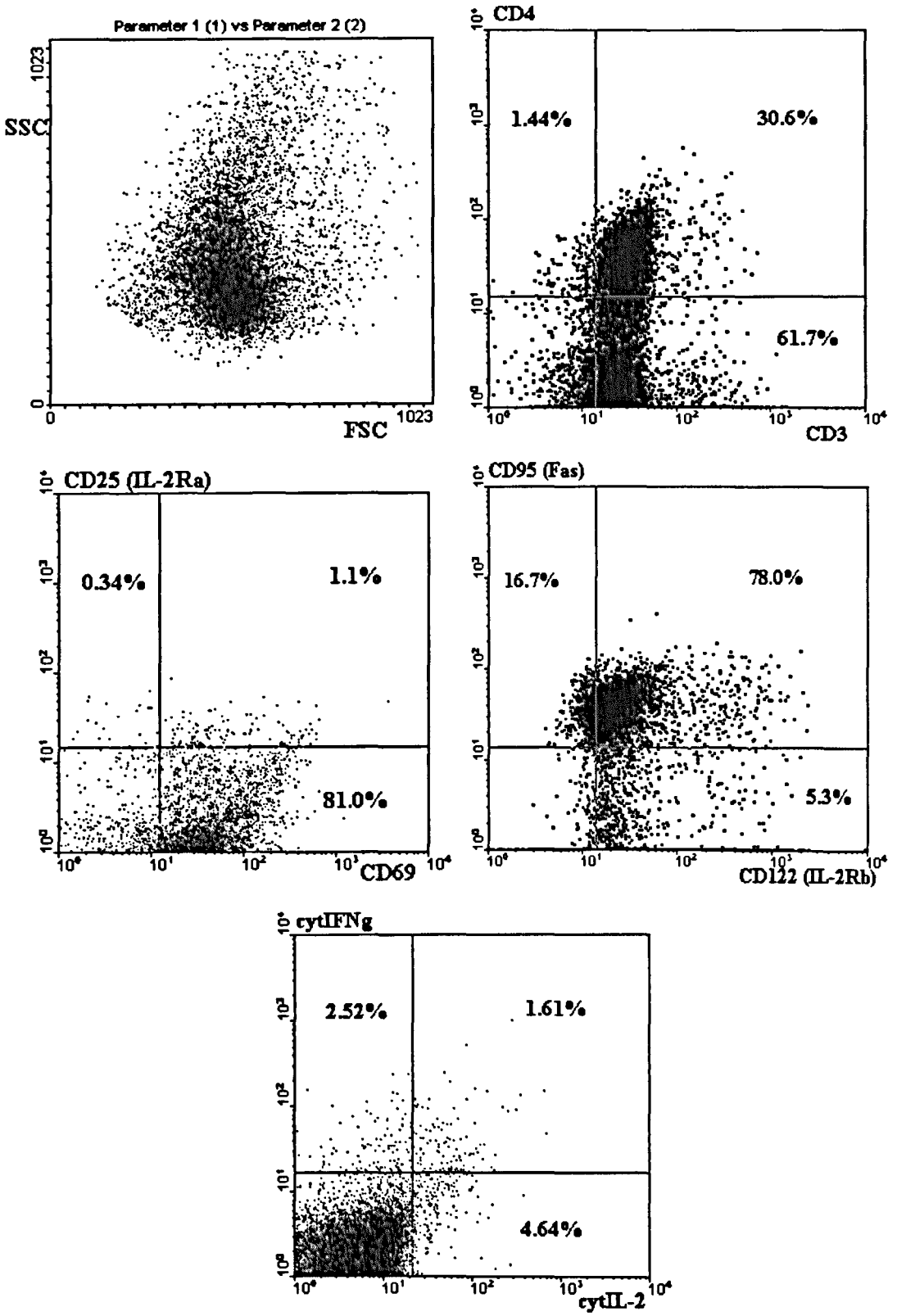


FIGURE 4.16. Surface phenotype of the human IL-2 dependent cell line, IDBL analysed by flow cytometry. **Key:** cytIFNg- intracellular interferon-γ, cytIL-2- intracellular IL-2

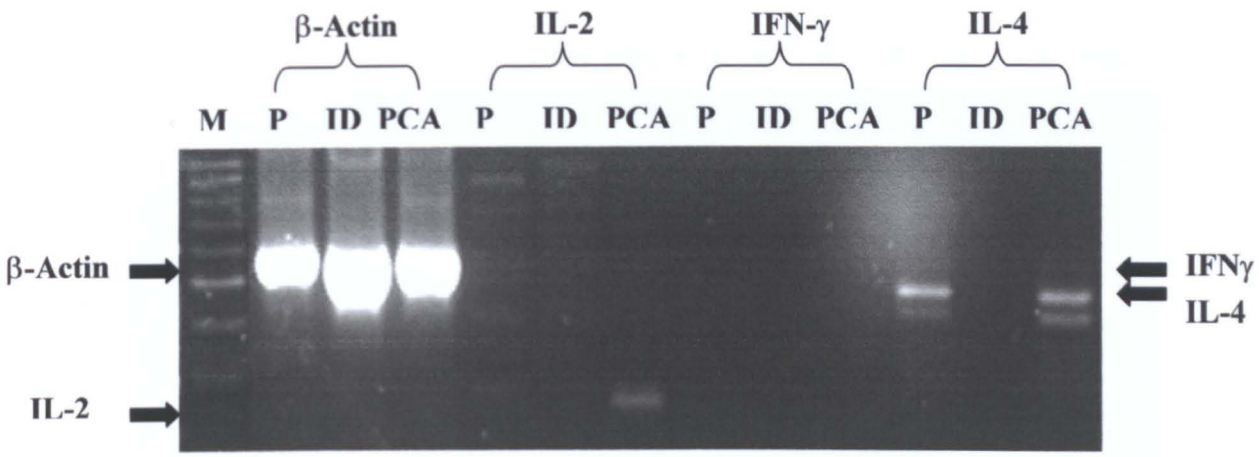


FIGURE 4.17. Expression of cytokine mRNA (IL-2, IL-4, IFN-γ) by IDBL cells (ID) after culture with rhIL-2. No IFNγ mRNA transcripts were detected in all 3 cell populations. β–Actin was used as an amplification and loading control. PBMNC cultured in the presence (PCA) and absence (P) of ConA for 72 hours were used as positive and negative controls, respectively.

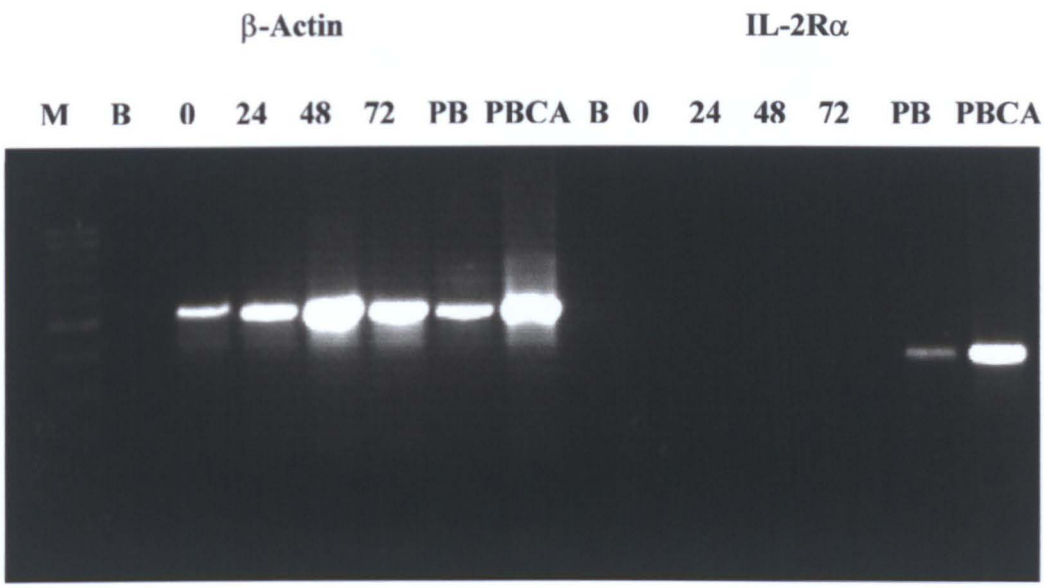


FIGURE 4.18. Expression of IL-2Rα mRNA in IDBL cells after 24, 48 and 72 hours of culture with rhIL-2 20U/ml. β–Actin was used as an amplification and loading control. Resting and ConA activated PBMNC from normal donors was used a controls. **Key:** M-markers; B-blank; 0- at time 0 hours; 24,48,72 – at the specified time in hours; PB-resting peripheral blood lymphocytes; PBCA- peripheral blood lymphocytes stimulated with ConA for 3 days.

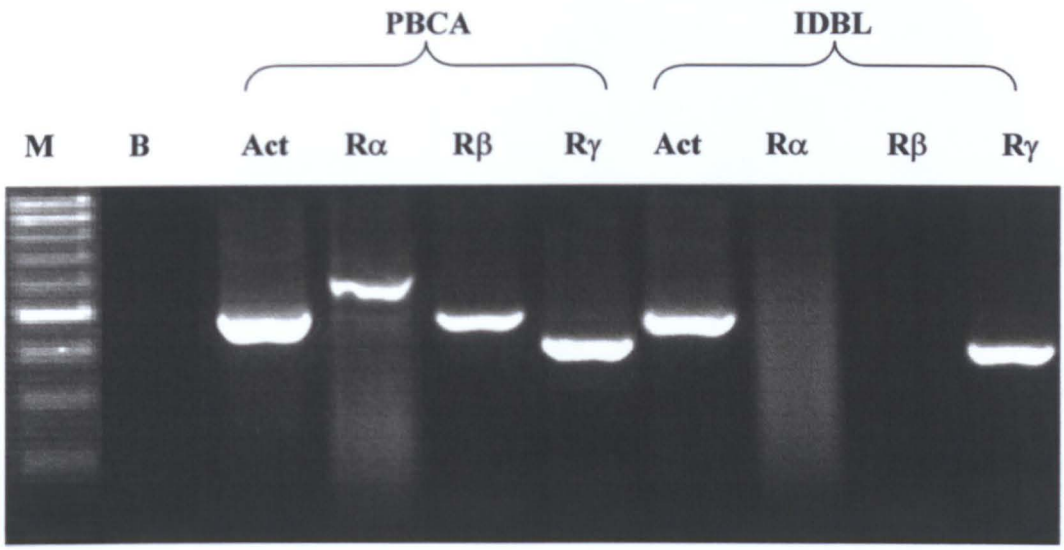


FIGURE 4.19. Expression of IL-2 receptor sub-unit mRNA by IDBL cells. RT-PCR detection of IL-2R sub-units after stimulation with rhIL-2 for 72 hours. β–Actin was used as an amplification and loading control. PBMNC stimulated ConA for 72 hours (PBCA) were used as positive controls. **KEY:** M-markers, B- negative control, Act- β–Actin, Rα- IL-2 receptor subunits α,β & γ.

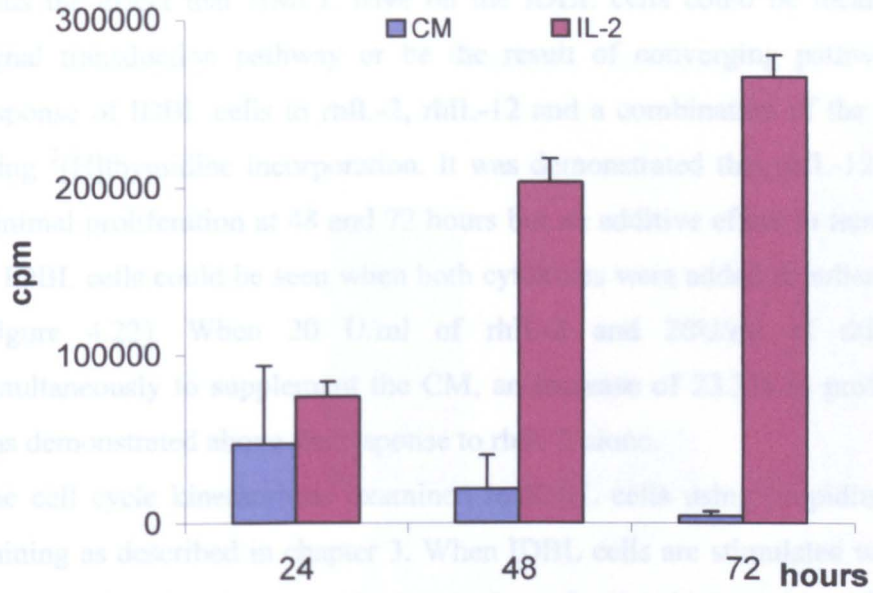


FIGURE 4.20. Proliferation of IDBL (10^6/ml) when stimulated with CM alone or CM supplemented with rhIL-2 for the time intervals indicated. IDBL cells demonstrate greater proliferation at 72 hours compared with 24 hours ($p<0.001$) and 48 hours ($p=0.02$). Proliferation was measured by ^{3}[H]thymidine uptake.

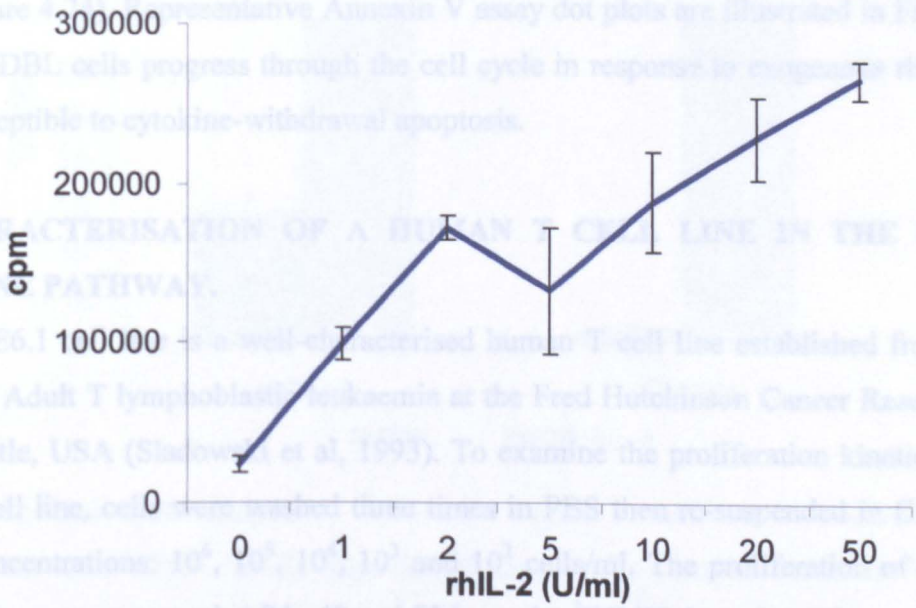


FIGURE 4.21. Proliferation of IDBL (10^6/ml) stimulated with varying doses of rhIL-2. Proliferation was measured by ^{3}[H]thymidine uptake ($r^2=0.616$, $p=0.007$).

Thus the effect that HMCL have on the IDBL cells could be localised to a specific signal transduction pathway or be the result of converging pathways. As such the response of IDBL cells to rhIL-2, rhIL-12 and a combination of the two was assessed using ^{3}[H]thymidine incorporation. It was demonstrated that rhIL-12 alone resulted in minimal proliferation at 48 and 72 hours but an additive effect in terms of proliferation of IDBL cells could be seen when both cytokines were added simultaneously to the CM (Figure 4.22). When 20 U/ml of rhIL-2 and 20U/ml of rhIL-12 were used simultaneously to supplement the CM, an increase of 23.3% in proliferative response was demonstrated above the response to rhIL-2 alone.

The cell cycle kinetics was examined in IDBL cells using propidium iodide – DNA staining as described in chapter 3. When IDBL cells are stimulated with rhIL-2 in CM, there is a significant increase in the number of cells which are identified as being in the G_2/S/M phase of cell cycle compared to those which were cultured in medium alone (35.6%$\pm$3.87 *cf.* 1.79%$\pm$0.421, p=0.0063; Figure 4.23). A significant difference in the number of cells in G_0/G_1 phase of cell cycle when stimulated with rhIL-2 compared to CM alone was observed (35.7%$\pm$2.06 *cf.* 14.5%$\pm$2.06, p=0.0106).

When apoptosis was analysed using the Annexin V assay, it was found that culturing IDBL cells in CM resulted in a significantly higher rate of apoptosis when compared to those cultured in CM supplemented with rhIL-2 (63.44%$\pm$20.13 *cf.* 15.99%$\pm$5.6, p=0.04, Figure 4.24). Representative Annexin V assay dot plots are illustrated in Figure 4.26. Thus IDBL cells progress through the cell cycle in response to exogenous rhIL-2 and are susceptible to cytokine-withdrawal apoptosis.

4.3.5 CHARACTERISATION OF A HUMAN T CELL LINE IN THE IL-2 AUTOCRINE PATHWAY.

The Jurkat E6.1 cell line is a well-characterised human T cell line established from a patient with Adult T lymphoblastic leukaemia at the Fred Hutchinson Cancer Research Centre, Seattle, USA (Sladowski et al, 1993). To examine the proliferation kinetics of the Jurkat cell line, cells were washed three times in PBS then re-suspended in CM at reducing concentrations: 10^6, 10^5, 10^4, 10^3 and 10^2 cells/ml. The proliferation of each cell suspension was measured at 24 , 48 and 72 hours by ^{3}[H]dThd uptake. When

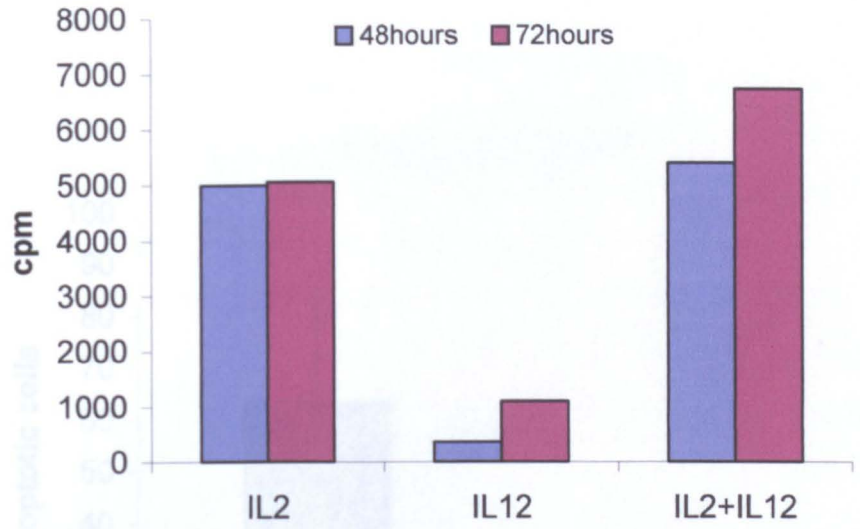


FIGURE 4.22. Proliferation of IDBL to stimulation with a combination of rhIL-2 and rhIL-12, as measured by ^{3}[H]thymidine uptake. IDBL at a concentration of 10^{5}/ml were stimulated with rhIL-2 20 U/ml and rhIL-12 100U/ml for 48 and 72 hours.

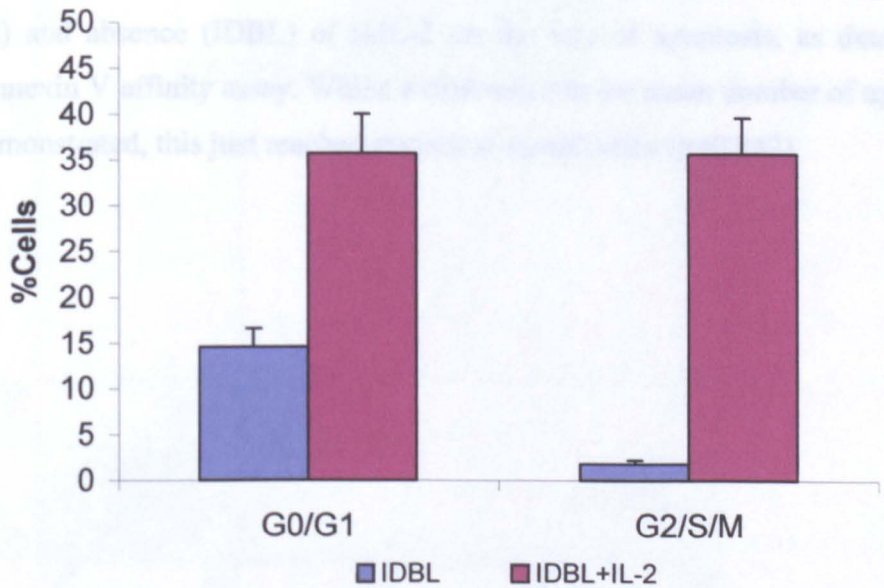


FIGURE 4.23. Cell cycle analysis of IDBL cells culture in medium alone (IDBL) or with rhIL-2 20 U/ml (IDBL+IL-2). Significantly more cells were identified in G_0/G_1 ($p=0.0106$) and G_2/S/M ($p=0.0063$) when rhIL-2 was added to the CM, compared to CM alone, the main deficit resulting from apoptosis owing to cytokine withdrawal.

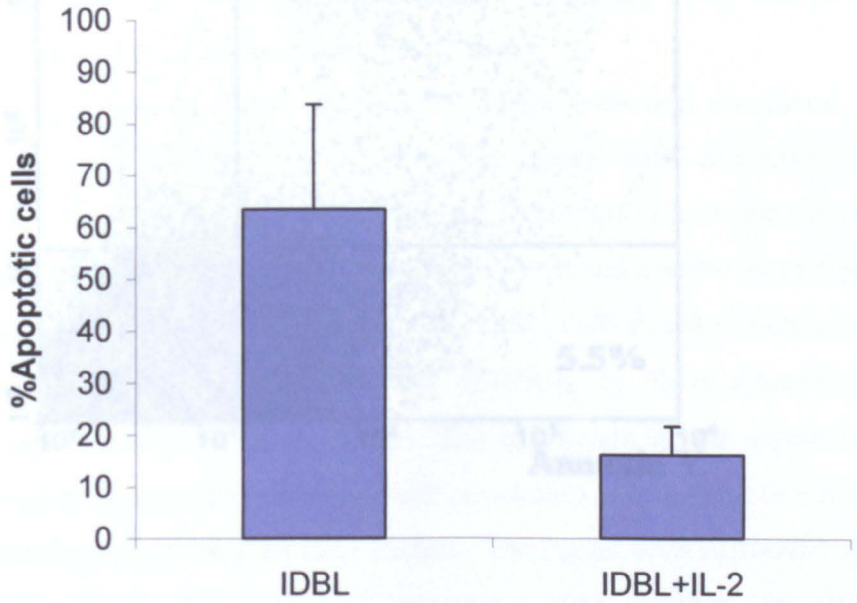
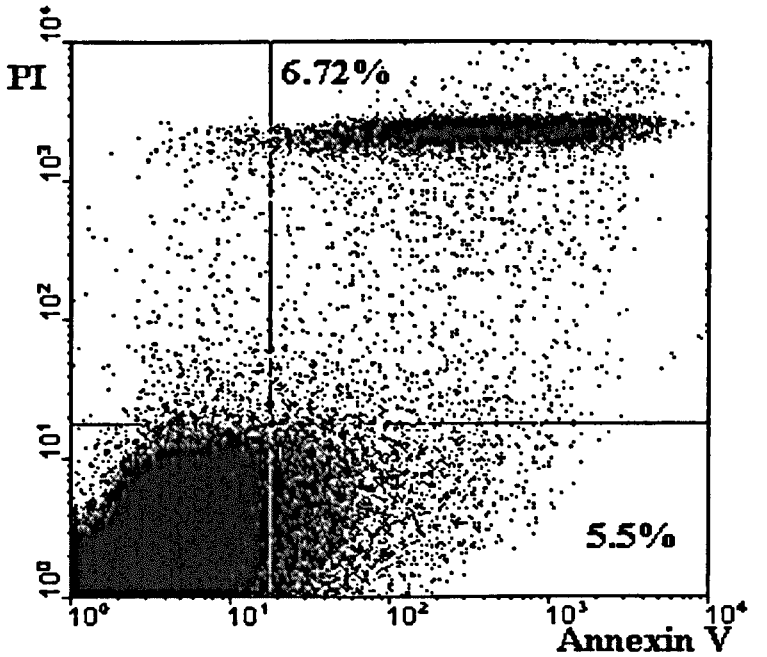


FIGURE 4.24. Analysis of the effect of culturing IDBL cells in the presence (IDBL+IL-2) and absence (IDBL) of rhIL-2 on the rate of apoptosis, as determined using the Annexin V affinity assay. Whilst a difference in the mean number of apoptotic cells was demonstrated, this just reached statistical significance (p=0.042).

A



B

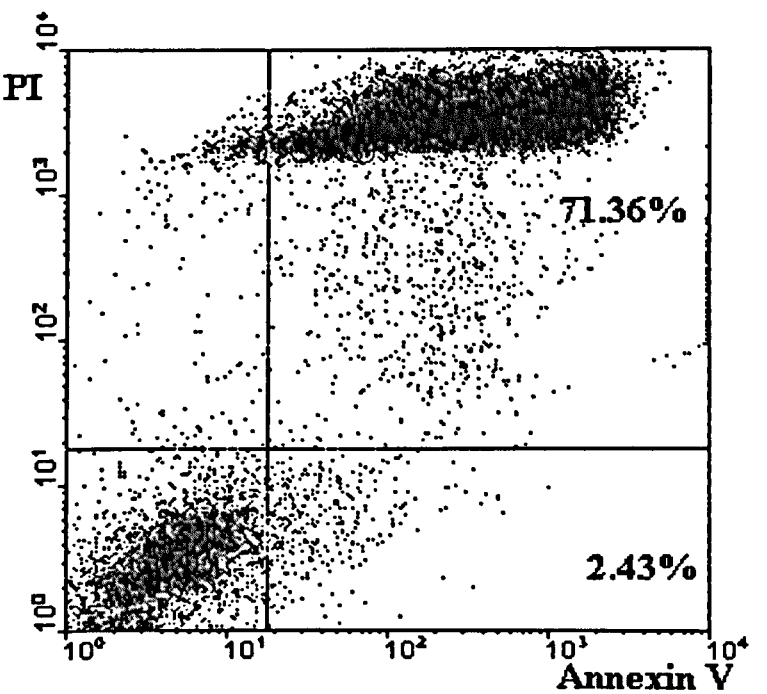


FIGURE 4.25. Representative Annexin V dot plots of IDBL cells cultured in the presence (A) and absence (B) of rhIL-2. Early apoptotic cells are identified as An^+/PI^- and late apoptotic and necrotic cells are identified as An^+/PI^+.

cells were cultured at the highest concentration (10^6 cells/ml) maximal proliferation was lower than 10^5 cells/ml , resulting from cell overgrowth and competition for CM (Figure 4.26). At 10^5cells/ml however, no appreciable change in proliferation was seen at the time points indicated unlike 10^4 cells/ml, which demonstrate a linear proliferation/time relationship (r^2=0.994, p=0.049). Therefore, all experiments using Jurkat E6.1 cells were conducted at a cell concentration of 10^4 cells/ml.

The surface expression of T cell markers by Jurkat cells was examined, including components of the IL-2R and Fas (CD95). The Jurkat cells demonstrated surface expression of CD2, CD3 and CD4 (92.25%±4.57, 80.98%±17.43 and 74.68%±17.58 respectively; Table 4.4). The majority of cells demonstrated a naïve phenotype through the expression of CD45RA (78.8%±9.66). The Jurkat cells failed to express the IL-2R components, CD25 (IL-2Rα) and CD122 (IL-2Rβ) by flow cytometry analysis (2.47%±1.4 and 4.54%±0.98, respectively). The expression of the activation marker, CD69 was more heterogeneous within the cell population with a mean of 55.66%±21.45 of cells expressing this marker on their surface. The Jurkat cells expressed surface Fas (APO-1/CD95) though the degree of expression was variable amongst the cell population (86.15%±12.79). Representative examples of Jurkat E6.1 cell surface phenotype are illustrated in Figure 4.27.

The Jurkat cells were examined for the mRNA transcripts of the IL-2R subunits. Compatible with absent surface expression of the α-subunit (CD25), no mRNA was detected by bulk-population RT-PCR (Figure 4.28). In contrast, the β subunit mRNA transcripts were detected by RT-PCR on bulk populations but were not detected at the protein level by FACS. This suggests that the β subunit of IL-2R may be modulated on these cells at a low level, depending on the cycling of individual cells. The γ_c subunit of IL-2R was detected by RT-PCR on bulk populations.

Jurkat E6.1 cells are cytokine-independent resulting from the autocrine production of IL-2. The cells were examined for the expression of IL-2 both at the mRNA and protein level. Using intra-cellular cytokine immuno-fluorescence staining, Jurkat cells demonstrated a uniform expression of IL-2 (94.57%±3.09; Table 4.4 (page 128) and Figure 4.29). Cytokine mRNA expression could be detected by RT-PCR (Figure 4.30). By comparison, the cells were examined for the production of interferon-γ (IFN-γ). The

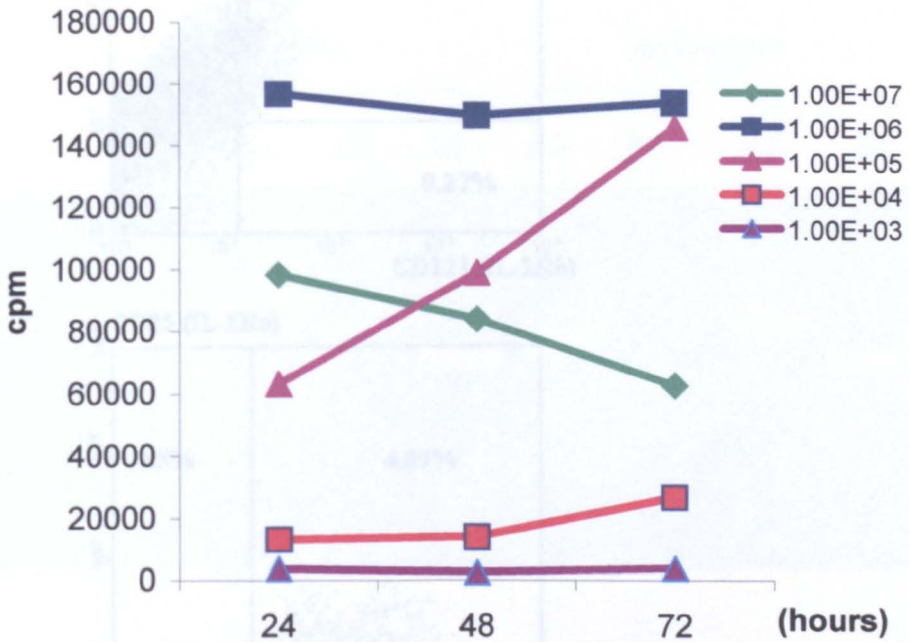


FIGURE 4.26. Proliferation kinetics of the human T cell line, Jurkat E6.1. Cell proliferation was measured by ^{3}[H]dThd uptake after 24, 48 and 72 hours culture at reducing cell concentrations. Key: 1.00E+07 – 10^{6}cells/ml, 1.00E+06 – 10^{5}cells/ml, etc.

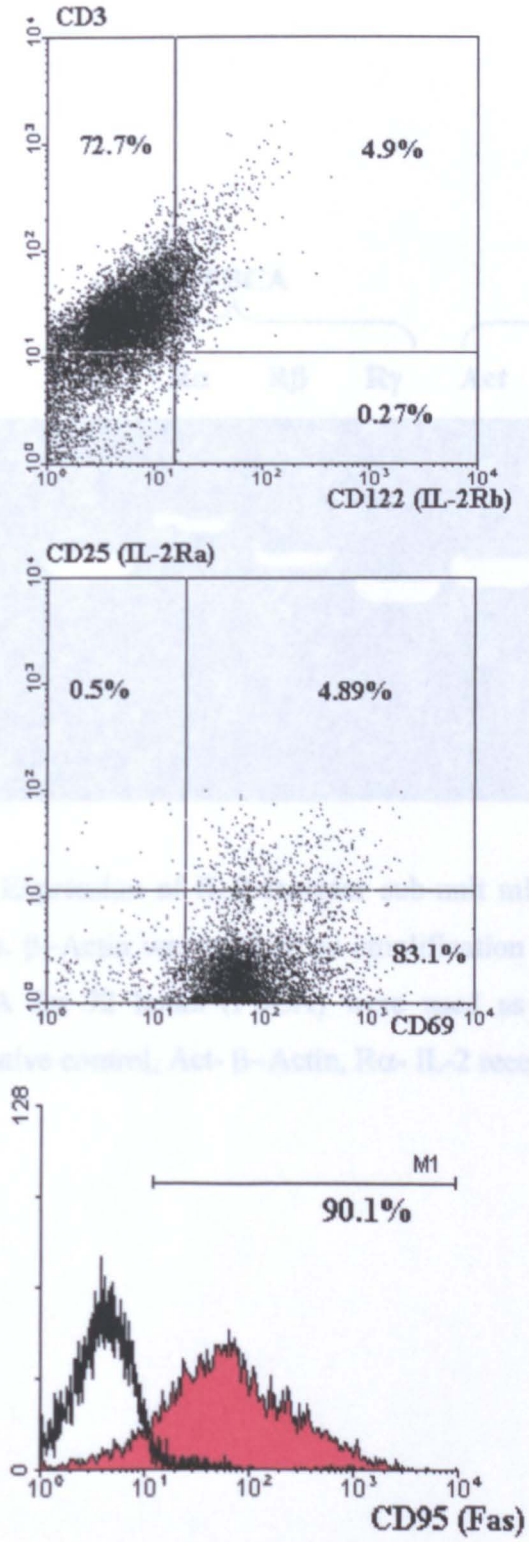


FIGURE 4.27. Surface phenotype of Jurkat cells. Cells are $CD3^+$ T cells with no detectable evidence of IL-2Rβ or IL-2Rα surface expression despite surface expression of CD69. High levels of Fas (CD95) can be demonstrated.

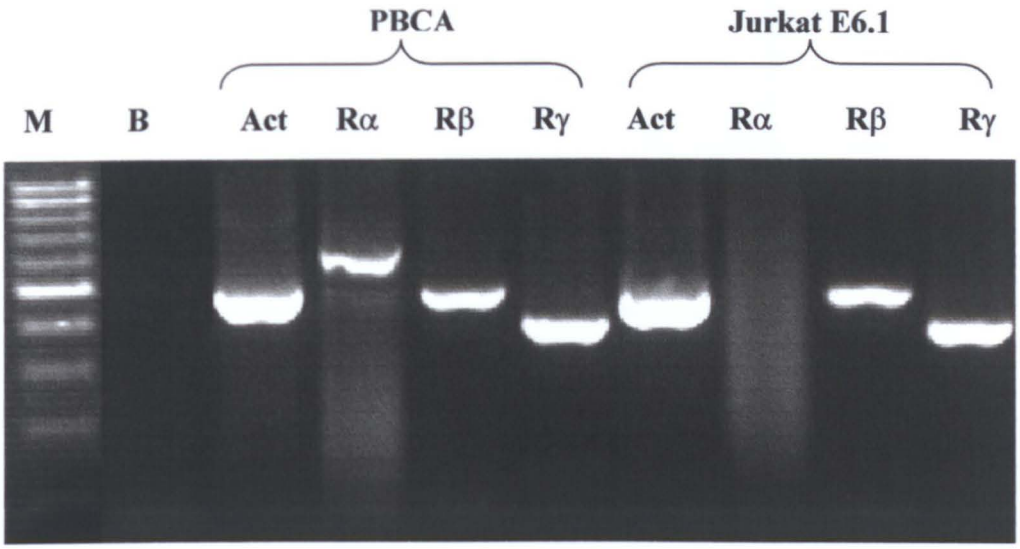


FIGURE 4.28. Expression of IL-2 receptor sub-unit mRNA detected by RT-PCR on bulk-populations. β–Actin was used as an amplification and loading control. PBMNC stimulated ConA for 72 hours (PBCA) were used as positive controls. **KEY:** M-markers, B- negative control, Act- β–Actin, Rα- IL-2 receptor subunits α,β & γ.

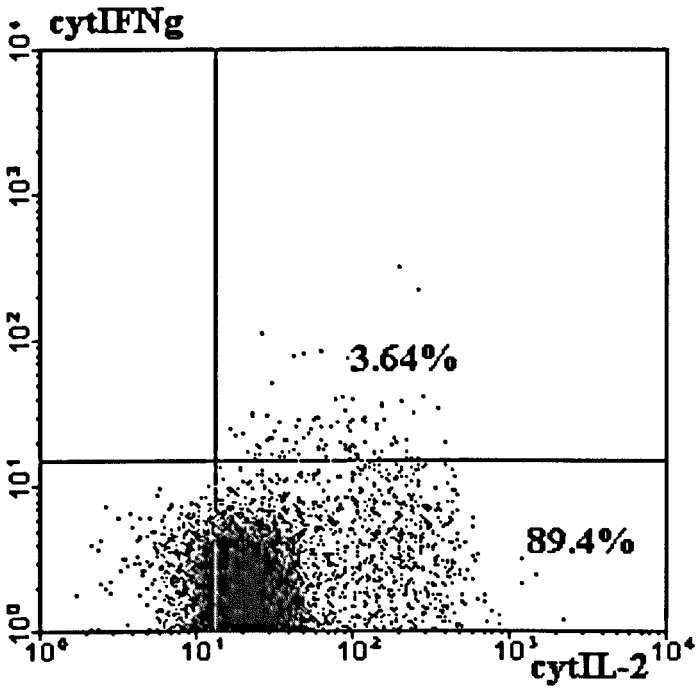


FIGURE 4.29. IL-2 (cytIL-2) and interferon-γ (cytIFNg) production at the single cell level by Jurkat cells detected by intra-cellular cytokine FACS analysis.

The cells were found neither to produce the protein or mRNA transcripts for this cytokine (3.37%±0.73, Table 4.4; Figure 4.30).

The cell cycle distribution of Jurkat cells cultured in CM were examined at various time points over a 72 cultured period suing PI-DNA staining. Analysis revealed that a mean of 26.03%±3.47 of cells were in the $S/G_2/M$ phase of cell cycle at any one time point. A representative DNA histogram is illustrated in Figure 4.31.

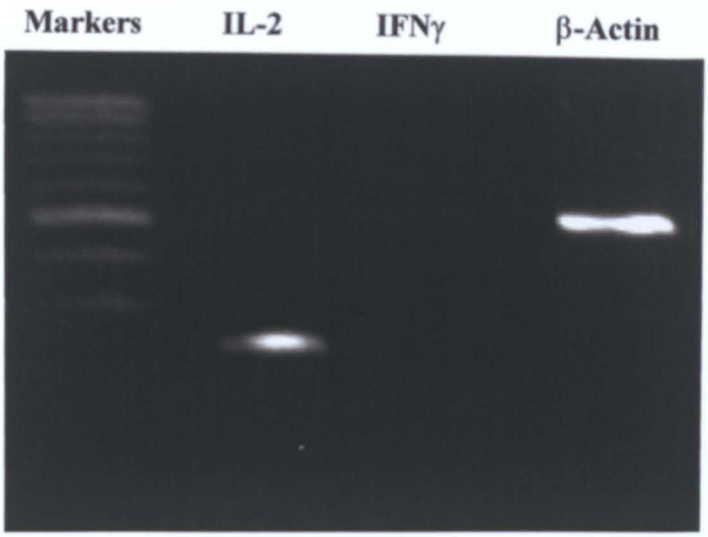


FIGURE 4.30. Expression of cytokine mRNA in Jurkat E6.1 cells, detected by RT-PCR. β–Actin was used as an amplification and loading control.

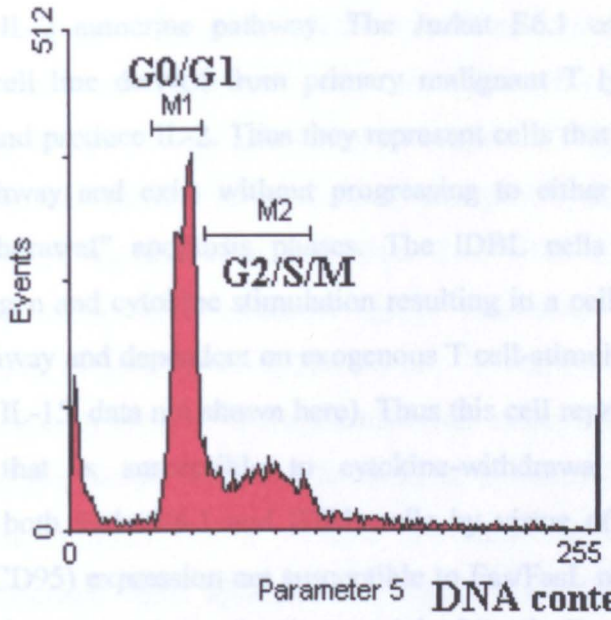


FIGURE 4.31. Representative cell cycle analysis of Jurkat cells using PI-DNA staining.

4.4 DISCUSSION

The data presented here characterise the phenotype, cytokine/cytokine receptor and proliferation profiles of three populations of human T lymphocytes – IL-2-responsive primary T cells and two T cell lines, Jurkat E6.1 and IDBL cells. These three cell types represent T cells at various stages of the activation process as illustrated in Figure 4.1. The IL-2-responsive primary T cells generated from the peripheral blood of healthy volunteers when stimulated with rhIL-2 represent an *in vitro* model of the initiation/activation process which results in cells up-regulating several key genes and entering the IL-2 autocrine pathway. The Jurkat E6.1 cells by virtue of being a transformed cell line derived from primary malignant T lymphocytes, are cytokine-independent and produce IL-2. Thus they represent cells that have entered into the IL-2 autocrine pathway and exist without progressing to either the "antigen-induced" or "cytokine-withdrawal" apoptosis phases. The IDBL cells have been generated by repeated mitogen and cytokine stimulation resulting in a cell which is beyond the IL-2 autocrine pathway and dependent on exogenous T cell-stimulating cytokines such as IL-2, IL-12 (and IL-15, data not shown here). Thus this cell represents an *in vitro* model of the T cell that is susceptible to cytokine-withdrawal or "passive" apoptosis. Furthermore, both JurkatE6.1 and IDBL cells by virtue of high levels of functional surface Fas (CD95) expression are susceptible to Fas/FasL mediated apoptosis and this can be utilised to represent an *in vitro* model of "active" apoptosis. Thus using these three T cell populations an *in vitro* model of the propriocidal model of T cell homeostasis in response to antigen can be generated, encompassing three key areas: activation accompanied by distinct molecular events, proliferation resulting from IL-2 autocrine pathway entry and apoptosis susceptibility resulting from both "active" and "passive" pathways (Crabtree, 1989; Boehme et al, 1993; Radvanyi et al, 1996).

Why is a three-cell model required to investigate the interaction of HMCLs and activating/proliferating T lymphocytes? As discussed in chapter 3, HMCLs suppress the proliferative responses of normal T lymphocytes to alloantigen and mitogen by inducing cell cycle arrest. However is not known which agent or indeed agents are responsible for these observed effects. In fact, the tumour cells in myeloma may produce several factors, which could act at several points in the homeostatic pathway of T lymphocytes. For example, FasL has been shown to be expressed by a number of malignant cells including melanoma (Hahne et al, 1996), hepatocellular carcinoma (Shiraki et al, 1997), lung cancer (Niehans et al, 1997) and myeloma cells (Villunger et al, 1997). By binding

to Fas (CD95) on T cells, FasL can induce activation-induced apoptosis particularly in the presence of antigen or IL-2. However, this form of activation induced cell death, or active apoptosis in the propriocidal regulation model, necessitates the T cells to become activated by antigenic stimulation or to have progressed into the second phase of the activation process. If HMCLs could both inhibit the activation process and augment active apoptosis mediated by FasL, then using primary T cells alone as the *in vitro* model would not permit the full dissection of the suppressor capacity of HMCLs. Thus using the two cell lines, IDBL and Jurkat E6.1, provide a suitable *in vitro* model to permit a closer examination of the effects of co-culturing with HMCLs.

Another known potent suppressor of immune effector cells is Transforming growth factor beta (TGF-β). TGF-β influences all stages of T lymphocyte development and differentiation through to their activation and proliferation, the exact effect being determined by the stage of maturation of the T cell. TGF-β inhibits T cell proliferation by down-regulating IL-2 mediated-signals mediated through reduced tyrosine phosphorylation of proteins of 120, 100, 85, 75 and 50 kDa and inhibition of IL-2 mediated phosphorylation of the retinoblastoma susceptibility gene product, which is pivotal in the progression of cell cycle (Wahl et al, 1989; Fontana et al, 1989; Ahuja et al, 1993). Furthermore, TGF-β prevents activation-induced apoptosis by preventing activation-associated FasL up-regulation, which results from TGF-β-induced down-regulation of *c-myc* (Genester et al, 1999). This, it is proposed, has an effect of augmenting the memory T cell pool. In addition, TGF-β has been shown to induce cell cycle arrest at the G_1 stage in T cells that have been stimulated with mitogen, superantigen or anti-CD3 MoAb (Ewen et al, 1993). Thus TGF-β does exert differing effects on T cells dependent on their position in the propriocidal regulation pathway of T cell homeostasis and using either transformed T cells such as Jurkat E6.1 or T cell hybridomas alone in studies to examine the effect of tumour-derived TGF-β may not permit full study of the effect of this pluripotent cytokine.

Both these examples demonstrate the need for an *in vitro* model that permits dissection of the stages of T cell activation and apoptosis as proposed in the propriocidal model of homeostasis. The three cell model developed in this chapter has the advantage of being able to permit such a dissection of immuno-suppressive effects which could be induced by tumour cells and thus lend itself to the further study of the interaction between HMCLs and T cells.

Transformed cells provide a readily available, often reliable and predictable cellular population for *in vitro* studies examining cell-to-cell interactions. The use of transformed T cell lines in the study of tumour cell-lymphocyte interactions have been reported widely in the literature (Villunger et al, 1997; Zaks et al, 1999). Investigators often use one cell type, which is sometimes modified by gene-transfection, in these reported studies, and this poses a problem. Transformed cell lines by the nature of their *in vitro* immortalisation may be limited in their representation of the *in vivo* activity and behaviour of primary T cells (Lu et al, 1999). These cell lines may have aberrant intra-cellular signalling pathways or may be exquisitely sensitive to apoptosis-inducing signals or indeed insensitive to similar signals and thus do not truly represent primary T cells. Furthermore, transformed T cell lines are cells which are actively traversing the cell cycle, either under the influence of exogenous or autocrine cytokines. They are thus limited in the study of those early molecular events involved in the initiation/activation phase of T cell activation including up-regulation of IL-2Rα gene and the oncogene, *c-myc*. This is crucial in the study of T cell-HMCL interactions as this is likely to be a key area of T cell suppression induced by the HMCLs. The model proposed in this chapter aims to circumvent these problems by utilising primary T cells to study these early activation events and to use two transformed T cell lines which is aimed at examining key areas of the propriocidal model of T cell homeostasis. It is accepted that, as stated above, the transformed cells whilst providing a useful study population are limited in terms of their true reflection of primary T cells, the advantage of the proposed model using the three cell types is that this system is not totally reliant on transformed cell lines and their use is specific to examine exact points in the activation/deletion pathways of T lymphocyte homeostasis.

The *in vitro* model system described in this chapter utilises IL-2-responsive primary T cells to study the initiation of activation and how this may be suppressed, if at all, by HMCL. These IL-2 responsive primary T cells have several advantages over the mitogen-activated PBMNC described in chapter 3. Firstly, by activating PBMNC from normal donors using a mitogen followed by a resting period and removal of non-viable cells by density gradient centrifugation, the PBMNC are purified into T cells (89.7%±4.1). This is advantageous, as I will explore the effect that HMCLs have on the activation, proliferation and survival of T lymphocytes in subsequent chapters. By purifying the T cells the influence that HMCLs can have on the functional integrity of

essential antigen-presenting cells required for primary activation using mitogens, antigens or super-antigens. Secondly, pre-activated T cells re-stimulated with IL-2 produces a more reliable response with less inter-donor variability that is seen with primary activation using mitogen alone: IL-2-induced proliferation mean 23,250±3,775 cpm and Con A-induced proliferation 70,459±13,670 cpm with significant variance ($p<0.001$, $n=13$). This is important; it has been shown that the degree of suppression of proliferative responses induced by HMCL directly correlates with the degree of activation (Cook & Campbell, unpublished results). This therefore generates a more reproducible *in vitro* assay system on which to examine potential tumour-derived suppressive factors and to study their effect at the sub-cellular level. Furthermore, these proliferative responses are seen following a shorter assay duration (36 hours versus 72 hours) which is important when setting up blocking experiments to identify potential suppressive factors as many of these blocking agents have short half-lives in culture conditions (24-48 hours).

The data presented in this chapter characterise a three cell *in vitro* model of the propriocidal model of T cell homeostasis with each cell type representing key stages of this process of activation and cell death which regulate peripheral T pools *in vivo*. This model will be useful for the identification of suppressive factors produced by HMCLs and to permit the study of the mechanisms by which these factors influence T cell proliferation.

CHAPTER 5

Human Myeloma Cell Line-derived Transforming Growth Factor β inhibits T lymphocyte IL-2 responsiveness.

5.1 INTRODUCTION

Data presented in chapter 3 demonstrate that HMCLs can suppress primary T cells responding to alloantigen and mitogens by inhibiting the progression into the S phase of cell cycle. Successfully stimulated T lymphocytes transmit signals from their surface membrane to the nucleus via pathways which involve numerous signal transduction molecules and protein tyrosine kinases (especially JAKs, STATs, $p56^{Lck}$ and ZAP70; reviewed by Robey & Allison, 1995). These act on many intra-cellular pathways culminating in the up-regulation of new genes required by T cells to become activated, including proto-oncogenes (*pim-1, c-fos, c-myc*) and cytokines involved in propagating the immune response e.g. IL-2, IL-3, IL-4, IL-5, IL-6 (Ullman et al, 1990). This early activation is followed by DNA synthesis, proliferation and the up-regulation of genes expressed in late T cell activation e.g. RANTES, Granzymes, Perforin, 519/granulysin (Oritz et al, 1997). Myeloma tumour cells could interfere with this activation process at any stage either by a single method or by an orchestrated manipulation of the effector mechanisms.

An array of cytokines and immune-modulating agents has been reported by many investigators in the field of tumour immunobiology (Johnston et al, 1996). In particular, B cells can produce a large number of cytokines both in disease and health (Pistola, 1997). These immune modulators include IL-10, the Fas (CD95/Apo-1) and FasL system, vascular endothelial growth factors (VEGF), the under-glycosylated surface peptide muc-1 and the transforming growth factor-β family of cytokines (TGF-β). IL-10, a pleiotropic cytokine produced by B cells, monocytes, macrophages and keratinocytes, inhibits synthesis of the pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-8, IL-12, TNF-α and GM-CSF) and is one of the most potent inducers of immunoglobulin synthesis, which may be important in the pathogenesis of multiple myeloma (Rousset et al, 1992). However, inhibition of T cell proliferation is not a recognised function of IL-10 (Weigh et al, 1995) though has been shown to inhibit the generation/differentation of cytotoxic T lymphocytes (Fiorentino et al, 1989; Becker et al, 1994).

The Fas/FasL system has been shown to be an important lymphocyte inactivation pathway in both physiological and pathological immune regulation (Alderson et al, 1995; Rensing-Ehl et al, 1995). It has been proposed that FasL on the myeloma cells can mediate T cell suppression by apoptosis induction (Villunger et al, 1997) and while

apoptosis does not play a significant role in mediating the myeloma-induce suppression described in chapter 3, other investigators have demonstrated that FasL can mediate cell cycle arrest in T cells (Desbarats et al, 1998). The role that Fas/FasL plays in the suppression of the immune response by myeloma tumour cells therefore remains to be clarified.

VEGF is a cytokine produced in large amounts by many tumours and its immunosuppressive effects are primarily restricted to manipulating the maturation of antigen presenting cells and are limited with respect to T cells (Gabrilovich et al, 1996; Ferrara et al, 1997). The role of muc-1 in the modulation of the immune system and, in particular, its role in suppressing anti-tumour responses remains unclear. Muc-1 expression may induce immunosuppression, though this has been shown to be mediated through apoptosis and is reversible by the actions of IL-2 (Gimmi et al, 1996; Agrawal et al, 1998). However, as indicated in chapter 3, the addition of exogenous IL-2 fails to restore mitogen-induce proliferation of primary T cells when co-cultured with HMCLs.

The TGF-β family of cytokines have been shown to inhibit T cell proliferation, CTL generation and down-regulate co-stimulatory molecules (Letterio & Roberts, 1998). Furthermore, in relation to the cytokine production of T cells in response to activation, TGF-β has been shown to induce a shift from the Th_1 to Th_2 phenotype, (Wahl et al, 1989; Bridoux et al, 1997). However, of the immune modulators indicated above, only TGF-β has been shown to inhibit T cell activation through down-regulating IL-2-mediated signals associated with reduced tyrosine phosphorylation of numerous proteins e.g. 120, 100, 85, 75 and 50 kDa (Fontana et al, 1989; Wahl et al, 1989) Furthermore, TGF-β inhibits IL-2-mediated phosphorylation of the retinoblastoma susceptibility gene product (Rb), which is pivotal in the progression of cell cycle (Ahuja et al, 1993).

Thus several potential modulators of T cell activation and cytokine production exist, many produced by malignant cells, though their modes of action vary considerably. A key element of tolerance induction, whether to "self" antigens or in the context of my studies, tumour cell evasion of the host immune system, is the inhibition of the effector arm of the immune response (Goodnow, 1996). The profile of the T cell suppressing effects of the TGF-β family of proteins are of interest in my model system in respect to my observations, as set out in chapter 3. The TGF-β family of proteins alter T cell function in different ways depending on their state of activation. In particular, for T

cells that are activating in response to an appropriate stimulus, they are capable of blocking IL-2-mediated signalling pathways and blocking gene transcription and proliferation. Therefore, in the search for potential mediator(s) of the T cell suppression induced by myeloma cells, this information forms the rationale for systematised investigations.

Experimental Aims

The aim of the studies presented here were to identify which T cell modulatory factor (s) are produced by HMCLs and to determine which of these is responsible for the suppressive effect HMCLs demonstrate on the activation of T cells. Furthermore, using the *in vitro* model developed in chapter 4, it was aimed to identify where in the process of T cell activation and homeostasis these modulators of T cell function, allowing further examination of the molecular events involved in subsequent studies.

5.2 MATERIALS AND METHODS

5.2.1 REAGENTS

Culture conditions and media are described in chapter 2. Recombinant human TGF-β1 was obtained from R&D systems (UK), which is produced by protein purification of acidified culture medium from Chinese hamster ovary cell (CHO) transfected with the human precursor protein DNA sequence. The anti-pan TGF-β monoclonal antibody was purchased from Sigma (USA) which was developed in rabbits using a mixture of recombinant human TGF-β1, porcine TGF-β1.2, porcine TGF-β2 and recombinant amphibian TGF-β5 as immunogens. Rabbit anti-pan TGF-β monoclonal antibody demonstrates blocking activity against all these TGF-β species. The ND_{50} of the antibody is defined as the concentration of antibody resulting in a one half maximal inhibition of bioactivity of recombinant, human TGF-β1 when the cytokine is present at a concentration high enough to elicit an inhibition of the murine IL-4-dependent cell line, HT2 (1.5μg/ml). Recombinant human Latency Associated Peptide (LAP) was purchased from Sigma (USA) who developed it from a DNA sequence corresponding to the 278 amino acid residues of the pre-pro- TGF-β1 terminating prior to the mature TGF-β1. The EC_{50} of recombinant LAP is defined as the effective concentration of growth factor that elicits a 50% inhibition of cell growth of Mv1Lu cells (8-40 ng/ml). The anti-human LAP (TGF-β1) monoclonal antibody was purchased from R&D systems (UK) who generated the antibody in goats immunised with purified, CHO cell-derived recombinant human LAP and chromatography purified the specific IgG. The ND_{50} of the antibody has been shown to approximately 0.05-0.15 μg/ml.

Cytokine and Cytokine Receptor Expression

The expression of cytokine and cytokine receptor mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) as described in chapter 2. All glassware, tubes aqueous solutions were autoclaved prior to use in RNA isolation and cDNA synthesis. Cells were washed twice in ice cold PBS and adjusted to 10^7/ml. Cell suspensions were transferred to 1.5ml autoclaved Eppendorf tubes and total RNA extracted as described before. Primers specific for human cytokine sequences were used in the PCR reactions and the sequences, predicted fragment sizes and melting temperatures (Tm) are listed in Table 5.1. These PCR primers were in-house sequences

	Strand	AT (^{0}C)	Size (bp)	5'-Sequence-3'
β-Actin	Sense	60	548	GTGGGGCGCCCCAGGCACCA
	Anti-sense			CTCCTTAATGTCACGCACGATTTC
IL-1α	Sense	60	421	GTCTCTGAATCAGAAATCCTTCTATC
	Anti-sense			CATGTCAAATTTCACTGCTTCATCC
IL-1β	Sense	60	802	ATGGCAGAAGTACCTAAGCTCGC
	Anti-sense			ACACAAATTGCATGGTGAAGTCAGTT
IL-6	Sense	60	628	ATGAACTCCTTCTCCACAAGCGC
	Anti-sense			GAAGAGCCCTCAGGCTGGACTG
IL-10	Sense	60	352	TCTCAAGGGGCTGGGTCATATCCCA
	Anti-sense			ATGCCCCAAGCTGAGAACCAAGACCCA
TNF-α	Sense	60	695	ATGAGCACTGAAAGCATGATCCGG
	Anti-sense			GCAATGATCCCAAAGTAGACCTGCCC
TGF–β1	Sense	60	181	AACATGATCGTGCGCTCTGCAAGTGCAGC
	Anti-sense			AAGGAATAGTGCAGACAGGCAGGA

TABLE 5.1 Oligonucleotide primers used for RT-PCR. Key: AT – annealing temperature used.

with known optimal reaction conditions. The primer sequence for the proto-oncogene, *pim-1*, are described in chapter 2 along with the PCR product verification by restriction enzyme digest. Using 2μl of cDNA solution (equivalent to 0.5μg of mRNA) the PCR reaction was carried out in 50μl volumes as described in chapter 2 and control samples, which contained all reaction constituents except cDNA, were used to exclude environmental contamination. PCR products were electrophoresed by 2% agarose gel electrophoresis at 100V and the imaged as described in chapter 2.

5.2.2 HMCL CLONING

Cell lines can be grown in a semi-solid culture system such as methyl cellulose, which is a gelling agent that immobilizes cells in discreet positions. As such, cells can be cloned by removing single cells from the methyl cellulose culture system and re-plating in methyl cellulose when subsequent colonies which appear after culture being derived from the initial single cells. Using this technique, the HMCL, U266 was sub-cloned by Mrs CE Carr with the resultant sub-clones being designated with unique clone suffixes. In brief, cells were recovered from suspension by centrifugation at 1500g for 5 minutes. 0.9mls of a 2.1% methyl cellulose stock solution (Life Technologies) was added to 2.4mls of CM to give a final concentration of 0.9% methyl cellulose. The cells were then washed three times in PBS and re-suspended in 0.9% methyl cellulose at a concentration of $1x10^3$/ml, with 3mls being loaded into each well on a 6 well plate. The plates were incubated for 72 hours when the six well plates were removed from the incubator and placed on the stage of an inverted microscope. Using a sterilised, filtered 2μl pipette tip, single cells were aspirated and transferred to sterile petri dishes containing 0.9% methyl cellulose. The cells were incubated for a further 72 hours and single colonies were aspirated suing the same sterile technique. The aspirated colonies were then disrupted and the cells were re-suspended in CM and transferred to 96 well plates and cloned using a limiting dilution technique. The cells were then expanded in CM and when bulk cultures were achieved the properties of each cell line sub-clone were assessed by their ability to stimulate MLR reactions, to suppress the mitogen-induced proliferative responses of normal T cells and their cytokine profile examined by RT-PCR.

5.2.3 SINGLE CELL PCR

Bulk cell population studies of cytokine mRNA expression in the HMCLs using RT-PCR was performed as described in chapter 2. For single cell and limited colony RT-PCR, the HMCL U266 were cultured in 0.9% methyl cellulose, as described in section 5.2.2. Cells grown in CM were recovered from suspension by centrifugation at 1500g for 5 minutes. The cells were then washed three times in PBS and re-suspended in 0.9% methyl cellulose at a concentration of $1x10^3$/ml, with 3mls being loaded into each well on a 6 well plate. The plates were incubated for the times specified in the results section. At the appropriate time points, the six well plates were removed from the incubator and placed on the stage of an inverted microscope. Using a sterilised, filtered 2μl pipette tip, single cells (x3) and colonies (x3) of <10 cells in diameter were aspirated in a volume of 1μl. Each 1μl aspirate was transferred to a separate autoclaved PCR tube.

To each 1μl of cell suspension, was added 4 μl of T lysis buffer {Tlysis buffer: 100μl made freshly consisted of 20μl of SSII 1st strand buffer (Gibco)/2.5%NP40 (Sigma), 66μl H_2O, 10μl 0.1M DTT (Sigma), 2μl RNase and 2μl dTN (10μg/μl oligo $dT_{(20)}$/ 0.5M dNTP)}. The samples were pulse spun (14,000g) in a microfuge and chilled on ice before being put into the PCR Express (Hybaid) thermocycler. The samples were then run on a cycle of 65°C for 1 minute followed by 20°C for 3 minutes, at which point the samples were then pulse spun in the microfuge and chilled on ice. To each sample, 0.5μl of reverse transcriptase (Gibco) was added and the samples were pulse spun in the microfuge and chilled on ice. The samples were run on a program as follows: 37°C for 15 minutes, 65°C for 10 minutes and 4°C for 4 minutes, following which the samples were pulse spun and chilled on ice. To each sample, 4 μl of Tail buffer {Tail buffer: 100μl freshly made consisted of 40μl (5x) TdT buffer, 2μl 100mM dATP, 57.6μl of H_2O and 0.4μl of 0.1M DTT} was added and the samples were pulse spun and chilled on ice. The samples were then run on a program as follows: 37°C for 20 minutes followed by 65°C for 10 minutes, which generated the poly A cDNA. 10μl of poly A cDNA solution was then added to a new autoclaved PCR tube and to this was added 10μl Taq buffer (Hybaid), 10μl $MgCl_2$, 10μl Brady primer (100 pM; 5' CATGTCGTCCAGGCCGCTCTGGACAAAATATGAATTCTTTTTTTTTTTTTTT TTTTTTT), 1μl dNTPs (10mM), 57μl of H_2O and 2μl of Taq (Hybaid). The samples were loaded on to the PCR Express and the following programme was run: 94°C for 2 minute, 42°C for 2 minutes and 72°C for 6 minutes with 10 second increments/cycle for

25 cycles. The samples were then removed, pulse spun and 1µl of Taq was added and the above program was repeated. Samples were then ready to be used in standard PCR reactions for amplification of cytokine cDNA or stored at -70^0C until required for analysis.

5.2.4 TGF-β1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The detection of active TGF-β1 in the supernates of HMCLs was analysed by Mrs CE Carr using a Promega TGF-β1 ELISA system. In brief, myeloma cell suspensions were centrifuged at 1500g for 5 minutes, washed three times in PBS and re-suspended in CM at a concentration of $1x10^6$/ml. CM with no added cells was set up to monitor the background TGF-β1 level in FCS. The cells were cultured at 37^0C/5% CO_2 for 72 hours. The supernates were removed and snap frozen immediately in liquid N_2 and stored at -70^0C until required for analysis. The supernates on recovery from frozen storage wer acid activated and diluted up to 1/100 in PBS to obtain results within the linear working range of the ELISA system (16-1000pg/ml). The samples were analysed using the Promega ELISA system, following the manufacturers instructions and the results from the cell line supernates were expressed minus the background level of CM.

5.2.5 IMMUNOHISTOCHEMISTRY

To detect the presence of latent TGF-β1 in the HMCLs, cell cytospins were stained immuno-histochemically using an anti-LAP antibody. Cell suspensions of the HMCL U266 were centrifuged at 1500g for 5 minutes and then washed three times in cold PBS. Following this, cells were fixed in 10% paraformaldehyde (Sigma) in PBS pH7.6 for 24 hours. Cells were then filtered and pelleted by centrifugation before being embedded in paraffin wax. 2.5µm sections were cut using a microtome (Leica RM2135) and the sections were floated in a water bath (50^0C) and collected upon Poly-l-lysine coated slides (Sigma). The slides were then baked at 56^0C for a minimum of 24 hours.

Prior to staining either for conventional histology or immunhistochemistry, the sections were de-paraffinized in xylene (3 changes) followed by rehydration through graded alcohols – 100%/95%/70%/50% ethanol/dH_2O. For conventional histology, the sections stained with Haematoxylin and Eosin (H&E) as follows: after rehydration, the sections were rinsed in running tap water before being immersed in Haematoxylin for 4 minutes (Sigma). The sections were rinsed in running tap water, immersed in "Scott's

Tap Water" (saturated lithium carbonate solution) for 1 minute and stained with Eosin for 30 seconds. The sections were then washed in running tap water before dehydrating through graded alcohols followed by clearing in xylene (2 changes) and being mounted in DPX mounting medium (BDH).

For immunohistochemical staining, the sections were de-paraffinised and hydrated as for the conventional staining. Following re-hydration, endogenous peroxidase activity was blocked to reduce false positive reaction by incubating sections in 3% hydrogen peroxide solution (3mls hydrogen peroxide in 100mls methanol) for 10 minutes. The sections were then washed in running tap water for 3 minutes. The sections were then blocked by incubating in 20% normal rabbit serum (NRS) in TBS for 30 minutes. The primary antibody (diluted 1:100 in 20% NRS) was added to the appropriate sections with an appropriate isotype control MoAb being used for background staining assessment. The sections were incubated in a sealed container at 4^0C overnight (18 hours) and were subsequently washed thoroughly in TBS. The secondary antibody, a biotinylated rabbit anti-goat immunoglobulin (Dako, Denmark), was diluted 1:500 in 20% NRS and added to the sections which were incubated for 30 minutes at room temperature. The sections were then washed thoroughly in TBS (5 minutes). Staining was visualised using the ABC Complex/HRP system (Dako) prepared according to the manufacturers instructions. One drop of avidin (in 0.001 mol/l PBS, pH 7.2) and biotinylated horseradish peroxidase (in 0.001 mol/l PBS, pH 7.2) were added to 5 mls of 0.05 mol/l Tris/HCL (pH 7.6) and mixed. The ABC complex was mixed and added to the sctions and incubated for 30 minutes. The sections were then washed thoroughly in TBS then incubated for 5-15 minutes with the enzyme substrate diaminobenzidine-H_2O_2 which was freshly made-up prior to use, according to the manufacturers instructions (Sigma Fast DAB). The sections were then washed in tap water, counter-stained using haematoxylin and dehydrated and mounted as described for conventional histology.

5.3 RESULTS

5.3.1 HMCL SUPPRESS THE PROLIFERATIVE RESPONSE OF PRIMARY T CELLS BUT NOT T CELL LINES JURKAT E6.1 AND IDBL.

Primary T cells from normal, healthy donors (n=8) re-stimulated with rhIL-2 demonstrated brisk proliferation at 36 hours (38820±8775 cpm). When co-cultured with the HMCLs U266, JIM1 and RPMI8226, inhibition of the proliferative responses was demonstrated: U266- 14917±6754 cpm (p=0.025), JIM1- 15899.7±3574 cpm (p=0.019) and RPMI8226- 15662.2±4475 cpm (p=0.020) (Figure 5.1). When expressed as the degree of inhibition, all three cell lines inhibited the proliferative responses of primary T cells (U266- 68.8%±8.8, JIM1- 60.2%±4.01 and RPMI8226- 62.9%±10.4; Figure 5.2). Primary T cells (n=3) expressing high levels ($CD25^{bright}$) of the α-subunit of the IL-2R (CD25) were sorted from those expressing low levels ($CD25^{dim}$) and both populations were re-stimulated with rhIL-2 in the presence and absence of HMCLs. There was a marked inhibition of proliferative responses of the $CD25^{bright}$ population from 21039±4386 cpm to 10720±6133 cpm, although this did not reach statistical significance (p=0.152), as opposed to the $CD25^{dim}$ T cells which essentially demonstrated no change in ^{3}[H]dThd uptake (4848±2438 cpm to 5468±5101 cpm, p=0.465; Figure 5.3). When the cell cycle distribution and apoptosis was examined using PI-DNA staining and Annexin V-affinity assays, respectively, primary T cells (n=8) when re-stimulated with rhIL-2 in the presence of U266 cells induced a significant increase in the number of cells remaining in the G_0/G_1 phase of cell cycle (74.2%±3.7 versus 49.7%±1.37, p=0.004; Figure 5.4). This was associated with a significant reduction in the number of cells progressing into the $S/G_2/M$ phase of cell cycle (12.9%±3.9 versus 24.9%±5.0, p=0.0398). No difference in the rate of apoptosis was seen when primary T cells were re-stimulated in the presence or absence of U266 cells (20.0%±5.0 versus 14.0%±4.4, p=0.2).

When the T cell lines, IDBL and Jurkat E6.1 were co-cultured with HMCL , a different effect was observed. No inhibition of IDBL proliferative response to rhIL-2 was evident when co-cultured with the HMCL, U266 at a ratio of 1HMCL:5 T cells after 72 hours (195382±36574 cpm *cf.* 205529±25769 cpm; n=3, p=NS). The results are illustrated in Figure 5.5. Similarly, when Jurkat E6.1 cells were co-cultured for 72 hours with U266 cells at a ratio of 1HMCL:5 T cells, no difference in the proliferation, determined by^{3}[H]thymidine uptake was evident (44339±1992 *cf* 54087±1656; n=3, p=NS).

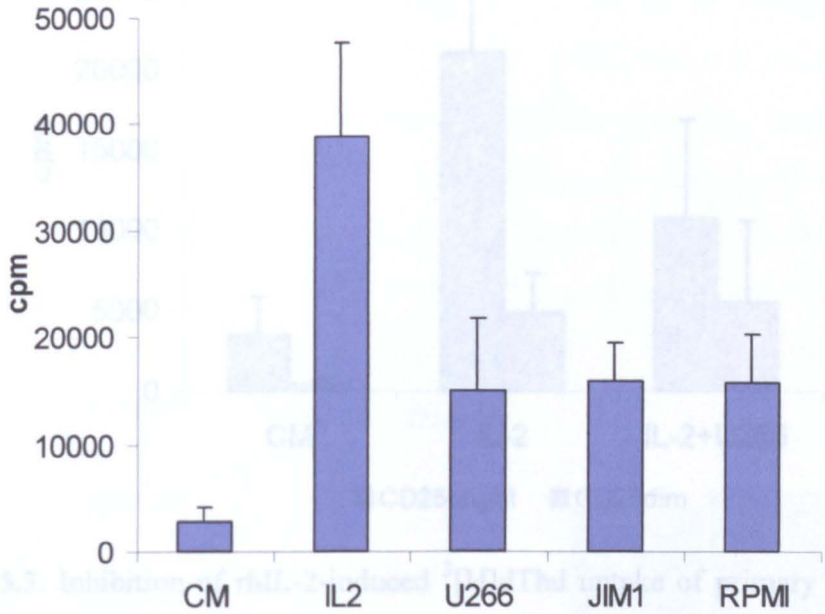


FIGURE 5.1. Suppression of primary T cell proliferative responses (n=8) to re-stimulation with rhIL-2 by co-culture with HMCLs, U266, JIM1 and RPMI8226. Proliferation was assessed by ^{3}H-Thymidine uptake, expressed as mean ±SEM counts per minute (CPM) and background T cell proliferation is indicated by "CM".

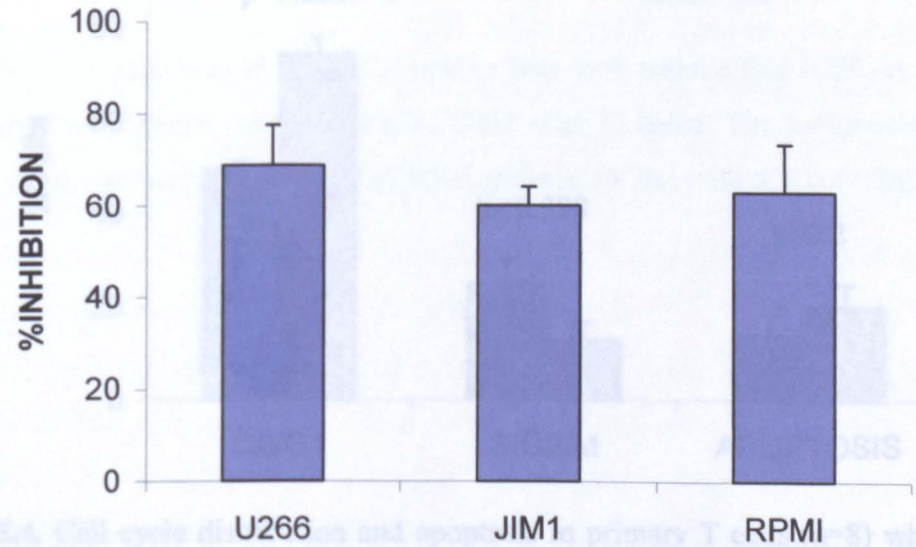


FIGURE 5.2. Inhibition of proliferative responses of primary T cells (n=8) after 36 hours re-stimulation with rhIL-2 when co-cultured with HMCLs.

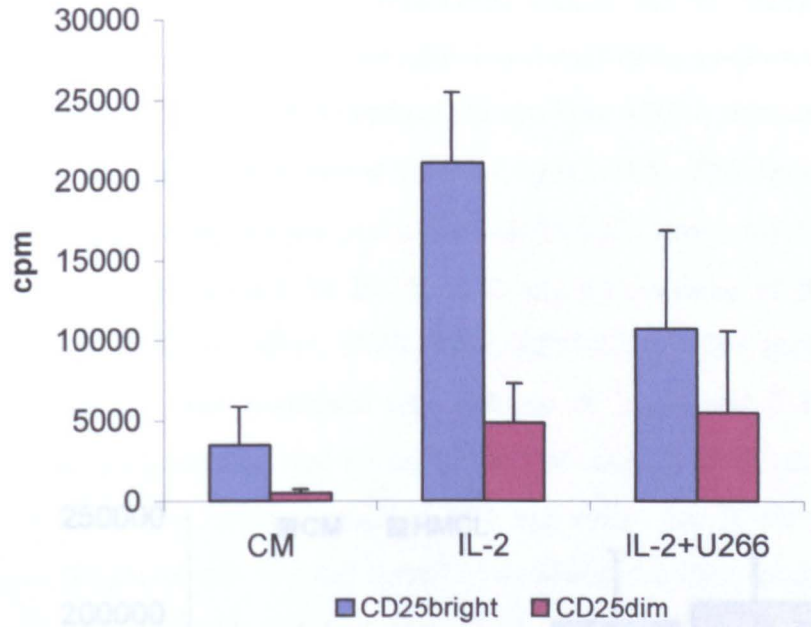


FIGURE 5.3. Inhibition of rhIL-2-induced ^{3}[H]dThd uptake of primary T cells (n=3) expressing high (CD25bright) and low (CD25dim) levels of surface CD25 when co-cultured with HMCL (U266) for 36 hours .

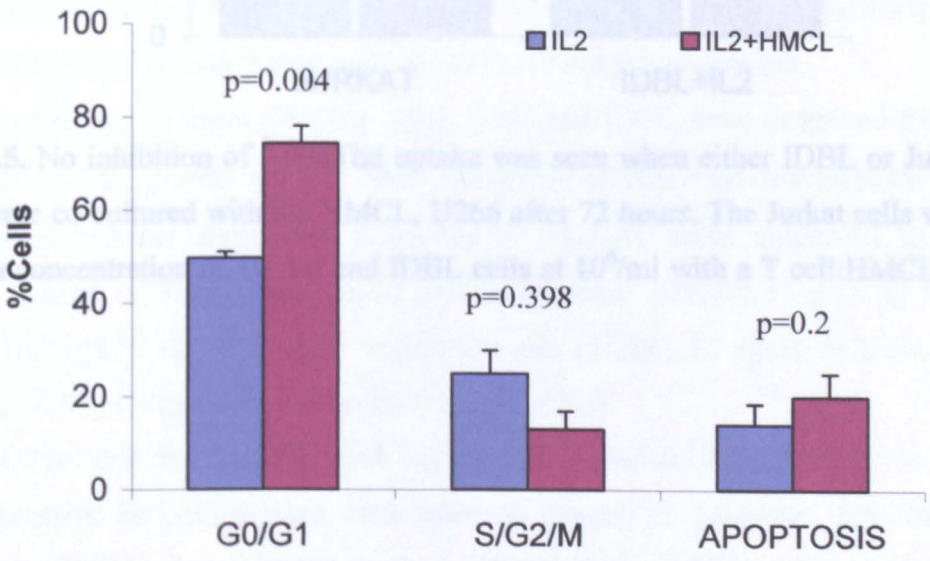


FIGURE 5.4. Cell cycle distribution and apoptosis in primary T cells (n=8) when re-stimulated with rhIL-2 in the presence and absence of U266 cells, as determined by PI-DNA staining and Annexin V-affinity assay, respectively.

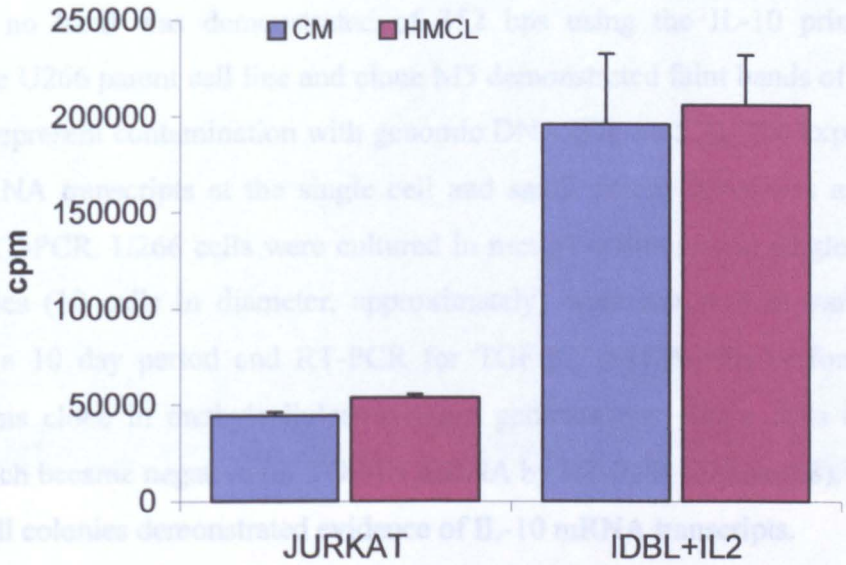


FIGURE 5.5. No inhibition of ^{3}[H]dThd uptake was seen when either IDBL or Jurkat E6.1 cells were co-cultured with the HMCL, U266 after 72 hours. The Jurkat cells were cultured at a concentration of 10^{5}/ml and IDBL cells at 10^{6}/ml with a T cell:HMCL of 5:1.

5.3.2 PRODUCTION OF T CELL-MODIFYING FACTORS BY HMCL.

It was important to attempt to identify potential T cell modifying cytokines produced by the HMCL. Firstly, U266 cells were examined for cytokine mRNA transcripts of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α and TGF-β1, by RT-PCR. This demonstrated that the tumour cells have strong mRNA transcripts for TGFβ1, strong mRNA transcripts for IL-6, faint mRNA transcripts for IL-1β, IL-2 and no evidence of IL-4, or IL-10 (Figure 5.6). Seven HMCLs - JIM1, JIM3. JJN3, RPMI8226, U266 (parent line) and clones M5 and 5R5 - were examined for evidence of IL-10 and TGF-β1 mRNA transcripts. It was demonstrated that all cell lines had strong transcripts for TGF-β1 mRNA but no band was demonstrated of 352 bps using the IL-10 primer pairs. However, the U266 parent cell line and clone M5 demonstrated faint bands of ~550 bps which may represent contamination with genomic DNA (Figure 5.7). The expression of TGF-β1 mRNA transcripts at the single cell and small colony level was assayed by single cell RT-PCR. U266 cells were cultured in methyl cellulose and single cells and small colonies (10 cells in diameter, approximately) were removed at various time points over a 10 day period and RT-PCR for TGF-β1 mRNA was performed. The culture of this clone in methylcellulose did not generate any single cells or 10-cell colonies which became negative for TGF-β1 mRNA by RT-PCR (Figure 5.8). No single cell or 10-cell colonies demonstrated evidence of IL-10 mRNA transcripts.

The supernates from 3 of these HMCLs, JIM1, JJN3 and U266, were examined for the production of TGF-β1 protein after 72 hours culture by ELISA. This demonstrated that these cell lines produce significant quantities of TGF-β1 when compared to the background content of FCS-supplemented culture medium: 10.63±0.39ng/ml in JJN3 supernates, 10.79±4.07 ng/ml in JIM1 supernates and 11.28±1.33 ng/ml in U266 M5 supernates *cf* 3.78 ±1.0 ng/ml in CM ($p<0.001$, Figure 5.9).

When the U266 cell line was cloned on methyl cellulose, heterogeneity in the immunosuppressive properties were seen between clones. In particular, two clones U266M5 and U266M6 demonstrated marked differences in their immunosuppressive properties on recovery from methyl cellulose into CM. U266M5 was able to suppress the Con A-induced proliferation of PBMNC from a healthy donor whereas U266M6 did not (Figure 5.10). Furthermore, whilst U266M6 clone demonstrated a typical MLR response U255M5 demonstrated a reverse MLR response.

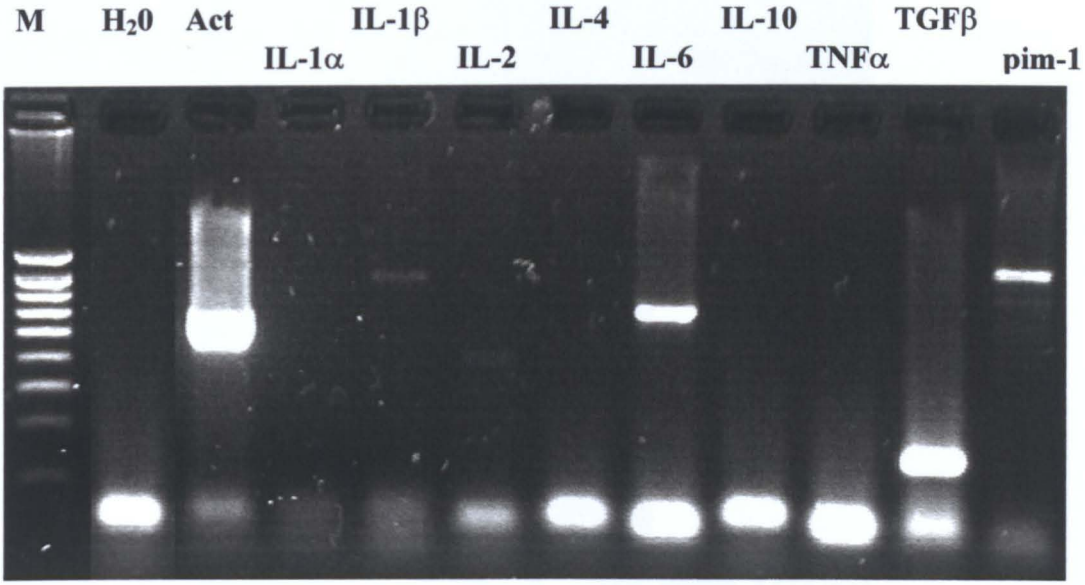


FIGURE 5.6. RT-PCR analysis of the HMCL, U266, for T cell-modifying cytokine mRNA. β-Actin mRNA was used as an amplification and loading control. PCR of total RNA revealed transcripts for IL-6 and TGF-β1 with faint levels of IL-1β and IL-2 mRNA transcripts being detected. The proto-oncogene, *pim-1*, was constitutively expressed in this cell line. Key: M- markers, Act- β-Actin, H_2O- negative control.

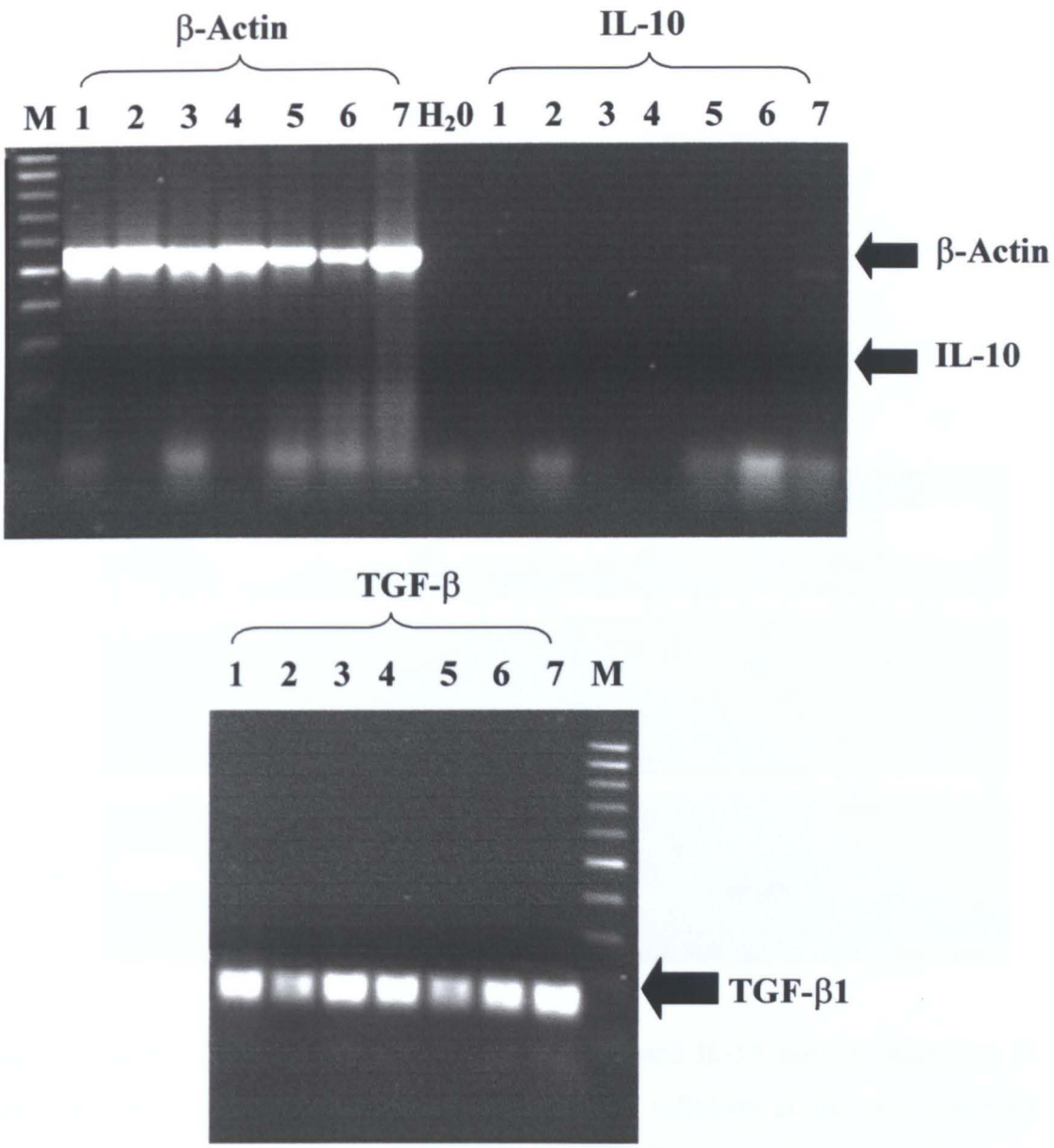


FIGURE 5.7. RT-PCR analysis of different HMCLs for the presence of IL-10 and TGF-β1 mRNA. Prominent TGF-β1 mRNA transcripts are seen in all cell lines but no evidence of IL-10 mRNA (352 bps) was detected. β-Actin mRNA was used as an amplification and loading control. **Key:** M- markers, H_2O - negative control, lane 1 – JIM1, lane 2- JIM3, lane 3 -JJN3, lane 4 - RPMI8226, lane 5 – U266 (parent line), lane 6- U266 5R5, lane 7- U266 M5.

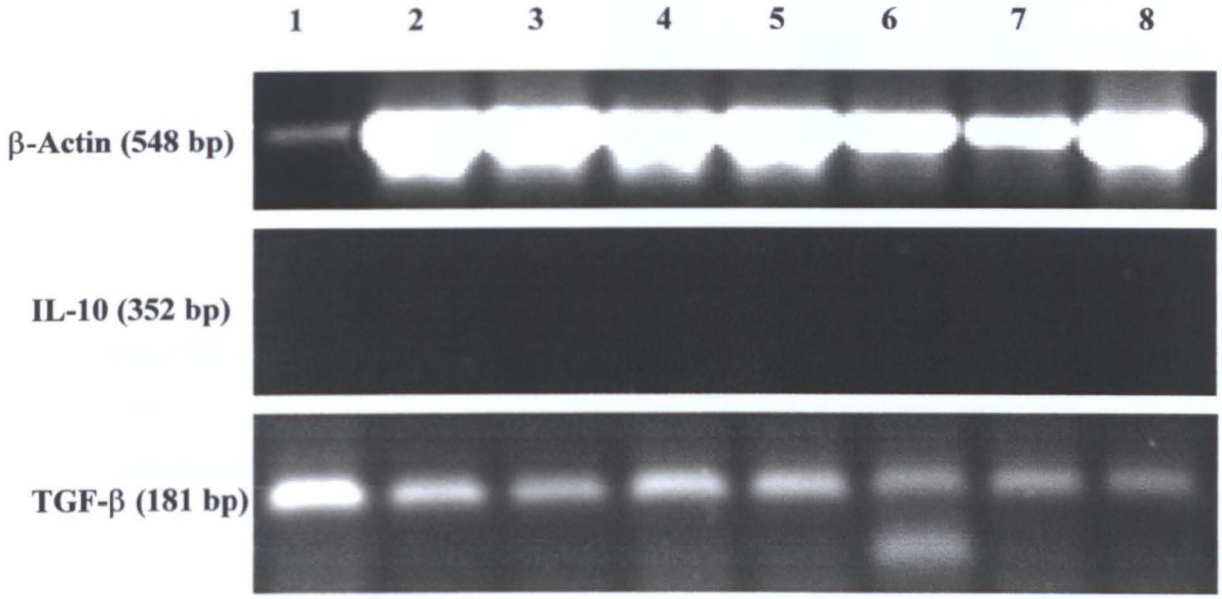


FIGURE 5.8. RT-PCR for the expression of TGF-β and IL-10 mRNA transcripts in single cells and 10-cell colonies cultured in methyl cellulose at the time intervals indicated. **Key:** lane 1-single cell, time 0; lane 2- 10 cell colony, time 0; lane 3- single cell, 24 hours; lane 4- 10 cell colony, 24 hours; lane 5- single cell, 72 hours; lane 6- 10 cell colony, 72 hours; lane 7- single cell, time 240 hours; lane 8- 10 cell colony, time 240 hours.

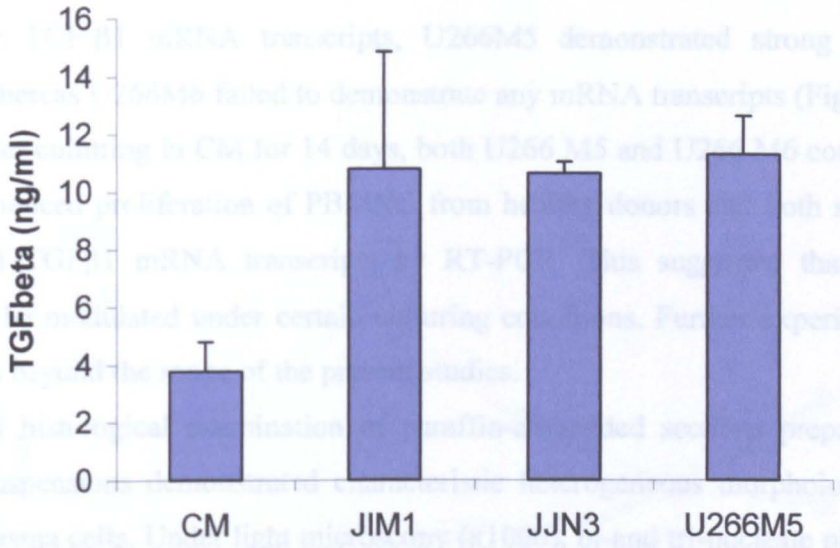


FIGURE 5.9. The HMCLs JIM1, JJN3 and U266 M5 produce significant quantities of TGF-β1 protein in their supernates after 72 hours culture, compared with FCS-supplemented CM ($p<0.001$).

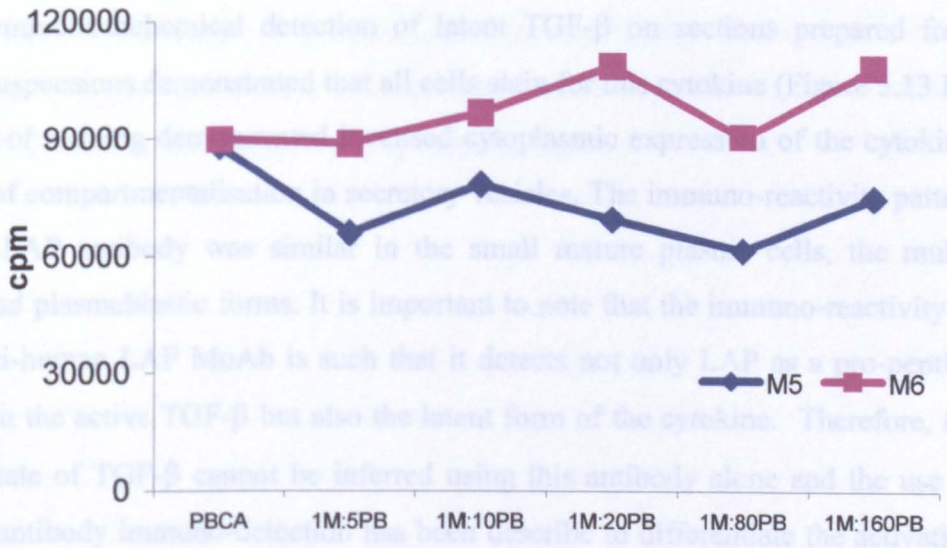


FIGURE 5.10. Suppression of Con A-induced proliferation of PBMNC from a normal donor when co-cultured with reducing concentrations of U266 clone M5. However, this is not evident when PBMNC are co-cultured with reducing concentrations of the U266 clone, M6. Proliferation was assessed by ^{3}[H]thymidine uptake and expressed as counts per minute. **Key:** PBCA- PBMNC - Con A, 1M:5PB- ratio of HMCL :PBMNC.

That is, with reducing concentrations of stimulator cells, an increase in the proliferation of the responders was demonstrated (Figure 5.11). When these clones were examined by RT-PCR for TGF-β1 mRNA transcripts, U266M5 demonstrated strong levels of transcripts whereas U266M6 failed to demonstrate any mRNA transcripts (Figure 5.12). However, after culturing in CM for 14 days, both U266 M5 and U266 M6 could inhibit the Con A-induced proliferation of PBMNC from healthy donors and both sub-clones demonstrated TGFβ1 mRNA transcripts by RT-PCR. This suggested that TGF-β1 mRNA may be modulated under certain culturing conditions. Further experimentation in this area is beyond the scope of the present studies.

Conventional histological examination of paraffin-embedded sections prepared from U266 cell suspensions demonstrated characteristic heterogeneous morphology of the malignant plasma cells. Under light microscopy (x1000), bi-and tri-nucleate plasma cell variants can be identified amongst the smaller plasma cells which demonstrate the characteristic features of mature plasma cells with eccentric mature nuclei, peri-nuclear golgi apparatus and a low nuclear:cytoplasmic ratio (Figure 5.13A & B). Amongst these smaller plasma cells, larger plasmablasts are identified demonstrating nucleolated nuclei. Apoptotic cells were easily identified in the sections with occasional mitotic figures. Immunohistochemical detection of latent TGF-β on sections prepared from U266 cell suspensions demonstrated that all cells stain for this cytokine (Figure 5.13 D). The pattern of staining demonstrated localised cytoplasmic expression of the cytokine, suggestive of compartmentalisation in secretory vesicles. The immuno-reactivity pattern of the anti-LAP antibody was similar in the small mature plasma cells, the multi-nucleated and plasmablastic forms. It is important to note that the immuno-reactivity of the goat anti-human LAP MoAb is such that it detects not only LAP as a pro-peptide cleaved from the active TGF-β but also the latent form of the cytokine. Therefore, the activation state of TGF-β cannot be inferred using this antibody alone and the use of differential antibody immuno-detection has been describe to differentiate the activation status of the cytokine in histological preparations (Barcelionos-Hoff et al, 1994).

5.3.3 RECOMBINANT TGF-β MIMICS HMCL-INDUCED T LYMPHOCYTE SUPPRESSION.

The effect of recombinant human TGF-β1 was investigated in the *in vitro* T cell model to determine whether this could mimic the growth retardation effect demonstrated by

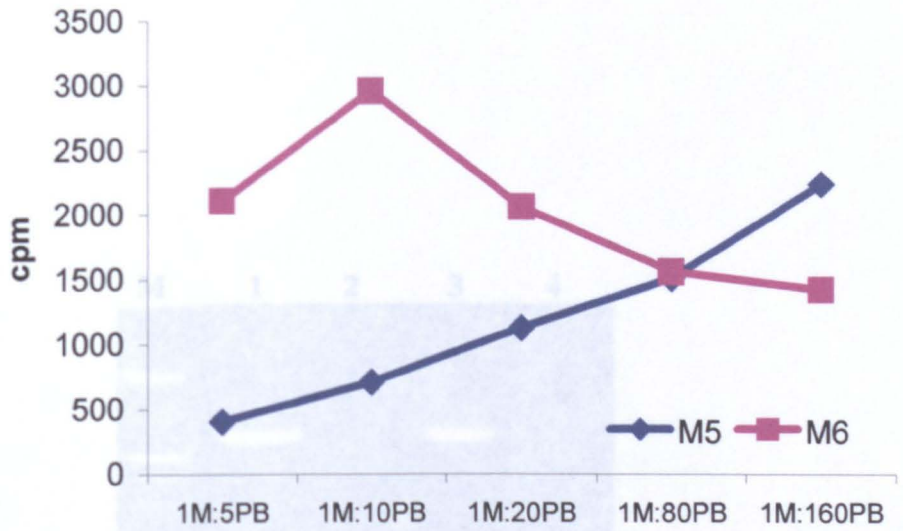


FIGURE 5.11. U266 clone M5 demonstrates a reverse MLR response with PBMNC from a normal donor after 5 days whereas U266 clone M6 exhibits a modest MLR response. Proliferation determined by ^{3}[H]thymidine uptake.

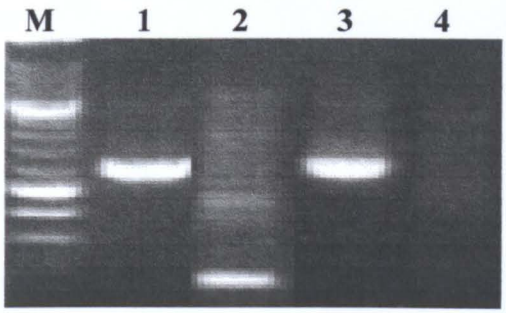


FIGURE 5.12. RT-PCR analysis of the two clones of U266, M5 and M6 for the presence of TGFβ1 mRNA. Prominent TGFβ1 mRNA transcripts are seen in M5 but not in M6. β-Actin mRNA was used as an amplification and loading control. **Key:** M-markers, lane 1 – M5 β-Actin , lane 2 - M5 TGF-β, lane 3 - M5 β-Actin, lane 4 – M5 TGF-β.

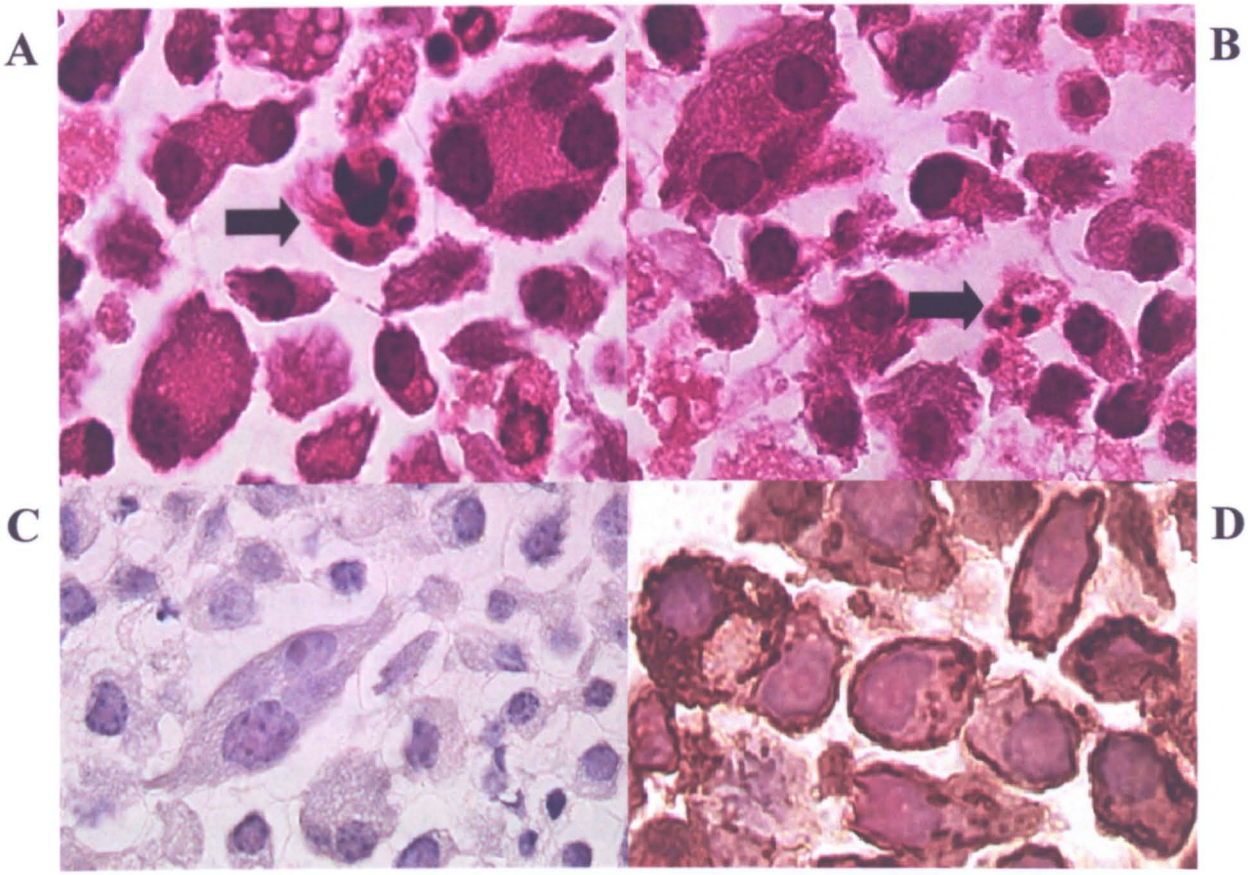


FIGURE 5.13. Immunohistochemical localisation of latent TGF-β1 in U266 cells, using a goat-anti-human LAP MoAb. Paraffin embedded sections of U266 cell suspension stained for histological examination with H&E (A & B; x1000), isotype control (C; x1000) and intra-cellular TGF-β1 using an anti-LAP MoAB (D; x1000). Characteristic malignant plasma cell features are seen in the H&E stained sections (A & B) including bi- and tri-nucleate variants and plasmablasts with occasional apoptotic cells seen (arrow).

myeloma-derived TGF-β. When IL-2-responsive PBL (n=8) were re-stimulated with rhIL-2 in the presence of increasing concentrations of recombinant TGF-β1, suppression of the proliferative responses was demonstrated. This suppression demonstrates a dose response effect, with the greatest effect being seen with TGFβ1 10 ng/ml with a reduction in ^{3}[H]dThd uptake from 33915±6,099 cpm to 16501±1,542 cpm (p=0.0142), as illustrated in Figure 5.14. A direct correlation was demonstrated between the degree of suppression and the TGF-β1 concentration (r^2=0.975, p=0.013). This suppression of IL-2 responsiveness was seen when 20 U/ml of rhIL-2 was used to stimulate the PBL. To determine whether increasing concentrations of IL-2 could overcome the suppression of proliferative responses, IL-2-responsive PBL were cultured for 36 hours in increasing concentrations of both rhIL-2 and TGF-β1 (Figure 5.15). Increasing IL-2 concentrations could not overcome the suppressive effect of TGF-β1 on PBL proliferation, even at the lowest TGF-β1concentration. However, incremental proliferative responses are still evident with increasing concentrations of rhIL-2. This agrees with data that I have presented in this thesis demonstrating that the addition of IL-2 to Con A could not overcome the suppression of proliferative responses of PBMNC by HMCLs (Figure 4.3).

Recombinant TGF-β1 can suppress the activation stage of the *in vitro* model, so to determine the effect of TGF-β1 on the IL-2 autocrine and cytokine-dependent stages, TGF-β1 was co-cultured with Jurkat E6.1 and IDBL cells, respectively. When TGF-β1 was added in increasing concentrations to the culture of Jurkat E6.1 cells, at a concentration of $1x10^4$/ml, no suppression of proliferation was detected after 72 hours of culture (n=3), by ^{3}[H]dThd uptake (Figure 5.16). Similarly, when TGF-β1 was added in increasing concentrations to IDBL cells cultured in 20 U/ml of rhIL-2, at a concentration of $1x10^5$/ml, no suppression of proliferation was demonstrated after 72 hours (n=3). Thus using the 3 cell model of T cell activation/homeostasis, recombinant TGF-β1, like HMCLs, specifically inhibit T cells entering the activation phase with little or no effect being demonstrated on cells which have traversed this phase and entered the IL-2 autocrine and cytokine dependent stages.

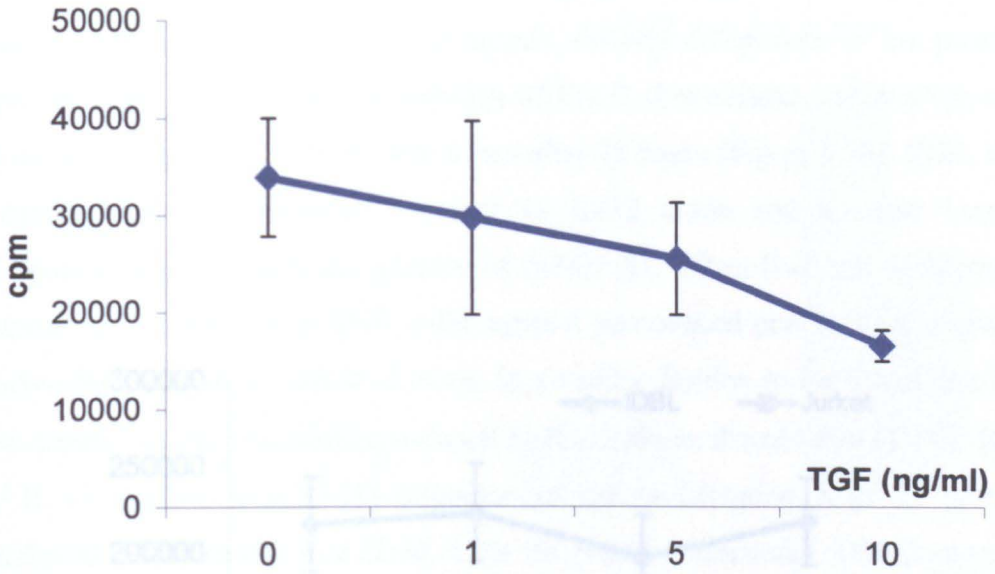


FIGURE 5.14. The effect of culturing mitogen activated PBL re-stimulated with 20 U/ml rhIL-2 (n=8) in increasing concentrations of TGFβ1 on proliferation, as measured by ^{3}[H]thymidine uptake after 36 hours. Only TGFβ1 10ng/ml demonstrated a statistically significant reduction in IL-2-induced proliferation (p=0.0142).

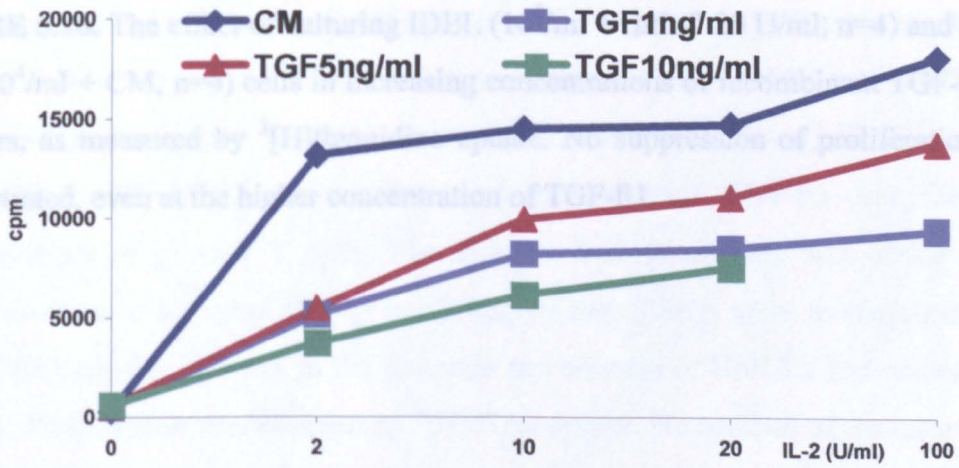


FIGURE 5.15. The effect of culturing IL-2 responsive PBL in increasing concentrations of rhIL-2 and TGF-β1 for 36 hours. Increasing the concentration of rhIL-2 does not completely reverse the suppressive effect of TGF-β1 though incremental proliferative responses are still seen. Proliferation is represented by ^{3}[H] thymidine uptake.

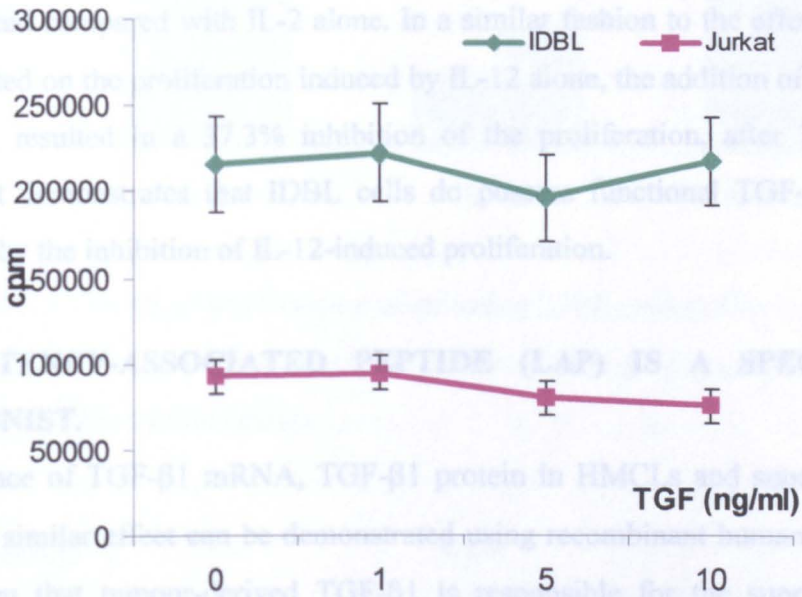


FIGURE 5.16. The effect of culturing IDBL (10^5/ml + rhIL-2 20 U/ml, n=4) and Jurkat E6.1 (10^4/ml + CM, n=4) cells in increasing concentrations of recombinant TGF-β1 for 72 hours, as measured by ^{3}[H]thymidine uptake. No suppression of proliferation was demonstrated, even at the higher concentration of TGF-β1.

When IDBL cells (10^5/ml, n=3) were stimulated with IL-2, IL-12 or IL-2 + IL-12 in the presence and absence of TGF-β1 (5 ng/ml), distinct differences in the proliferative responses were observed. No suppression of the IL-2–mediated proliferative response was demonstrated when TGF-β1 was added after 72 hours (Figure 5.17). IDBL cells fail to demonstrate a proliferative response to IL-12 alone and a slight increase in proliferation was seen with the addition of rhTGF-β1. When IL-2 and IL-12 were used to stimulate proliferation of IDBL cells, again a pronounced proliferative response was demonstrated compared with IL-2 alone. In a similar fashion to the effect that TGF-β1 demonstrated on the proliferation induced by IL-12 alone, the addition of TGF-β1 to IL-2 + IL-12 resulted in a 37.3% inhibition of the proliferation, after 72 hours. This experiment demonstrates that IDBL cells do possess functional TGF-β receptors as evidenced by the inhibition of IL-12-induced proliferation.

5.3.4 LATENCY-ASSOCIATED PEPTIDE (LAP) IS A SPECIFIC TGFβ ANTAGONIST.

The presence of TGF-β1 mRNA, TGF-β1 protein in HMCLs and supernates and the fact that a similar effect can be demonstrated using recombinant human TGF-β1, does not confirm that tumour-derived TGF-β1 is responsible for the suppressive effects exerted by HMCL on activating T cells. Experiments were set up to directly block TGF-β1 and examine the effect this has on immune suppression. A rabbit anti-pan TGF-β monoclonal antibody was used to reverse the suppressive effect of HMCLs (JIM1, JIM3, JJN3, RPMI8226 and U266) and recombinant TGF-β1 on the IL-2 responsiveness of primary T cells. The anti-pan TGF-β antibody was added to the culture medium at 1.5μg/ml (ND_{50}) and 2.0μg/ml and T cells were re-stimulated with rhIL-2 (20U/ml) for 36 hours in the presence and absence of HMCLs and recombinant TGF-β1. Proliferation was assessed by ^{3}[H]dThd uptake. No reversal of the suppression of IL-2 responsiveness was demonstrated when the monoclonal antibody was added at either concentration to primary T cells stimulated in the presence of

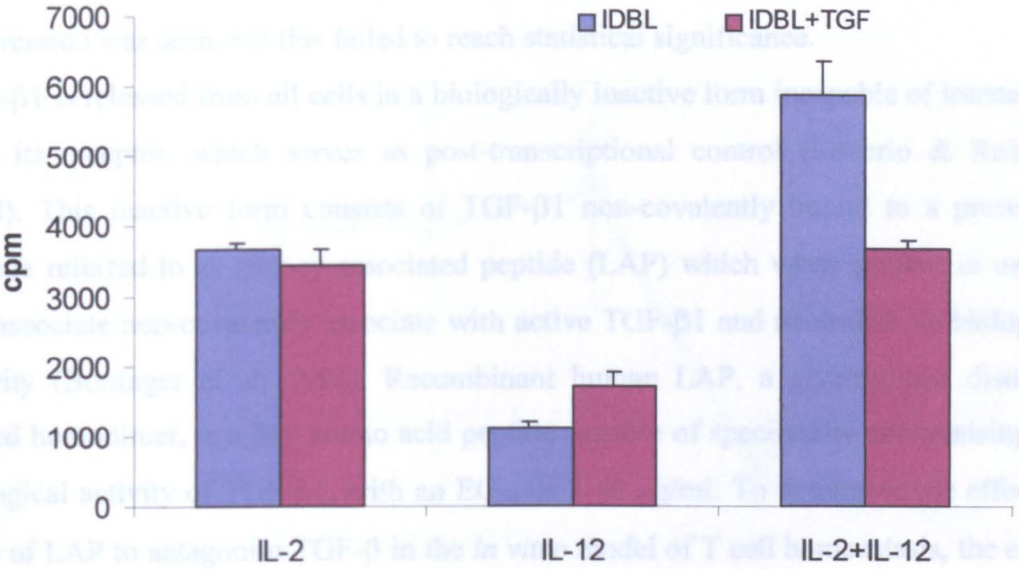


FIGURE 5.17. The effect proliferation of culturing IDBL cells (10^5/ml, n=3) with IL-2, IL-12 and IL-2+IL-12 in the presence and absence of TGF-β1 (5 ng/ml) for 72 hours, as measured by ^{3}[H]thymidine uptake.

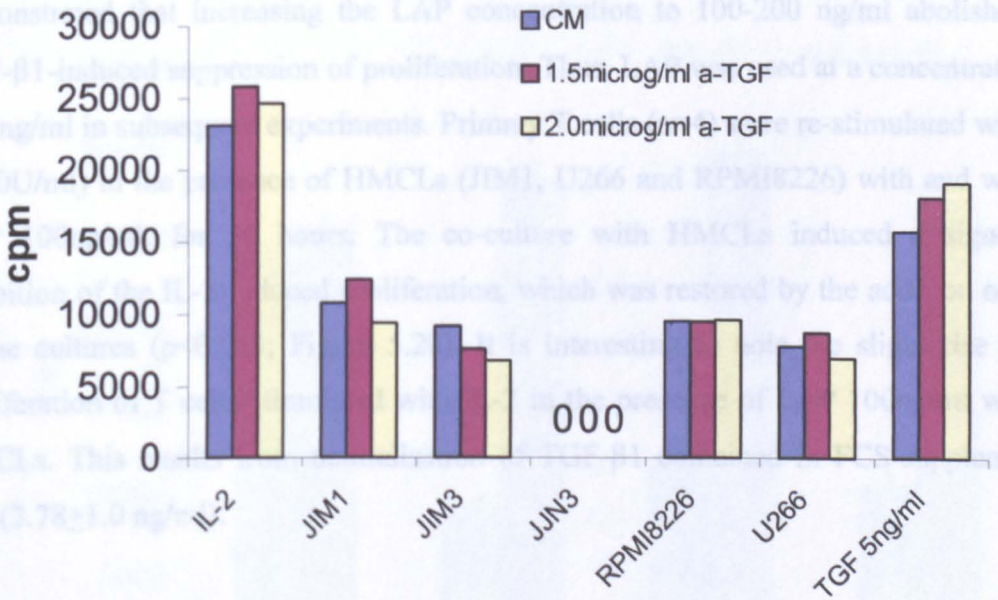


FIGURE 5.18. The addition of rabbit anti-pan TGF-β (a-TGF) at the ND_{50} dose (1.5μg/ml) and higher has no effect on the immunosuppressive effect of HMCLs on the IL-2 responsiveness of primary T cells, As determined by ^{3}[H] thymidine uptake. Slight improvement of suppression induced by recombinant TGF-β1 was seen both at 1.5μg/ml and 2.0μg/ml, which was not statistically significant.

HMCLs (Figure 5.18). A slight improvement in recombinant TGF-β1-induced suppression was seen, but this failed to reach statistical significance.

TGF-β1 is released from all cells in a biologically inactive form incapable of interaction with its receptor, which serves as post-transcriptional control (Letterio & Roberts, 1998). This inactive form consists of TGF-β1 non-covalently bound to a proregion peptide referred to as latency associated peptide (LAP) which when present in excess can associate non-covalently associate with active TGF-β1 and neutralise its biological activity (Bottinger et al, 1996). Recombinant human LAP, a glycosylated disulfide linked homodimer, is a 249 amino acid peptide capable of specifically antagonising the biological activity of TGF-β1, with an EC_{50} of 8-40 ng/ml. To determine the effective dose of LAP to antagonise TGF-β in the *in vitro* model of T cell homeostasis, the effect of increasing concentrations of LAP on the suppression of IL-2-induced proliferation of primary T cells by 5 ng/ml of TGF-β1 was assessed. It was shown that at 100ng/ml of LAP, the suppressive effect of TGF-β1 was abolished in a 36 hour culture, as determined by ^{3}[H] thymidine uptake (Figure 5.19). When the effect of TGF-β1 on IL-2-induced proliferation was expressed as percentage inhibition of proliferation, it clearly demonstrated that increasing the LAP concentration to 100-200 ng/ml abolishes the TGF-β1-induced suppression of proliferation. Thus, LAP was used at a concentration of 100 ng/ml in subsequent experiments. Primary T cells (n=4) were re-stimulated with IL-2 (20U/ml) in the presence of HMCLs (JIM1, U266 and RPMI8226) with and without LAP 100ng/ml, for 36 hours. The co-culture with HMCLs induced a significant inhibition of the IL-2-induced proliferation, which was restored by the addition of LAP to the cultures ($p<0.001$; Figure 5.20). It is interesting to note the slight rise in the proliferation of T cells stimulated with IL-2 in the presence of LAP 100ng/ml without HMCLs. This results from neutralisation of TGF-β1 contained in FCS-supplemented CM (3.78±1.0 ng/ml).

5.3.5 THE EFFECT OF TGF-β BLOCKADE ON HMCL GROWTH AND VIABILITY.

TGF-β has been shown to inhibit B lymphocyte proliferation and induce apoptosis in both B cells and mature plasma cells, which is thought to be an important negative regulatory feedback loop to limit expansion of activated cells (Letterio & Roberts, 1998). However, the importance of this inhibitory autocrine loop in malignant B cells

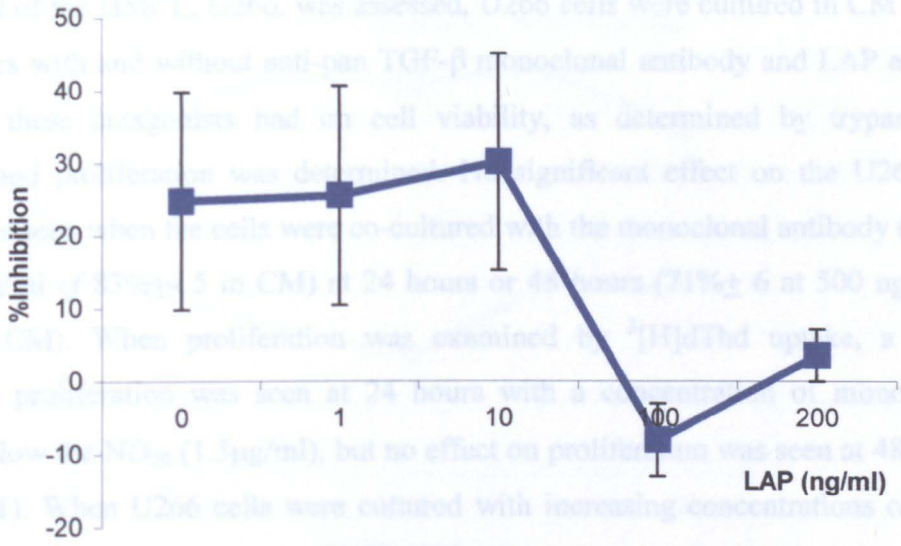


FIGURE 5.19. The effect of increasing concentrations of LAP on the TGF-β-induced (5ng/ml) inhibition of the proliferative response to IL-2 by primary T cells, as depicted in Figure 5.18.

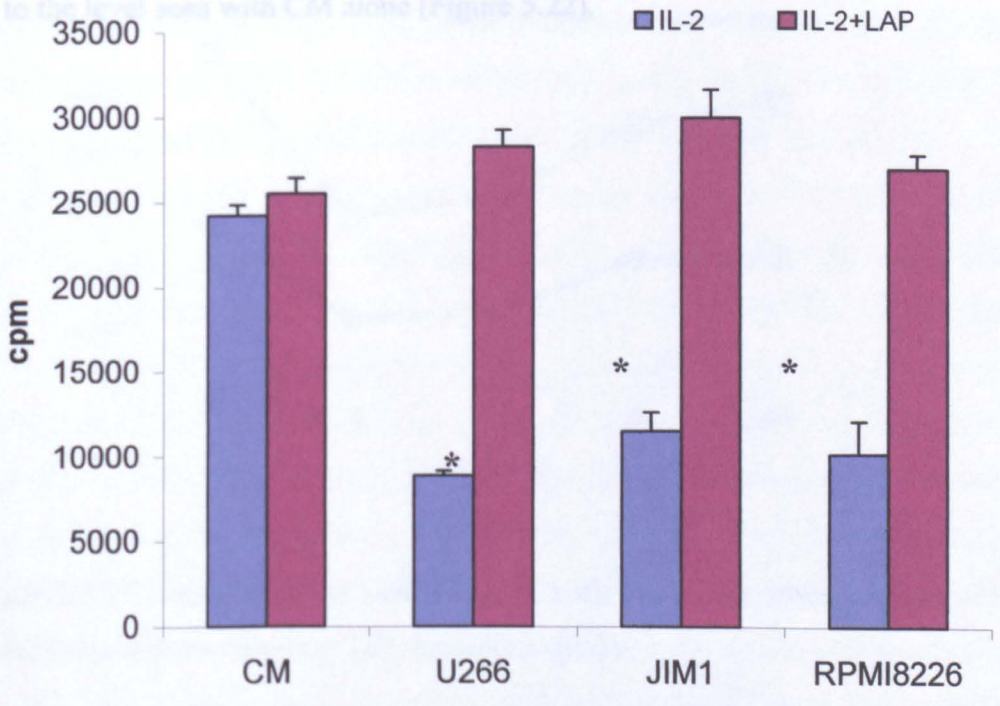


FIGURE 5.20. The addition of LAP reverses the suppression of IL-2-induced proliferation of primary T cells (n=4) by HMCLs, JIM1, U266 and RPMI8226. (*= $p<0.001$).

remains to be determined. The effect of TGF-β1 autocrine secretion on the proliferation and survival of the HMCL, U266, was assessed. U266 cells were cultured in CM for 24 and 48 hours with and without anti-pan TGF-β monoclonal antibody and LAP and the effect that these antagonists had on cell viability, as determined by trypan blue exclusion, and proliferation was determined. No significant effect on the U266 cell viability was seen when the cells were co-cultured with the monoclonal antibody (77%± 3 at 500 ng/ml *cf* 83%±4.5 in CM) at 24 hours or 48 hours (71%± 6 at 500 ng/ml *cf* 85%±2 in CM). When proliferation was examined by ^{3}[H]dThd uptake, a slight decrease in proliferation was seen at 24 hours with a concentration of monoclonal antibody below the ND_{50} (1.5μg/ml), but no effect on proliferation was seen at 48 hours (Figure 5.21). When U266 cells were cultured with increasing concentrations of LAP for 24 and 48 hours, similarly, no significant alteration in the cell viability was seen at either time point (81%± 5 at 40 ng/ml *cf* 83%±4.5 in CM at 24 hours and 73%± 6 at 40 ng/ml *cf* 83%±4.5 in CM at 48 hours). When the proliferation of U266 cells was examined at 24 and 48 hours of co-culture with increasing concentrations of LAP, it was shown that there is a suppression of proliferation at a concentration of LAP below the EC_{50} (10ng/ml), which recovers with increasing LAP concentration though does not return to the level seen with CM alone (Figure 5.22).

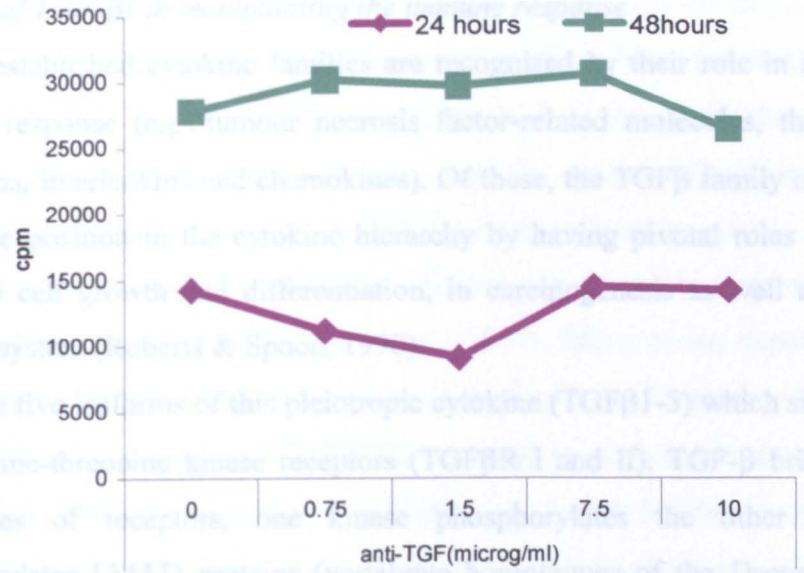


FIGURE 5.21. Co-culture of U266 with increasing concentrations of the anti-pan TGF-β monoclonal antibody. Proliferation was determined by ^{3}[H] thymidine uptake.

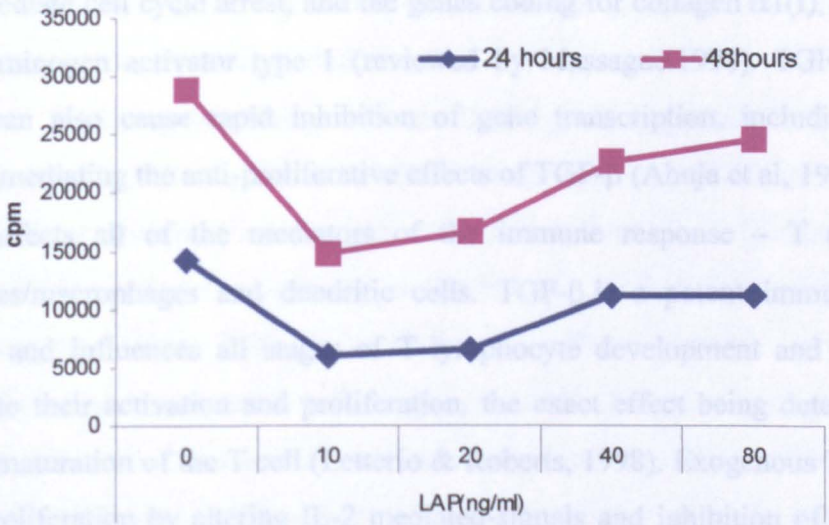


FIGURE 5.22. Co-culture of the HMCL, U266 with increasing concentrations of LAP. Proliferation was determined by ^{3}[H] thymidine uptake.

5.4 DISCUSSION

The role of TGF-β1 in manipulating the immune response

Several established cytokine families are recognised by their role in manipulating the immune response (e.g. tumour necrosis factor-related molecules, the TGFβ family, interferons, interleukins and chemokines). Of these, the TGFβ family of cytokines have an unique position in the cytokine hierarchy by having pivotal roles in development, epithelial cell growth and differentiation, in carcinogenesis as well as regulating the immune system (Roberts & Spoon, 1990).

There are five isoforms of this pleiotropic cytokine (TGFβ1-5) which signal through the same serine-threonine kinase receptors (TGFβR I and II). TGF-β brings together the two types of receptors, one kinase phosphorylates the other which in turn phosphorylates SMAD proteins (vertebrate homologues of the *Drosophila* gene *Mad* (*mothers against dpp*)) which are novel signal transducers (Massagué.1998). The SMADs, upon phosphorylation, migrate to the nucleus and generate transcriptional complexes of specific DNA-binding ability. Numerous gene responses to TGF-β have been described and include the cyclin-dependent kinase inhibitors $p15^{Ink4b}$ and $p21^{Cip1}$ which mediate cell cycle arrest, and the genes coding for collagen α1(I), collagen α2(I) and plasminogen activator type 1 (reviewed by Massagué.1998). TGF-β and related factors can also cause rapid inhibition of gene transcription, including *c-myc* and cdc25A, mediating the anti-proliferative effects of TGF-β (Ahuja et al, 1993).

TGF-β affects all of the mediators of the immune response – T cells, B cells, monocytes/macrophages and dendritic cells. TGF-β is a potent immunosuppressive cytokine and influences all stages of T lymphocyte development and differentiation through to their activation and proliferation, the exact effect being determined by the stage of maturation of the T cell (Letterio & Roberts, 1998). Exogenous TGF-β inhibits T cell proliferation by altering IL-2 mediated-signals and inhibition of IL-2 mediated phosphorylation of the retinoblastoma susceptibility gene product (Fontana et al, 1989; Wahl et al, 1989; Ahuja et al, 1993;Becker et al, 1994). Furthermore, TGF-β has been shown to reduce activation-induced apoptosis in T cells by down-regulating the surface expression of FasL in association with the down regulation of *c-myc* gene expression (Genestier et al, 1999).

The generation of distinct T cell subsets, based on their cytokine production profiles, from naïve peripheral T cells is a process that is poorly understood in humans. There are

multiple pathways that facilitate the polarisation of T_h subsets and certain cytokines have a role as inducers of either the T_{h1} (IFN-γ and IL-2) or the T_{h2} (IL-4) response (Seder et al, 1994). Different experimental systems have produced conflicting and variable roles for TGF-β in T_h differentiation. TGF-β1 can inhibit the production of, and response to cytokines associated with each T_H subset. TGF-β is produced by an identified population of antigen-specific T cells, referred to as T_{H3} cells, which may represent a subset of cells that regulate the intensity of an immune response to specific antigen (Chen et al, 1994; Bridoux et al, 1997). More recent experimental evidence implicating a role for TGF-β1 in manipulating the T_H differentiation in human systems suggests that the effects of TGF-β1 are mediated through mechanisms associated with abrogation of IL-12 production (Pardoux et al, 1997). Neither exogenous IL-12 nor IFN-γ can reverse this antagonistic effect of TGF-β1. Furthermore, it has been shown in a murine experimental system, that the immuno-suppressive effect of TGF-β (tumour-derived) is more marked on $CD4^+$ T cells and that observed defects in CTL generation result from failure of the T_H cells to orchestrate a suitable response (Tada et al, 1991).

In the *in vitro* model system used in the experiments described in this chapter, TGF-β1, both tumour-derived and exogenous cytokine, reduced primary T cell responses to IL-2. Other investigators have suggested that TGF-β1 suppresses IFN-γ and IL-4 production by human T cells by interfering with the IL-2-signalling pathway (Ahuja et al, 1993; Holter et al, 1994). This is similar to effects demonstrated by IL-10, though this effect is mediated by suppressing antigen-presenting cell-derived IL-12 (Korholz et al, 1997). IL-10 mRNA was not demonstrated in the HMCLs used in these experiments. It has been suggested that TGF-β1 mediates its effects through tyrosine and Rb phosphorylation in association with suppression of IL-2Rα gene up-regulation (Ahuja et al, 1993). However, it has not been determined whether such effects are mediated through the TCR and IL-2R pathways or IL-2R pathway alone. In summary, TGF-β1 regulates T cell proliferation, cell cycle progression and cytokine production through the inhibition of phosphorylation of molecules involved in cell cycle regulation. It particularly inhibits IL-2-mediated proliferative signals through as yet unknown pathway(s). T cells both produce active TGF-β1 and express TGF-β1 receptors and thus under physiological conditions it represents a regulator of the immune response. However, produced in excess by a tumour, TGF-β1 could be responsible for tumour-associated immuno-suppression. TGF-β has been shown to be produced by many

different tumour cell types (Fischer et al, 1994; Urishima et al, 1996; Merogi et al, 1997). Many defects in T cell responsiveness have been reported in these settings, but in multiple myeloma, no such mechanism has been characterised to explain the observed T cell anergy.

The role of TGF-β1 in the pathogenesis of Multiple Myeloma.

The synthesis of TGF-β1 by B cells in health relates to their state of activation and differentiation. For example, when tonsillar B cells are stimulated by mitogens (staphylococcal antigen Cowan; SAC), a slight up-regulation of TGF-β1 mRNA is seen but this is associated with a 7-fold increase in protein, most of which (>90%) remains in the latent/inactive form (Kehrl et al, 1986). TGF-β typically inhibits proliferation in both B cells and mature plasma cells and therefore may act as a negative feedback to regulate the extent of the immune response (Lomo et al, 1995). It has been proposed that some B cell malignancies (chronic lymphocytic leukaemia; CLL) have retained this negative feedback loop that might account for their reduced proliferative capacity (Lotz et al, 1994). However, it is worthy of note that like many epithelial malignancies, several B cell malignant disorders have been identified as "insensitive" to the inhibitory effects of TGF-β, perhaps resulting from loss or dysfunction of one or more components of the TGF-β receptor (Tada et al, 1991; Decoteau et al, 1997). This has been proposed to contribute to the suppression of immune surveillance and associated with tumour progression. TGF-β was originally thought to suppress the secretion of immunoglobulins (reviewed in Stavnezer, 1996). However, it has been shown that the addition of anti-TGF-β MoAb, which blocks the biological effect of TGF-β, to cultures of LPS-activated B cells, a reduction in the secretion of IgG1, IgG2a, IgG3 and IgE is seen with no effect on the production of IgM nor IgA (Snapper et al, 1993). This suggests that TGF-β may have differing effects on B cell function at varying levels and may enhance the production and secretion of immunoglobulin under certain conditions.

TGF-β has been shown not only to regulate the response of T cells but also of B cells to IL-2 (Kerhl et al, 1986). It was demonstrated that TGF-β1inhibits IL-2-dependent DNA synthesis in B cells though this effect was limited in EBV-transformed B cell lines, owing to the absence of TGF-β receptors on the transformed cell line. The production of TGF-β by B cells may be important in the cell-cell and cell-microenvironment interactions. TGF-β has been shown to accelerate wound healing and stimulate

fibroblasts and has been associated with the production of IL-6 by bone marrow stromal cells (Letterio & Roberts, 1998). As such TGF-β may function as an important B cell cytokine for the orchestration of the immune response in health, both in establishing and limiting the extent of the response in an autocrine fashion. However its role in the malignant variant of normal B cell physiology is clearly quite different and variable according to the malignant transformation.

In multiple myeloma, a malignant disorder of terminal B cells, several studies have shown TGF-β1 mRNA in tumour cells and derived cell lines which correlates with secretion of active TGF-β1, as demonstrated in this chapter. (Matthes et al, 1993; Portier et al, 1993; Matthes et al, 1995; Urashima et al, 1996). Some investigators have shown that the level of TGF-β1 secreted by MM cells is greater than normal B cells and CD40L-activated B cells and that BM stromal cells from MM patients secrete more TGF-β1 than BM stromal cells from normal control subjects (Urashima et al, 1996). Other groups have demonstrated high serum and urinary levels of TGF-β1 in patients with MM using ELSIA assays and there is a suggestion that serum levels correlate with the extent of myeloma bone disease (Jiang et al, 1995; Kroning et al, 1997).

In contrast to its effects on normal B cells, TGF-β1 does not decrease the proliferation of MM cells and may even augment IL-6 secretion and related proliferation (Urashima et al, 1996). It has been demonstrated that the lack of inhibition of proliferation is associated with a failure to inhibit Rb phosphorylation or DNA synthesis in both HMCLs and fresh tumour cells from patients with multiple myeloma. This insensitivity may in part be related to abnormalities of both Rb and p53 tumour suppressor gene products, mutations of which have been reported in 35-40% and up to 52% of fresh myeloma tumour cells, respectively (Corradini et al, 1994). TGF-β1 has been shown to induce both autocrine IL-6 production by fresh myeloma tumour cells and HMCLs (Urashima et al, 1996). The same authors demonstrated that TGF-β1 induced paracrine IL-6 production from the stromal cells derived from bone marrow samples of patients with multiple myeloma. In addition, using anti-TGF-β1 MoAb, the authors demonstrated a reduction in the IL-6 production by both stromal cells and fresh myeloma tumour cells, perhaps mediated through alterations in cell-cell adhesion, though no alteration in surface density of adhesion molecules has been demonstrated. In this chapter I have shown preliminary experimental data suggesting that blocking TGF-β1 using LAP altered the proliferation kinetics of the HMCL, which may be influenced

by alterations in IL-6 production and signalling. However, further investigation of the role of TGF-β1 in propagating the viability and growth of myeloma plasma cells in beyond the remit of this thesis. Clearly there is a need to examine in greater detail what effect TGF-β1 has on myeloma plasma cell cytokine and chemokine production, cell division kinetics especially in relation to the cell cycle regulators Rb, *c-myc*, p53 and E2F and the cyclin-dependent kinase inhibitors $p15^{Ink4b}$ and $p21^{Cip1}$. Furthermore, the relationship of TGF-β1 and the homing/re-circulation of myeloma plasma cells including alterations in adhesion molecule profile and chemokines production and responsiveness remains to be explored.

TGF-β is responsible for myeloma-induced immunosuppression.

The data presented in this chapter strongly support the theory that myeloma tumour cells suppress the activation of T lymphocytes through the production of the pleitropic cytokine, TGF-β1. In consideration of this theory, there are a number of facts illustrated by this data worth noting. Firstly, both HMCLs and recombinant TGF-β1 suppress the IL-2 responsiveness of T cells from normal volunteers that have previously been activated by the Con A. Such suppression results in the accumulation of cells in the G_0/G_1phase of cell cycle with no significant increase in the apoptosis rate compared to T cells re-stimulated in the absence of HMCLs. As the T cells are blocked from entering the activation phase, susceptibility to apoptosis is no different from that seen in the absence of HMCLs. Fas/FasL interactions may cause T lymphocyte cell cycle arrest rather than apoptosis dependent on the stage of activation, though this failure to progress through the stages of cell cycle can be reversed by IL-2 (Desbarats et al, 1998). Secondly, TGF-β1 was identified as the main mediator of the suppression T cell activation. The identification of cytokine mRNA in the tumour cells is in itself not proof of involvement of suppression of T cell responses. The primary level of control of TGF-β1 production is not regulated at the mRNA level but is subjected to post-transcriptional control by the process of secretion and activation of the TGF-β1 protein (Letterio & Roberts, 1998). The HMCLs do in fact secrete high levels of total TGF-β1, as evidenced by the ELISA assays that showed that levels up to 4 fold greater than that recorded in FCS-supplemented CM. The involvement of TGF-β1 as a major mediator of HMCL-induce T cell suppression was further confirmed by the blocking experiments using LAP. TGF-β1 and LAP are components of a pro-peptide which is cleaved in the

post–golgi compartment prior to secretion (Massagué, 1998). LAP is a highly specific antagonist for TGF-β1, which binds the active cytokine and holds it in an inactive conformation, thus blocking its biological effects (Bottinger et al, 1996). LAP demonstrates superior binding capacity to TGF-β1 compared to the soluble extra-cellular domain of the TGF-β receptor type II with a 15-fold (8nM versus 120nM) lower equilibrium dissociation constant.

Thirdly, using the three-cell model of T cell activation/homeostasis, the data demonstrate that the effects of HMCLs and rhTGF-β1 are limited to the activation phase and not the IL-2-dependent phases as evidenced by the absence of suppression of Jurkat E6.1 and IDBL cell proliferation. It is accepted that using transformed cell lines in an *in vitro* cellular model in this setting is subject to limitations. The cellular characteristics, particularly in respect to suppressive factors, may not truly represent the *in vivo* behaviour of T cells at these phases of proliferation and cytokine homeostasis. Many potential reasons may exist to explain these including absent or dysfunctional receptors for immune modulating cytokines and chemokines, absent or defective intra-cellular signalling pathways for transduction of immune mediator interactions with their respective receptors and altered activation signal transduction pathways. It is important to emphasise that IDBL cells were demonstrated to have functional TGF-β1 receptors by virtue of the suppression of IL-12-mediated proliferative signals by exogenous, recombinant TGF-β1. Phenotypic analysis using immunofluorescent flow cytometry to detect TGF-β receptors was not performed on either cell line.

Amongst the other possible explanations for the observed insensitivity of these cell lines to TGF-β1 is the fact that both Jurkat E6.1 and IDBL cells do not express the IL-2Rα subunit, neither at the protein level nor the mRNA level, and thus do not signal through the high affinity receptor (IL-2Rαβγ_c). Signalling through the high affinity IL-2R facilitates the entry into the IL-2 autocrine pathway (Minami et al, 1993). However, both Jurkat E6.1 and IDBL cells are beyond the entry point to the IL-2 autocrine pathway: Jurkat cells being immortalised in the IL-2 autocrine pathway and IDBL cells existing beyond this phase, demonstrating cytokine-dependant susceptible apoptosis upon the withdrawal of exogenous IL-2. As such both Jurkat E6.1 and IDBL cells may be resistant to the inhibitory effects of HMCL-derived and recombinant TGF-β1 may relate to the absence of signalling through the IL-2Rα and this warrants further investigation.

The exact role of TGF-β1 in the pathogenesis of multiple myeloma, including host tolerance of the malignant clone, remains to be clarified. The data presented in this chapter clearly indicates it is a major cytokine involved in modulation of T cell activation whilst other investigators have demonstrated its role in the maintenance and survival of the malignant B cell clone. It is also possible that TGF-β1 contributes to the identified suppression of normal haematopoiesis frequently associated with the disease, especially in advanced stages. The molecular mechanisms which dictate the inhibition of T cells which are in the early activation phase prior to entry into the IL-2 autocrine pathway warrants closer examination including the relative resistance that transformed T cell lines which represent cells in the autocrine IL-2 pathway and at the cytokine-dependent stage of T cell homeostasis demonstrate. This series of investigations will form the basis of the chapter 6.

CHAPTER 6

Myeloma-derived TGF-β requires the α chain of the IL-2 receptor for T cell suppression

6.1 INTRODUCTION

In chapter 3 and 5 it was demonstrated that HMCLs prevent the full activation of T lymphocytes associated with cell cycle arrest. This effect is mediated through the production of the potent immuno-suppressive cytokine, TGF-β1. The effect of HMCL-derived and recombinant TGF-β1 is not observed on T cells which have committed to the IL-2 autocrine pathway or have progressed beyond this into the cytokine-dependent phase of T cell homeostasis. Other investigators have shown that TGF-β1 can inhibit IL-2 production and the inhibitory effects have been mapped to the octamer element of the IL-2 promoter (Brabletz et al, 1993; Rooney et al, 1995). Furthermore this does not explain the exemption from suppression by TGF-β1 of the cells in the IL-2 autocrine and cytokine dependent phases of activation. Therefore, as yet, the influence that TGF-β1 exerts on the IL-2 gene up-regulation and entry of T cells into the IL-2 autocrine pathway remains to be determined.

Whilst there are many molecular differences between the three distinct phases of T cell activation, as delineated in the *in vitro* model I have described, one major difference of note is the variation in the requirement of IL-2Rα subunit in supporting the high affinity interaction of IL-2 and subsequent progression into the IL-2 autocrine pathway. Primary T cells can up-regulate IL-2Rα in response to IL-2 as a prelude to autocrine production of IL-2. This is in contrast to both IDBL cells and Jurkat E6.1 which have no requirement for high affinity interactions with IL-2 and neither express nor up-regulate IL-2Rα. Most primary T cells rendered IL-2 responsive by mitogen activation express IL-2Rα but these cells still need to up-regulate IL-2Rα in order to progress to the IL-2 autocrine pathway in response to exogenous cytokine.

Interleukin-2

The T cell growth factor, IL-2, is produced by T cells upon antigenic stimulation and is vital for clonal expansion and survival (Minami et al, 1993; Nelson & Willerford, 1998). IL-2 is a 15-18 kD (depending on the degree of glycosylation), 133 amino acid glycoprotein encoded by a single gene on chromosome 4 and acts in a monomeric form in its ligation with its receptor (Smith, 1993). IL-2 is a potent growth factor responsible for the promotion of cell cycle progression, in particular G_1-S phase transition, in T cells. T cell antigen recognition by the TCR with appropriate co-stimulation results in the de novo transcription and translation of IL-2, mediated by the protein tyrosine

kinase, $p56^{lck}$ (Minami et al, 1993B). The production of IL-2 results in the up-regulation of the high affinity receptor, IL-2Rα, which mediates further IL-2 production by an autocrine loop (Nelson & Willerford, 1993). In addition, IL-2-mediated signal transduction results in functional uncoupling of the TCR complex through altering the sub-cellular distribution of kinase active lck, indicating the role of IL-2 in a negative feedback regulatory mechanism (Haughn et al, 1998; Lenardo et al, 1999).

The exact signalling pathways involved in the actions of IL-2 remain unclear, especially in the context of inducing cell cycle progression and protection from apoptosis. In part, some of the uncertainty results from initial studies that examined sub-cellular signalling in transformed T cells. These indicated that IL-2-induced cell cycle progression and protection from apoptosis resulted from the up-regulation of *c-fos* and *c-jun* which resulted in the formation of the AP-1 complex and the activation of NF-κB (Guizani et al 1996; Xie et al, 1997). However, AP-1 and NF-κB are not essential for the effect that IL-2 has on primary T cells, at least in respect to cell cycle progression and anti-apoptosis (Iacobelli et al, 1999). The differences may be explained by subversion of signalling pathways in *in vitro* transformed cells. Instead, the Jak/STAT pathway may play a pivotal role in mediating the IL-2/IL-2R interactions in primary T cells. Janus tyrosine kinases (Jak) are activated by phosphorylation mediated by receptor/ligand interaction. Subsequently, they phosphorylate the signal transducer and activator of transcription (STAT) molecules which translocate to the nucleus and initiate transcription of target genes (reviewed by Leonard & O'Shea, 1998; Table 6.1). In particular, Jak1 and Jak3 are constitutively associated with the intra-cellular domains of IL-2Rβ and IL-2Rγ_c chains, respectively (Sugamura et al, 1996). STAT5 and STAT3 interact with the cytokine receptors via their SH2 domains and are subsequently phosphorylated by Jak1 and Jak3, respectively. The phosphorylated STAT5 and STAT3 molecules then form homo- and heterodimers and migrate to the nucleus and activate genes conataining STAT-binding sites, referred to as interferon-γ activated sequences (GAS elements).

Another potential mediator of IL-2-induced proliferation and survival signalling in primary T cells is the cell cycle regulator E2F (Brennan et al, 1997). E2F, so named as the factor for adenovirus E2 gene transcription, heterodimerises with the hypophosphorylated retinoblastoma susceptibility gene product, Rb (Weinberg, 1995). As the Rb protein is hyperphosphorylated, mediated by a number of kinases and cyclin

complexes, E2F is released and activated. The now activated E2F heterodimerises with members of the DP family of proteins thus facilitating the transcription of target genes involved in cell cycle progression. IL-2 has been shown to activate phosphatidylinositol 3-kinase (PI-3K) that in turn activates AKT/PKB (protein kinase B), which is one of the key kinases involved in E2F activation (Karnitz & Abraham, 1996; Naheed et al, 1997). Essential to the activation process of T cells is the entry into the IL-2 autocrine pathway and this is facilitated by the up-regulation of the α subunit of the IL-2R. Data presented in previous chapters indicate that HMCL inhibit the IL-2 responsiveness of peripheral T cells. The effect that HMCLs exert on activating T cells, mediated by TGF-β1, may result from interference with the assembly of the high affinity IL-2 receptor, thus blocking the entry into the IL-2 autocrine pathway. The effect that tumour-derived TGF-β1 has on the regulatory control of IL-2Rα expression and associated signalling pathways remains to be established.

Experimental Aims

The aim of the work presented in this chapter is to examine the regulation of IL-2Rα in relation to the inhibitory effect of HMCL-derived TGF-β1. The effect that TGF-β1 has on the signal transduction of the IL-2R will also be investigated. The experiments presented here aim to explain, at the molecular level, the mechanisms which are involved in the mediation of T cell suppression induced by myeloma-derived TGF-β1.

Type I Cytokines	Jaks	STATs
Cytokines whose receptors share γ_c		
IL-2, IL-7, IL-9, IL-15	Jak1, Jak3	STAT5a, STAT5b, STAT3
IL-4	Jak1, Jak3	STAT6
IL-13	Jak1, Jak2, Tyk2	STAT6
Cytokines whose receptors share β_c		
IL-3, IL-5, GM-CSF	Jak2	STAT5a, STAT5b
Cytokines whose receptors share gp130		
IL-6, IL-11, OSM, CNTF, LIF, CT-1	Jak1, Jak2, Tyk2	STAT3
IL-12	Jak2, Tyk2	STAT4
Leptin		STAT3
Cytokines with homodimeric receptors		
Growth hormone		STAT5a, STAT5b, STAT3
Prolactin	Jak2	STAT5a, STAT5b
Eryhtropoietin	Jak2	STAT5a, STAT5b
Thrombopoietin	Jak2	STAT5a, STAT5b
Type II Cytokines		
Interferons		
IFNα, IFNβ	Jak1, Tyk2	STAT1, STAT2
IFNγ	Jak1, Jak2	STAT1
IL-10	Jak1, Tyk2	STAT3

TABLE 6.1. The activation of Jaks and STATs by different cytokine families (Leonard & O'Shea, 1998).

6.2 MATERIALS AND METHODS

6.2.1 REAGENTS & MATERIALS.

Monoclonal Antibodies

All cells were cultured in CM supplemented with recombinant human cytokines as indicated in the text. Anti-human CD25 and IL-2 antibodies conjugated with PE were obtained from Pharmingen (UK). Recombinant human IL-2 and LAP were obtained from Sigma-Aldrich (USA) and rhTGF-β1 was obtained from R&D systems. Anti-STAT3, anti-phosphorylated STAT3 and STAT5 antibodies were obtained from Santa Cruz Biotech and anti-phosphorylated STAT5 was obtained from New England Biolabs. The anti-*bcl-2* monoclonal antibody for intra-cellular FACS analysis was purchased from Dako (UK).

Oligonucleotide Primers

As indicated in chapter 2, the IL-2Rα sense and anti-sense oligonucleotide primers were designed to give a larger PCR product than the primer pairs reported in the literature. The verification of PCR products is discussed in chapter 2. Similarly, I designed and verified the sense and anti-sense primers for the IL-2-responsive protoncogene *pim-1* as described in chapter 2. All other sense and anti-sense primer sequences have been described elsewhere except the protoncogene, *c-abl* (A3):

sense 5' TTCAGCGGCCAGTAGCATCTGACTT

anti-sense 5' GGTACCAGGAGTGTTTCTCCAGACTG

which yielded a PCR product of 273 bps.

Clinical Samples

Serum from bone marrow aspirate samples was prepared as follows: following informed consent, 2 mls of bone marrow blood was aspirated at the end of the diagnostic procedure from the posterior superior iliac crest under local anaesthetic. The sample was immediately decanted into preservative-free heparin and transported to the laboratory. The serum and cellular elements were separated by centrifugation at 1,500G for 5 minutes and the serum was stored in a 1.8 ml Nunc vial at -70^0C until required. The cellular component was re-suspended in RPMI 1640 supplemented with 10% autologous serum for 24 hours, then non-viable cells were removed by density gradient centrifugation. Recovered cells were then washed in PBS and re-suspended in

10%DMSO/FCS and stored in liquid N_2 until required. Serum from one of these patients, a 56-year old female with IgG myeloma, was taken at diagnosis (BM6) and used in the experiments described in this chapter.

6.2.2 SEMI-QUANTITATIVE PCR FOR CYTOKINE RECEPTOR mRNA.

Duplex PCR is a modification of the standard sequential amplification of DNA species where two distinct loci are simultaneously amplified from the one DNA species in a single reaction chamber, the subsequent products being distinguishable by differing sizes on gel electrophoresis (De Santis & Azzi, 1996). The PCR reaction is composed of three distinct phases in relation to the accumulation of oligonucleotide products: an initial exponential phase, a linear phase and a plateau phase. During the linear phase, as a result of the reaction kinetics and reduced competition of the primer pairs, semi-quantitative comparisons of target sequences can be made using a duplex PCR assay (Sambrook et al, 1989). For the study of IL-2Rα up-regulation a "partner gene" which is constitutively expressed in T lymphocytes was chosen. Three genes were selected: the housekeeping gene β-Actin, the oncogene *c-abl* and the IL-2Rγ_c. The linear phase cycle range for all four primer pairs had to be established so that a suitable "partner gene" could be selected. cDNA synthesized from total RNA isolated from mitogen-activated lymphocytes was used in these assays. For each primer pair, 10 simultaneous samples were set up as follows: 5μl 10X PCR buffer (Hybaid,UK), 2μl 50mM $MgCl_2$ (Hybaid,UK), 2μl cDNA, 1μl 25 μM Sense primer, 1μl 25 μM Anti-sense primer, 1μl 10mM dNTP (Life technologies Ltd), 0.4μl (2U) AGS Gold Taq polymerase (Hybaid,UK) and 37.6μl μl dH_2O. Limited cycle PCR reactions were conducted as follows: 95^{0}C for 5 minutes (double strand dissociation), *n* cycles (where *n* represents 17 and with each successive sample *n* increases by 1) of 95^{0}C for 1 minute (denaturation), 60^{0}C for 1 minute (primer annealing), 72^{0}C for 1 minute (extension) with a final incubation at 72^{0}C for 5 minute to ensure complete extension of the PCR products. The 10 serial PCR samples were then visualized by electrophoresis on a 2% agarose gel and the volume of the PCR product band was calculated using UViphoto software.

As a result of these experiments, a partner gene was selected and the sensitivity of this reaction was determined by serial dilutions of cDNA derived from mitogen activated T cells (expressing both genes in the duplex PCR) in cDNA derived from total RNA

isolated from the human leukaemia cell line, K562, which expresses the IL-2Rγ_c but not the IL-2Rα. The duplex PCR reactions were set up as follows: 5µl 10X PCR buffer, 2µl 50mM $MgCl_2$, 2µl cDNA, 1µl 25 µM IL-2Rα sense primer, 1µl 25 µM IL-2Rα anti-sense primer, 1µl 25 µM IL-2Rγ_c sense primer, 1µl 25 µM IL-2Rγ_c anti-sense primer 2µl 10mM dNTP, 0.4µl (2U) AGS Gold Taq polymerase and 34.6µ1 µl dH_2O. Limited cycle PCR reactions were run as follows: 95^0C for 5 minutes, 23 cycles of 95^0C for 1 minute, 60^0C for 1 minute, 72^0C for 1 minute with a final incubation at 72^0C for 5 minute to ensure complete extension of the PCR products. Products were visualized by electrophoresis on a 2% agarose gel and the volume of each PCR product band was calculated using UViphoto software. A ratio of the two PCR product bands was then calculated to give the relative expression of the IL-2Rα in response to re-stimulation.

6.2.3 GENERATION AND PURIFICATION OF TRANSFECTANT CELL LINE.

The Chinese hamster ovary fibroblast cell line (CHO-K1) was transfected with the human IL-2Rα subunit by Dr Trevor Paterson, Academic Transfusion Medicine Unit, University of Edinburgh. In brief, the cDNA for human IL-2Rα (ATCC clone 39890) was cloned as a *Pst*I(partial)-*Xba*I fragment into the polylinker of pCDNA3 expression plasmid (Invitrogen) under the control of the CMV IE promoter/enhancer. Cell surface expression of IL-2Rα was detectable by FACScan (Becton Dickinson) analysis of transiently transfected (Superfect, Qiagen) CHO-K1 cells upon staining with anti-CD25 (IL2Rα) FITC conjugated monoclonal antibody (MCA1319F, Serotec). Stable CHO-K1 cell lines were selected with 750µg/ml G418 after transfection with circular or *Rca*I-linearized plasmid DNA. Both approaches produced stable populations in which approximately 25% of cells stained positively for surface IL2Rα. Single cell derived clones were isolated by limiting dilution of the G418 resistant populations in multiwell plates. The CHO-K1 cells transfected with IL-2Rα (CHO-K1/25) and wild type/mock transfectants (CHO-K1/wt) were transferred to me at this stage for further selection.

Stable CD25 transfectant cells were purified by surface expression of the α subunit using the using the MACS magnetic cell sorting system by Miltenyi , as described in chapter 4. The resulting purified cells (CHO-K1/25) demonstrated stable high levels of CD25 surface protein (Figure 6.16). Cells were maintained in CM that was replenished once per week and the cells were sub-cultured once per week. For use in experiments and for sub-culturing, the plastic adherent cells were treated with x1 trypsin/EDTA at

37^0C for 10 minutes to detach them. The cells were then diluted in x10 volume of warm CM to inactivate the trypsin. The cells were then pelleted by centrifugation. For sub-culturing, the cells were then split and re-suspended in CM, with plastic adherence being achieved within 15 minutes at 37^0C. For use in the experimental studies, cells were pelleted by centrifugation, fixed in 2% formaldehyde at 4^0C for 10 minutes then washed three times in CM before using in the experiments at the cell dilutions indicated.

6.2.4. WESTERN BLOTTING FOR SIGNALLING MOLECULES.

For the study of STAT signal transduction molecules, T cells activated by mitogen (Con A 2.5μg/ml for 5 days), washed x3 in CM and serum starved for 24 hours by culturing the cells in RPMI1640/0.5%FCS. Viable cells were recovered by density gradient separation (Ficoll-Hypaque) and washed x3 in CM. The cells were re-suspended in CM at a concentration of $2x10^6$/ml and re-stimulated with 20 U/ml rhIL-2 in the presence and absence of HMCL at a concentration of 1U266:5 PBL. Cells were incubated for 45 minutes then washed x3 in ice-cold CM. Cell lysates were prepared from the cell pellet after centrifugation by the addition of 75μl of RIPA buffer containing 10μl of protease inhibitors (x50), followed by vigorous pipetting. The samples were incubated on ice for 15 minutes then subjected to 3 rounds of freeze/thawing alternating between dry ice and a 37^0C water bath. The samples were centrifuged at 4^0C at 14,000G and the supernates were decanted into a fresh tube and stored at -70^0C until required.

Samples were recovered from storage and thawed at room temperature. 5 μl of western loading buffer was added to 20μl of protein solution and the samples were denatured by heating at 100^0C for 5 minutes before the samples were pulse spun and stored at 4^0C until required. Denatured samples were then loaded onto a SDS-PAGE gel (10% running acrylamide gel and 5% stacking acrylamide gel) with pre-stained molecular weight markers. Proteins were then separated by electrophoresis in western blot running buffer (~100Mv for 60-120 minutes). The gel was then removed from the stacking system, trimmed using a clean scalpel and the bottom right-hand corner was notched for orientation. The gel was then transferred on to BioTrace PVDF transfer membrane (Pall Gelman Sciences, USA) pre-soaked in 100% methanol and transfer bufferusing a semi-dry Transfer Cell (BioRad Electro-Transfer SD).

After successful gel transfer, the membrane was washed in distilled water and unoccupied protein binding sites were blocked with western blocking solution for 2

hours and then stained with the primary antibody (diluted in 20 mls of 1%BSA/TBST) at 4^0C overnight (18 hours). The membrane was then washed in TTBS for 5 minutes and then stained with a HRP-conjugated secondary antibody (diluted 1:2000 in 20 mls of 1%BSA/TTBS) for 1 hour. HRP-conjugated anti-biotin antibody (diluted 1:1000 in 1%BSA/TTBS) was used to detected biotinylated markers. The membrane was then washed in TTBS for 5 minutes before the membrane was incubated with 10mls of LumiGLO (0.5mls 20X LumiGLO, 0.5ml 20X peroxide and 9.0mls Milli-Q water) and mixed by gentle agitation for 1 minute at room temperature. Excess LumiGLO solution was removed and whilst the membrane was moist, it was rapped in Saran Wrap and exposed to x-ray film.

6.2.5. CELL TRACKING.

PKH26 (PKH26-GL, Sigma-Aldrch, USA) is a red fluorescent cell linker which incorporates aliphatic reporter molecules into cell membrane by selective partitioning (Horan & Slezak, 1989). It has been found to be useful in *in vitro* cell labelling and *in vitro* and *in vivo* cell tracking. It was used in the experiments presented in this chapter for general cell labelling to identify secondary phenotypic changes in specified cells in mixed cellular populations e.g. *bcl-2* expression in IDBL (labelled) cells co-cultured with U266 (unlabelled) cells. The labelling is not a saturation reaction, the extent of surface labelling is a function of both dye and cell concentration. Over-labelling resulted in loss of membrane integrity and cell recovery. Therefore, the optimal cell/dye concentration was determined using PI exclusion and fluorescent intensity of IDBL cells stained by this protocol. It was found that the optimal cell concentration was 1-5x10^6/ml and the optimal dye concentration was 5x10^{-6}M PKH26 (results not shown).
Cells for labelling were harvested by centrifugation (1500G for 5 minutes) and the cell pellets were then washed in RPMI1640 medium (serum-free) three times before being resuspended at 1-5x10^6/ml in 1 ml of Diluent C (supplied with kit). Immediately prior to staining, 4 µl of PKH26 dye stock solution (1x10^{-3} M in ethanol) was added to 1ml of Diluent C and kept at room temperature. The 1 ml of dye was added to 1 ml of cell suspension (final concentration 5x10^{-6}M) and gently mixed at room temperature for 10 minutes. Adding an equal volume of heat-inactivated FCS and mixing gently terminated the reaction. Cells were then recovered by centrifugation and washed in CM three times. Cells were then re-suspended in the appropriate medium for subsequent experimental

studies. An aliquot of cells was immediately fixed in 2% formalin to represent the extent of surface staining prior to cell division under experimental conditions. Cells were stained with appropriate monoclonal antibodies after the experimental period with due care and attention being paid to compensation, as described in chapter 2.

6.3 RESULTS

6.3.1 HMCL PREVENT ACTIVATING T CELLS FROM ENTERING THE IL-2 AUTOCRINE PATHWAY

In the previous chapter, the IL-2 responsiveness of mitogen-activated lymphoblasts from healthy donors is impaired in the presence of HMCL. This was demonstrated using proliferation assays as a measure of cell cycle progression and cell division. A key feature of this activation process in T cells is the entry into the IL-2 autocrine pathway. Using intra-cellular cytokine immunofluorescent staining and flow cytometry, the entry into the IL-2 autocrine pathway of primary T cells in response to exogenous IL-2 was examined. When compared to resting T cells, the percentage of cells which were positive for intra-cellular IL-2 by flow cytometry after 36 hours of culture were significantly increased by the stimulation of exogenous, recombinant human IL-2 20 U/ml (27.20%$\pm$6.74 *cf* 70.6%$\pm$5.38, n=4, p=0.0011; Table 6.2). When the primary T cells from healthy donors (n=4) were re-stimulated with rhIL-2 (20 U/ml) for 36 hours in the presence of the HMCL, U266, the bone marrow serum from a patient with multiple myeloma at diagnosis (BM6) and rhTGF-β-1, a reduction in the percentage of cells entering the IL-2 autocrine pathway was demonstrated (Table 6.3). Co-culturing with U266 cells resulted in a mean 33.7%$\pm$10.5 reduction in the number of cells entering into the IL-2 autocrine pathway, whilst co-culturing with the BM serum from a patient with myeloma resulted in a mean 30.2%$\pm$8.2. Two of the donor T cells (D1 & D2) demonstrated a non-significant increase and two donors (D3 & D4) demonstrated only a slight reduction in the percentage of cells that stained for intra-cellular IL-2, after 36 hours of stimulation with rhIL-2 in the presence of TGF-β. This may reflect the half-life of rhTGF-β in this culture system that un-like HMCL-produced TGF-β, is not constantly replenished in the culture medium. The fact that suppression is still seen with BM serum may reflect the relatively high level of the cytokine found in BM sera from patients with myeloma (5-12 ng/ml; results not shown).

6.3.2 INHIBTION OF IL-2 AUTOCRINE PATHWAY ENTRY BY HMCL IS ASSOCIATED WITH FAILURE TO UP-REGULATE IL-2Rα.

The entry of primary T cells into the IL-2 autocrine pathway is preceded by the up-regulation of the high affinity chain of the IL-2R, IL-2Rα and thus, IL-2Rα controls, at

	D1 (%)	D2 (%)	D3 (%)	D4 (%)	MEAN±SEM (%)	p
RESTING	33.38	37.49	36.48	7.46	27.20±6.74	0.001
IL-2	64.57	67.85	63.48	86.49	70.6±5.38	
IL-2+HMCL	49.39	52.44	22.09	66.18	47.53±9.27	0.042
IL-2+BM6	47.07	50.84	28.1	9.53	33.89±9.52	0.01
IL-2+TGF (5ng/ml)	75.65	78.59	60.2	61.3	68.9±4.8	0.41

TABLE 6.2. The effect of co-culturing primary T cells re-stimulated with IL-2 in the presence of HMCL (U266), BM6 and rhTGF-β-1 on IL-2 autocrine pathway entry. P values represent the change in percentage cells positive for intracellular IL-2 compared with T cells re-stimulated with rhIL-2 20 u/ml.

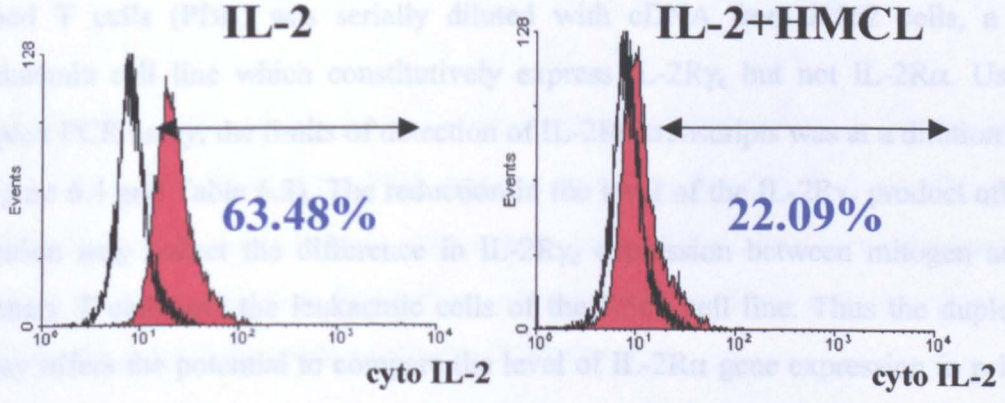


FIGURE 6.1. Representative histograms demonstrating the effect of co-culturing U266 cells on the IL-2 autocrine pathway entry of primary T cells when re-stimulated with rhIL-2. The hollow histogram represents the isotype control MoAb and the filled histogram represents the cytoplasmic IL-2 immunofluorescent staining.

least in part, the IL-2 responsiveness of T cells (Lowenthal et al, 1985). IL-2Rα chain expression is regulated at the transcriptional level (Meyer et al, 1997). Therefore to examine the effect that HMCL-derived TGF-β-1 exerts on the up-regulation of IL-2Rα, a semi-quantitative duplex PCR assay was used. The linear phase of the PCR reaction for the IL-2Rα primers and each potential constitutively expressed partner gene primer pairs was determined using simultaneous, parallel samples set up as described in the methodology section. The volume of each PCR product band was assessed using UViphoto software and was plotted on a linear graph against the cycle number. For the IL-2Rα primer pairs, the linear phase of the PCR reaction was found to be between 20-26 cycles (Figure 6.2A). Three constitutively expressed genes in T cells were tested for the linear phase of the PCR reaction for each of their respective primer pairs: β-Actin, *c-abl* and IL-2Rγ_c. The linear phase for the β-Actin primer pairs was found to be <19 cycles (Figure 6.2B), the linear phase of the *c-abl* primer pars was found to be >25 cycles (Figure 6.2C) and the linear phase of the IL-2Rγ_c primer pairs was found to be between 21-25 cycles (Figure 6.2D and representative PCR product gel electrophoreses are illustrated in Figure 6.3). Thus, for the duplex semi-quantitative PCR assay, expression of the IL-2Rα and IL-2Rγ_c genes was measured and compared at 23 cycles.

To determine the sensitivity of this assay, cDNA from mitogen activated peripheral blood T cells (PBL) was serially diluted with cDNA from K562 cells, a human leukaemia cell line which constitutively express IL-2Rγ_c but not IL-2Rα. Using the duplex PCR assay, the limits of detection of IL-2Rα transcripts was at a dilution of 1:32 (Figure 6.4 and Table 6.3). The reduction in the level of the IL-2Rγ_c product after 1:16 dilution may reflect the difference in IL-2Rγ_c expression between mitogen activated primary T cells and the leukaemic cells of the K562 cell line. Thus the duplex PCR assay offers the potential to compare the level of IL-2Rα gene expression in primary T cells under differing experimental conditions with a modest level of sensitivity, avoiding the use of Northern blotting and the applications of radio-nucleotides.

Using the duplex assay, the effect of re-stimulating mitogen-activated T cells with rhIL-2 in the presence and absence of rhTGF-β1, HMCL and bone marrow serum from a patient with myeloma was examined in relation to IL-2Rα gene expression. PBL from normal donors (n=3: 10^6 cells/ml) were re-stimulated with rhIL-2 (20 U/ml) in 24-well plates, with and without rhTGF-β1 (5 ng/ml) for 24 hours. Total RNA was isolated and

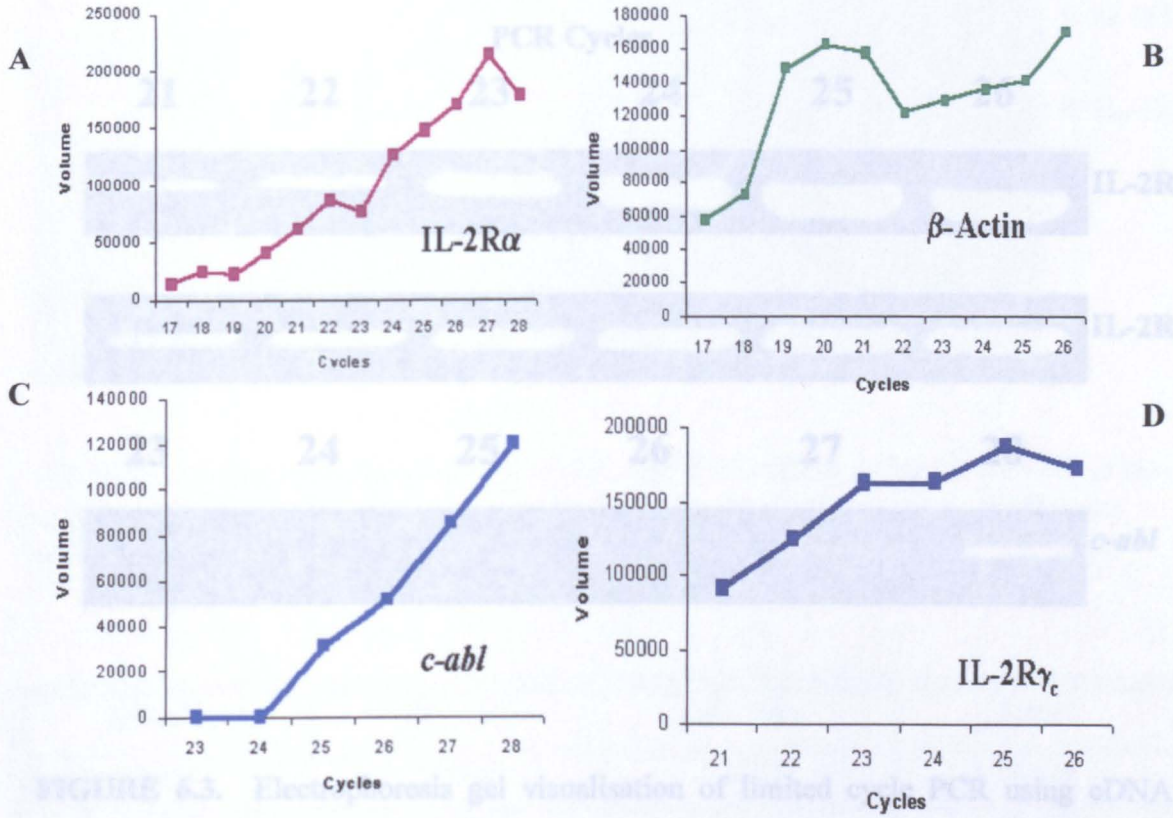


FIGURE 6.2. Determining the linear phase of PCR amplification using limited cycle PCR. The linear phase of the PCR reaction using IL-2Rα (A), β-Actin (B), *c-abl* (C) and IL-2Rγ_c sense and anti-sense primer pairs. Each PCR product was visualised by 2% agarose gel electrophoresis. The volume of each PCR band was determined in arbitrary units by the gel documentation software (UViphoto).

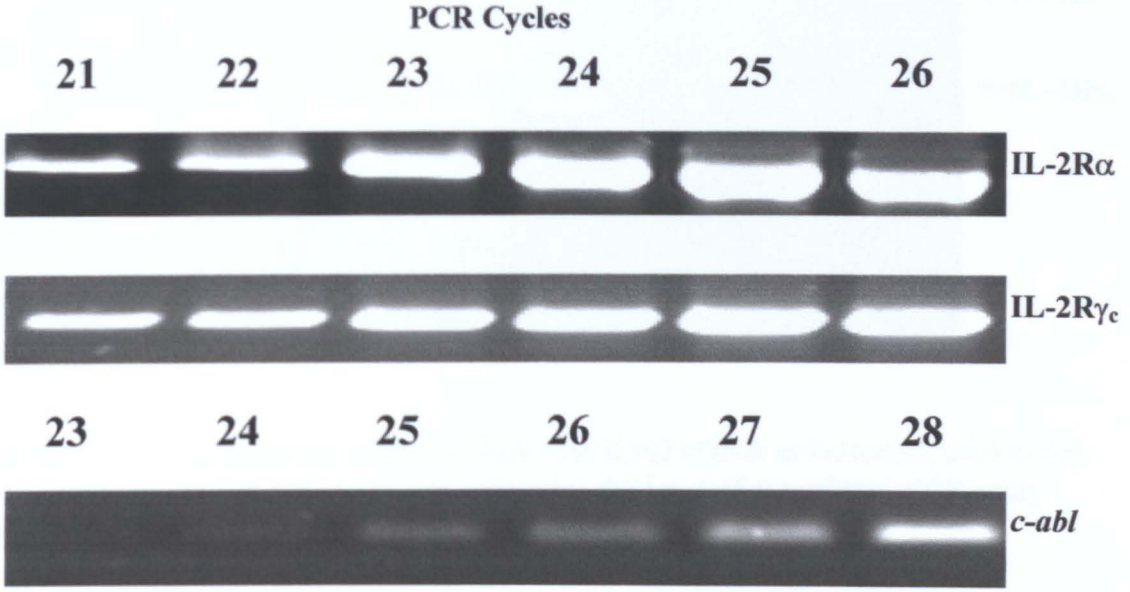


FIGURE 6.3. Electrophoresis gel visualisation of limited cycle PCR using cDNA synthesised from mitogen-activated T cells from a healthy donor. Sequential cycle PCR products amplified with IL-2Rα, IL-2Rγc and *c-abl* sense and anti-sense primer pairs respectively, as described.

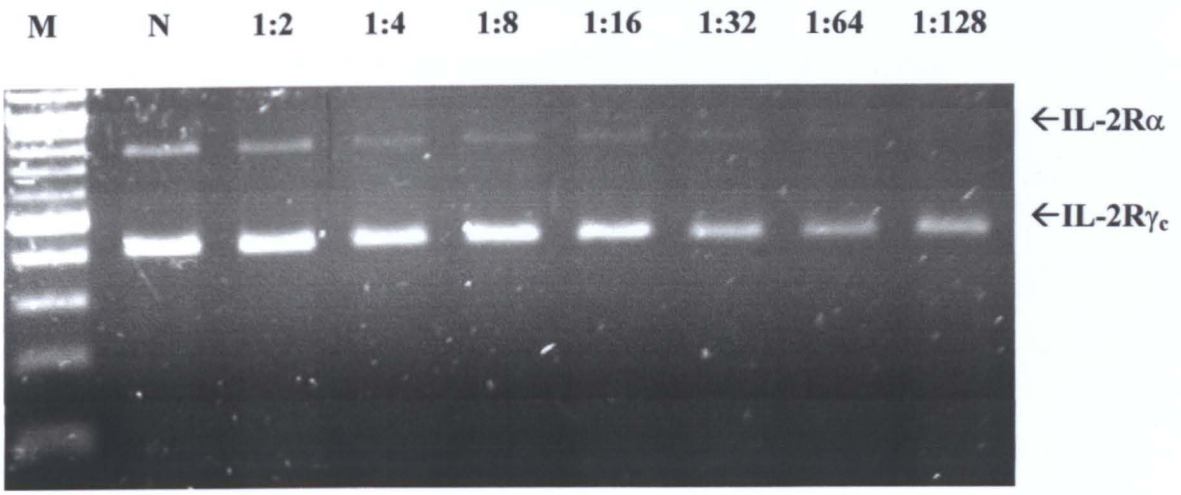


FIGURE 6.4. Serial dilutions of PBL cDNA with K562 cDNA as indicated, used in the duplex PCR (IL-2Rα/IL-2Rγc) assay to determine the level of sensitivity. PCR assay was performed at 23 cycles of amplification.

Dilution	IL-2Rα	IL-2Rγc	Ratio
Neat	57848	135721	0.426
1:2	42164	129061	0.327
1:4	43788	132198	0.479
1:8	7847	138451	0.316
1:16	975	118412	0.066
1:32	0	79158	0.012
1:64	0	59395	0
1:128	0	59375	0

TABLE 6.3. Serial dilutions of PBL cDNA with K562 cell cDNA used in the duplex PCR assay to semi-quantitate the level of IL-2Rα transcripts.

cDNA synthesised. The cDNA was then used in the duplex PCR assay to compare the level of expression of IL-2Rα and IL-2Rγc genes. The presence of rhTGF-β1 reduced the up-regulated expression of the IL-2Rα gene in response to re-stimulation with rhIL-2 whilst no appreciable effect was seen on the expression of the IL-2Rγc (Table 6.4 and Figure 6.5). When the ratio of gene expression was calculated, a downward trend in the IL-2Rα/IL-2Rγc ratio was seen when re-stimulated with rhIL-2 in the presence of rhTGF-β1. A representative example of the duplex PCR assay is illustrated in Figure 6.6. When β-Actin was amplified under similar PCR conditions as that used in the duplex reaction, similar levels of product were detected, as a measure of amplification and loading control (Figure 6.5 and 6.6).

The effect of re-stimulating mitogen-activated T cells with rhIL-2 in the presence and absence of the U266 cells and bone marrow serum from a patient with myeloma (BM6) was examined in relation to IL-2Rα gene expression using the duplex PCR assay. PBL from normal donors (n=3: 10^6 cells/ml) were re-stimulated with rhIL-2 (20 U/ml) in 24-well plates, with and without mitomycin-C-treated U266 cells or BM6 at a 1:10 dilution in CM. Total RNA was isolated, cDNA synthesized and then used in the duplex PCR assay. The presence of either U266 cells or the diluted bone marrow serum, BM6, resulted in a failure to up-regulate the IL-2Rα with a resultant reduction in the IL-2Rα/IL-2Rγc ratio (Table 6.5 & Figure 6.7). A representative example of the duplex PCR is illustrated in Figure 6.8. For confirmation, the cDNA from each of the respectively treated PBL samples were tested in a standard 30 cycle PCR reaction for the expression of IL-2Rα, IL-2Rγc, β-Actin and the IL-2 responsive protoncogene, *pim-1* gene. The results indicate that rhTGF-β1, HMCL and bone marrow serum from a patient with myeloma inhibit the up-regulation of both IL-2Rα and *pim-1* in PBL when re-stimulated with rhIL-2 (Figure 6.9 & Figure 6.10). The positive control used was CHO-K1 cell line transfected with human cDNA encoding the IL-2Rα and interestingly whilst the β-Actin and IL-2Rγc primers amplify products of similar size to the human cells, the pim-1 primers amplifying two products both being smaller than the human PCR product.

Corroboration of the PCR results by assessing IL-2Rα protein synthesis was hampered by the fact that surface expression analyses were inherently difficult. As described in chapter 4, 62.3%±2.3 of $CD3^+$ cells already expressed IL-2Rα protein on their surface,

	D1	D2	MEAN±SEM
IL-2Rα			
Resting	0	0	0
IL-2	60977	70556	65767±4803
IL-2+TGFβ	27939	10961	19450±8514
Control	57848	70958	64403±6574
IL-2Rγc			
Resting	37662	70558	54110±16496
IL-2	157186	86067	121627±35665
IL-2+TGFβ	87719	42332	65026±22761
Control	135721	105408	120565±15201
IL-2Rα/IL-2Rγc ratio			
Resting	0	0	0
IL-2	0.388	0.820	0.717±0.17
IL-2+TGFβ	0.319	0.469	0.313±0.18
Control	0.426	0.673	0.579±0.08

TABLE 6.4. The comparative expression of IL-2Rα and IL-2Rγc genes in primary T cells re-stimulated with rhIL-2 using a semi-quantitative duplex PCR assay.

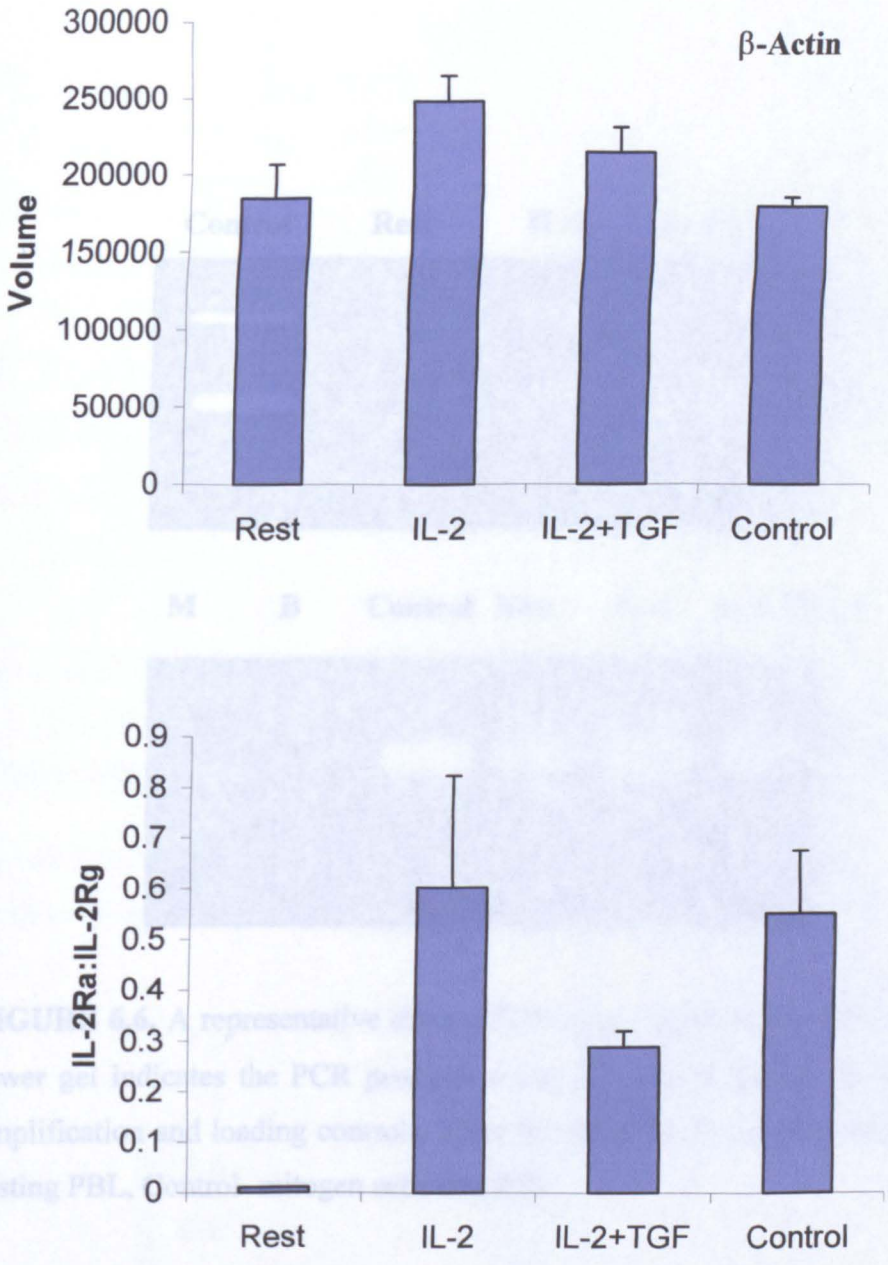


FIGURE 6.5. The effect of re-stimulating primary T cells (n=2) with rhIL-2 20U/ml in the presence of TGF-β1(5ng/ml) on the relative expression of the α chain of the IL-2R. The volume of PCR products amplified using the β-Actin sense and anti-sense primer pairs is illustrated as a amplification and loading control.

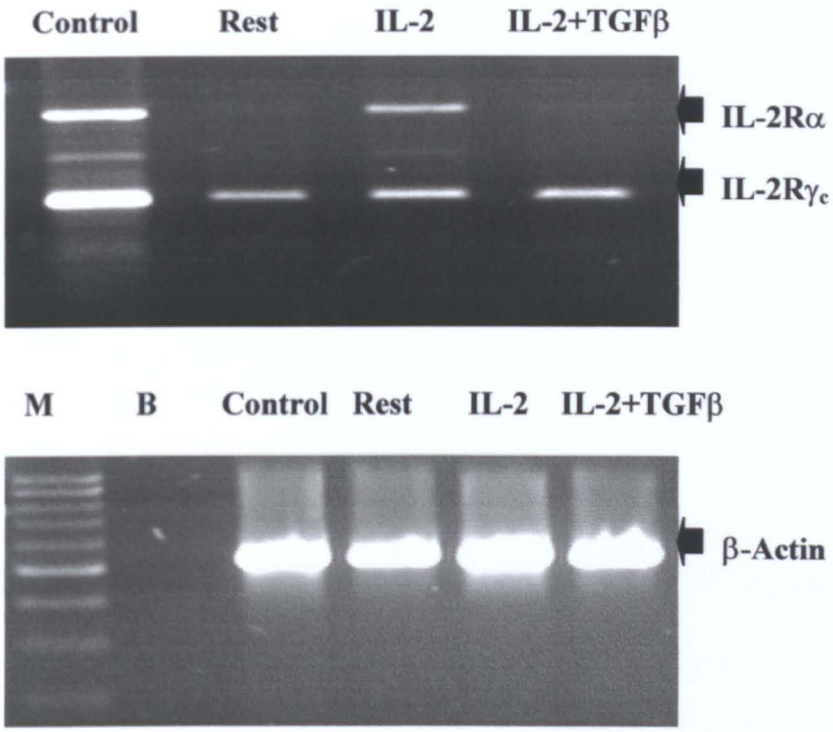


FIGURE 6.6. A representative duplex PCR assay visualised on a 2% agarose gel. The lower gel indicates the PCR products using β-Actin sense and anti-sense primers, as amplification and loading controls. **Key:** M- markers, B- negative (H_2O) control, Rest- resting PBL, Control- mitogen activated PBL.

	D1	D2	D3	MEAN±SEM	p
IL-2Rα					
Resting	0	0	0	0	
IL-2	64201	68527	61971	64900±1927	
IL-2+HMCL	1854	407	0	754±563	<0.001
IL-2+BM6	225	279	205	236±22	<0.001
IL-2Rγc					
Resting	63117	50997	45962	50025±2124	
IL-2	64936	65734	68214	66294±988	
IL-2+HMCL	59874	61001	58606	59827±692	NS
IL-2+BM6	55105	56442	50967	54171±1650	NS
IL-2Rα/IL-2Rγc Ratio					
Resting	0	0	0	0	
IL-2	0.989	1.043	0.908	0.98±0.039	
IL-2+HMCL	0.013	0.016	0.012	0.011±0.003	<0.001
IL-2+BM6	0.004	0.005	0.004	0.004±0.001	<0.001

TABLE 6.5. The comparative expression of IL-2Rα and IL-2Rγc genes in primary T cells re-stimulated with rhIL-2 using a semi-quantitative duplex PCR assay.

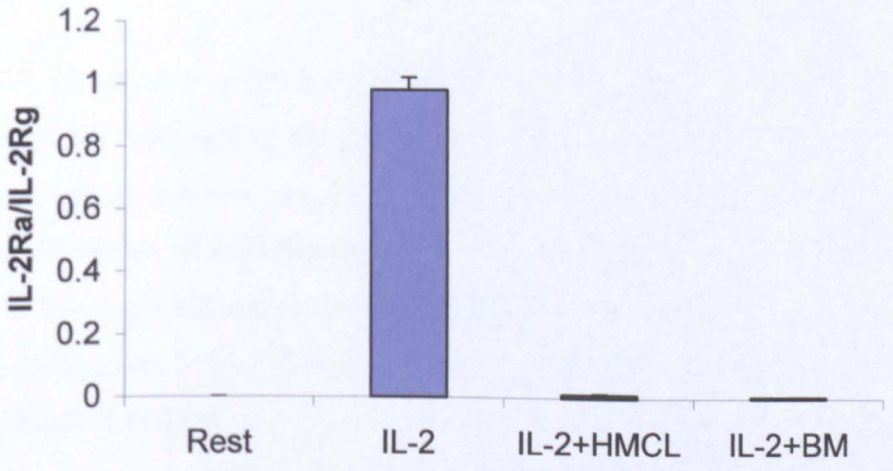


FIGURE 6.7. The effect of re-stimulating primary T cells (n=3) with rhIL-2 in the presence of U266 cells (HMCL) and bone marrow serum from a patient with myeloma (BM) on the relative expression of the α chain of the IL-2R.

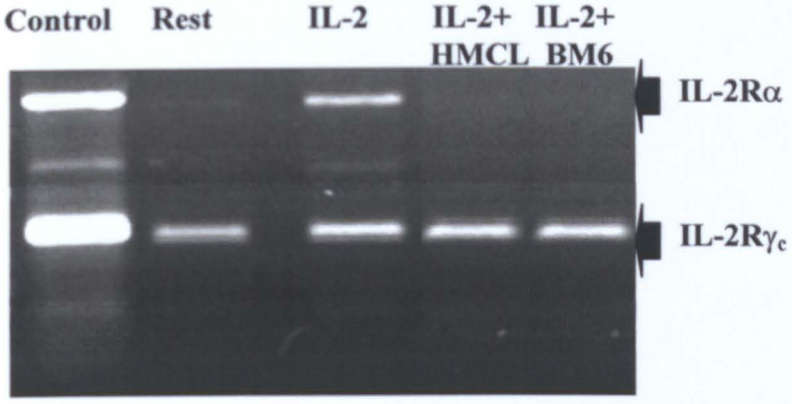


FIGURE 6.8. A representative duplex PCR assay visualised on a 2% agarose gel. **Key:** Control- mitogen activated PBL, Rest- resting PBL, HMCL- U266 cells, BM6- bone marrow serum.

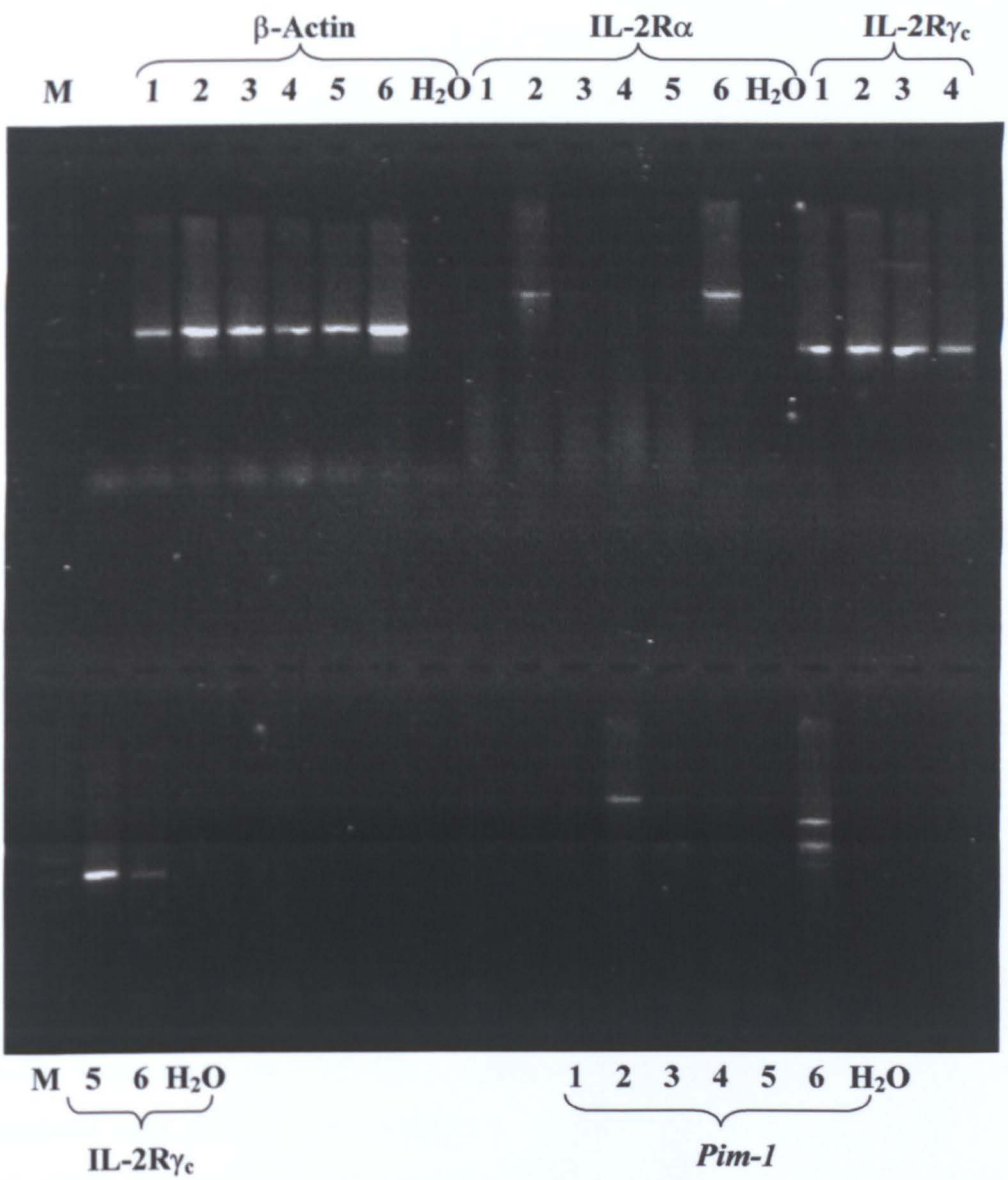


FIGURE 6.9. Expression of the IL-2 responsive genes, IL-2Rα and *pim-1*, in T cells when re-stimulated with rhIL-2 for 24 hours in the presence or absence of TGF-β1, U266 cells or bone marrow serum from a patient with myeloma. PCR products generated by 30 cycles of amplification and visualised by 2% agarose electrophoresis. **Key:** Lane 1- Resting T cells; Lane 2- IL-2 stimulated; Lane-3 IL-2+rhTGF-β; Lane 4 – IL-2+U266 cells; Lane 5- IL-2+BM serum; Lane 6- Positive control (CHO-K1/25 cell line); H_20- Negative control.

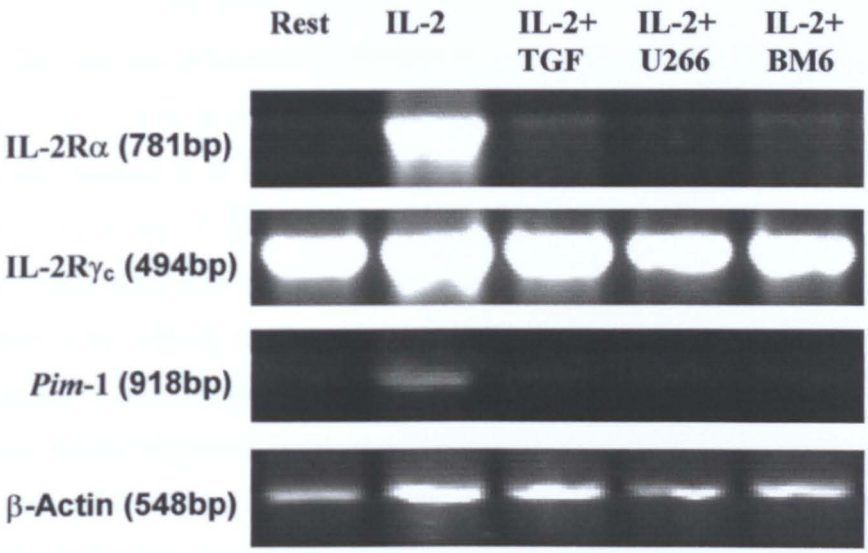


FIGURE 6.10. Summary of the expression of the IL-2 responsive genes in primary T cells re-stimulated with rhIL-2 in the presence or absence of TGF-β1, U266 cells or bone marrow serum from a patient with myeloma. PCR products generated by 30 cycles of amplification and visualised by 2% agarose electrophoresis.

thus invalidating up-regulated surface expression analysis as a methodology for *de novo* protein synthesis.

6.3.3 INHIBITION OF IL-2Rα UP-REGULATION IN T CELLS INDUCED BY HMCL IS ASSOCIATED WITH FAILURE OF STAT3 AND STAT5 PHOSPHORYLATION.

Ligation of IL-2 with its receptor is associated with several different signalling pathways, of which the Jak/STAT pathways have pivotal importance. Jak3/STAT3 activation through phosphorylation appears to be associated with the γ_c subunit whilst Jak1/STAT5 activation is associated with the intra-cellular domain of the β subunit (reviewed by Leonard & O'Shea, 1998). The effect on phosphorylation of STAT3 and STAT5 when primary T cells were re-stimulated with rhIL-2 in the presence and absence of U266 cells was studied using western blotting. When primary T cells were re-stimulated with rhIL-2, the presence of U266 cells resulted in failure of STAT3 phosphorylation when compared to those T cells re-stimulated in the absence of U266 cells (Figure 6.11). The total level of STAT3 was unchanged in re-stimulated T cells either co-cultured in the presence of U266 cells or in CM only. The addition of LAP, the TGF–β1 antagonist, completely restored the STAT3 phosphorylation in response to rhIL-2. Similarly, when re-stimulated T cells were co-cultured with U266 cells, there was failure of STAT5 phosphorylation despite similar levels of total STAT5 when compared to T cells re-stimulated with rhIL-2 in the absence of U266 cells (Figure 6.12). Once again, the addition of LAP completely restored the STAT5 phosphorylation in response to rhIL-2. These results corroborate the RT-PCR data as STAT3 is central to the transcriptional regulation of the IL-2-induced protoncogene, *pim-1*, and STAT5 has a pivotal role in the activation of the PRRIII of the IL-2Rα gene (John et al, 1996; Meyer et al, 1997, Shirogane et al, 1999). As has already been demonstrated, the co-culturing of T cells with HMCLs results in failure to up-regulate both *pim-1* and IL-2Rα genes when responding to re-stimulation with rhIL-2.

6.3.4 INTER-CELLULAR IL-2Rα ACTING *in trans* INDUCES HMCL AND TGF-β SUSCEPTIBILITY IN IDBL CELLS.

Data presented in chapter 5 demonstrate that IDBL cells are resistant to the inhibitory effects of both rhTGF-β1 and HMCLs, in terms of cell cycle progression and

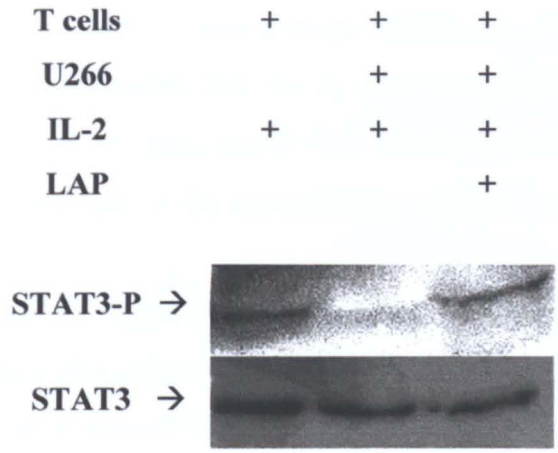


FIGURE 6.11. Phosphorylation of STAT3 in primary human T cells in response to re-stimulation with rh-IL-2 in the presence and absence of U266 cells.

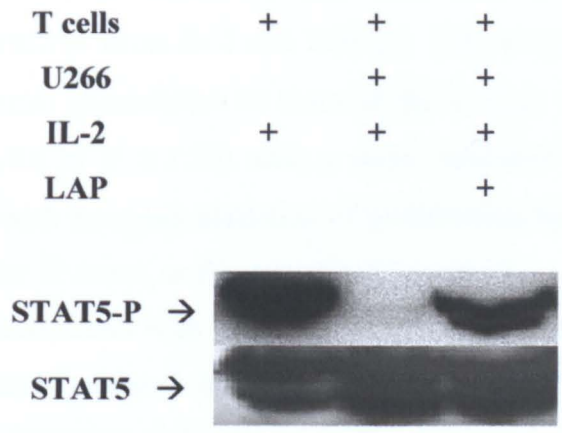


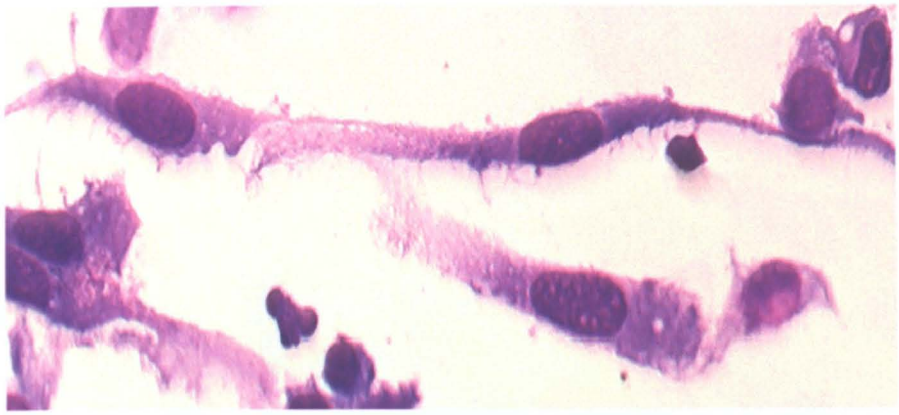
FIGURE 6.12. Phosphorylation of STAT5 in primary human T cells in response to re-stimulation with rh-IL-2 in the presence and absence of U266 cells.

proliferation. As indicated, these cells have progressed beyond the IL-2 autocrine stage and depend on exogenous IL-2 for proliferation and survival. These cells did not express the high affinity IL-2R but did express the IL-2Rβ and IL-2Rγ_c subunits. It has been shown that IL-2Rα on one cell can present IL-2 to the β and γ_c subunits on another cell and this in *trans* action results in inter-cellular cytokine augmentation which may influence the behaviour of cells which lack the IL-2Rα subunit (Eicher & Waldmann, 1998). Therefore, the effect of providing the IL-2Rα subunit to IDBL cells was studied, in particular the influence this had on the susceptibility to HMCL and TGF-β1-induced suppression.

CHO-K1/25 are illustrated in Figure 6.13 demonstrating their typical morphological (Figure 6.13A) and surface phenotypic characteristics (Figure 6.13B). When this cell line, fixed in formaldehyde to prevent ligand binding and internalisation, was added to IDBL cells (1CHO-K1/25:20 IDBL cells) cultured in rhIL-2, slight stimulation of proliferation was seen after 72 hours (Figure 6.14). When increasing concentrations of rhTGF-β1 was added to IDBL cells alone, no suppressive effect was seen, as has been demonstrated earlier. However, when CHO-K1/25 cells were added to the culture, this rendered the IDBL cells susceptible to the suppressive effect of TGF-β1 (Figure 6.14). Only a slight suppression of proliferation was seen when IDBL cells were co-cultured with fixed control CHO-K1/wt cells, IL-2 and TGF-β1. This susceptibility to TGF-β1 induced by the inter-cellular presentation *in trans* of the α-chain was seen at varying concentrations of CHO-K1/25 (1:5-1:20) with a mean inhibition of proliferation of 62.46%±2.03 compared with the mean inhibition of proliferation by TGF-β1 alone of –1.0%±9.45 ($p=0.002$) after 72 hours, as illustrated in Figure 6.15.

When IDBL cells were stimulated with rhIL-2 in the presence of mitomycin-C-treated U266 cells for 24 hours, minimal inhibition of proliferation was demonstrated (2.45%±6.47, n=4), as determined by ^{3}[H]dThd uptake. However, when formalin-fixed CHO-K1/25 cells were added to IDBL cells stimulated with rhIL-2 for 24 hours, marked inhibition of proliferation was demonstrated, with a mean inhibition of ^{3}[H]thymidine uptake of 44.65%±10.5 ($p=0.005$; Figure 6.16). The effect on apoptosis of IDBL cells by co-culturing with formalin-fixed CHO-K1/25 cells in the presence of rhIL-2 and U266 cells was assessed using the annexin V affinity assay. The apoptosis rate of IDBL cells was measured by gating on PKH26$^+$ cells and determining the surface staining of Annexin V-FITC.

A



B

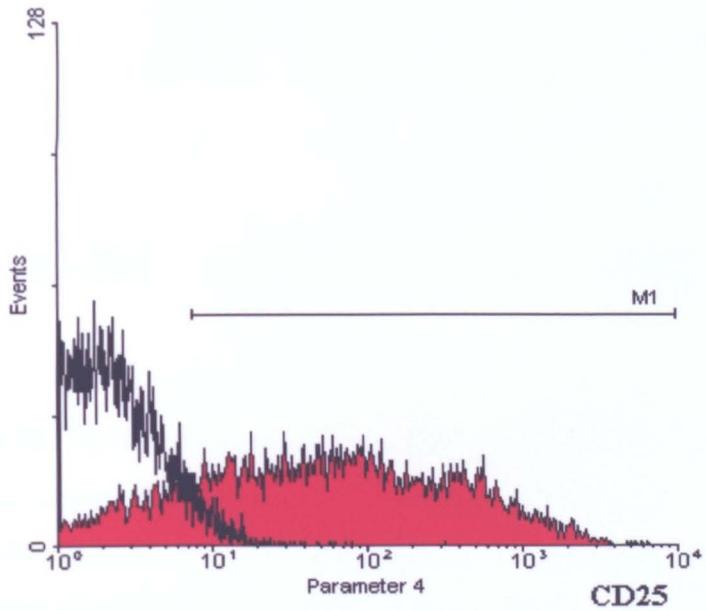


FIGURE 6.13. CD25 (IL-2Rα) surface expression on CHO-K1/25 cells (Geimsa, x1000, A) as determined by flow cytometry (B).

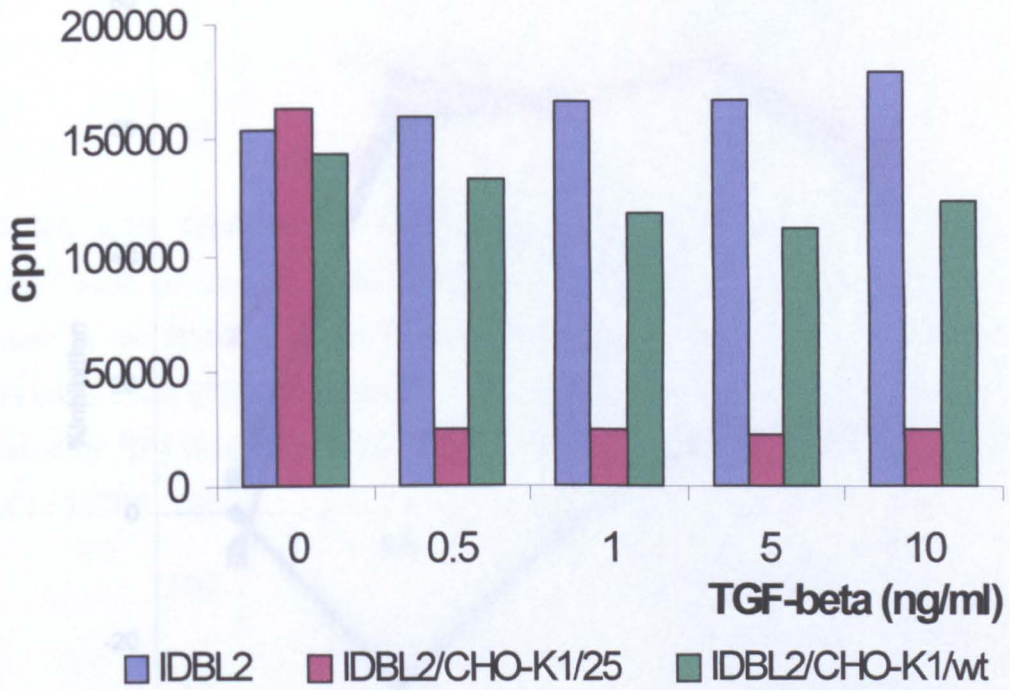


FIGURE 6.14. The effect of co-culturing fixed CHO-K1/25 or CHO-K1/wt cells on the IL-2 responsiveness of IDBL cells (10^5/ml) in increasing concentrations of rhTGF-β1 (1CHO-K1/25: 20IDBL cells). Proliferation was determined by [^{3}H] thymidine uptake after 72 hours of culture. Key: IDBL2- IDBL cells with IL-2 20 U/ml, IDBL2/CHO-K1/25- IDBL cells with IL-2 & fixed CD25-transfected CHO cells, IDBL2/CHO-K1/wt- IDBL cells with IL-2 & fixed control-transfected CHO cells.

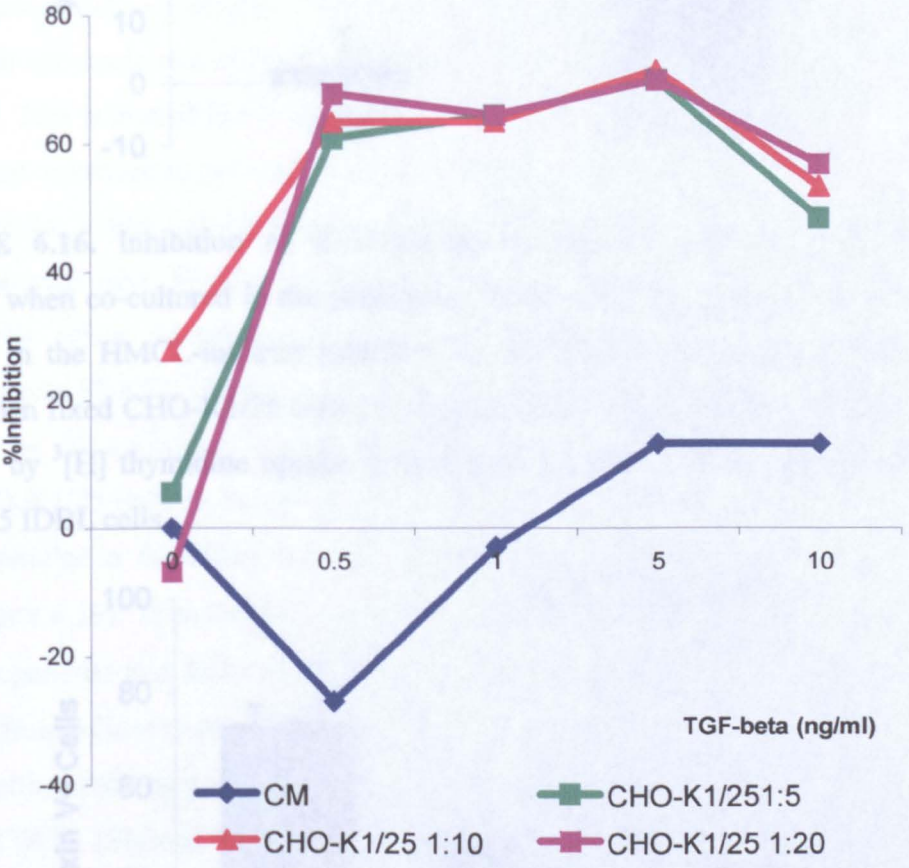


FIGURE 6.15. Inhibition of the proliferative response of IDBL cells (10^5/ml) to rhIL-2 20 U/ml with increasing concentrations of rhTGF-β1in the presence and absence of the IL-2Rα-transfected cell line, CHO-K1/25 (fixed). Proliferation was determined by [^{3}H]thymidine uptake after 72 hours of culture. Ratios (e.g.1:20) represent the number of CHO-K1/25 cells to IDBL cells.

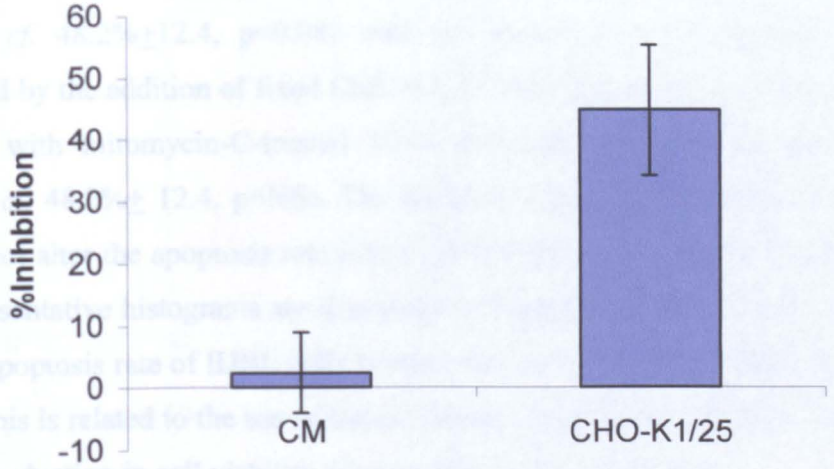


FIGURE 6.16. Inhibition of IL-2-induced proliferative responses of IDBL cells (10^5/ml) when co-cultured in the presence of U266 cells. A significant (n=4, p=0.005) increase in the HMCL-induced inhibition of proliferation was demonstrated after 48 hours when fixed CHO-K1/25 cells (1:10) were added to the cultures. Proliferation was assessed by ^{3}[H] thymidine uptake. Mitomycin-C treated HMCL used at a ration of 1 HMCL: 5 IDBL cells.

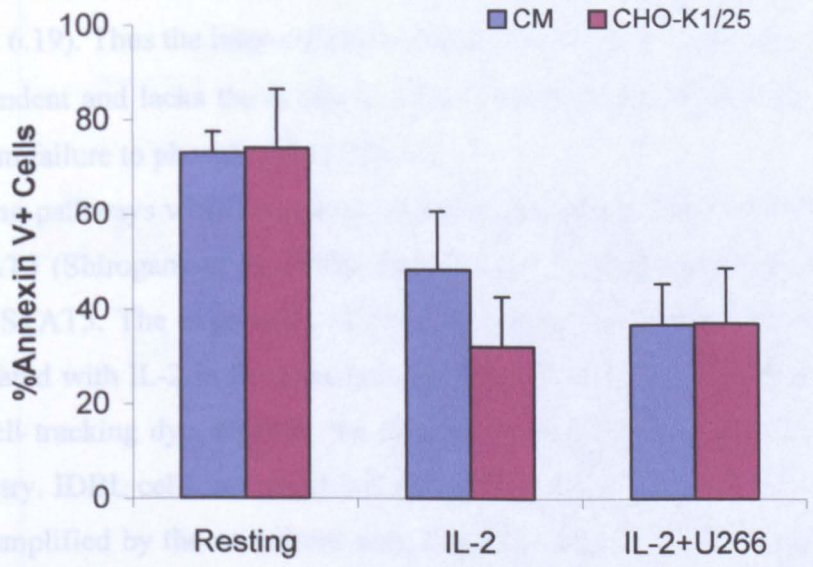


FIGURE 6.17 The effect on apoptosis of stimulating IDBL cells (n=3) with rhIL-2 in the presence and absence of U266 cells. The addition of the transfectant cell line, CHO-K1/25 to this culture system demonstrates a reduction (non-significant) in the apoptosis rate but does not alter the apoptosis rate of IDBL cells when stimulated in the presence of U266 cells.

A reduction in the resting apoptosis rate was seen upon stimulation with rhIL-2 (72.9%±4.5 *cf.* 48.2%±12.4, p=0.08) with no impact on the apoptosis rate being demonstrated by the addition of fixed CHO-K1/25 cells (Figure 6.17). The co-culture of IDBL cells with mitomycin-C-treated U266 cells did not alter the apoptosis rate (36.6%±8.5 *cf.* 48.2%± 12.4, p=NS). The addition of CHO-K1/25 cells to the culture system did not alter the apoptosis rate either (36.6%±8.5 *cf.* 36.9%±11.5, p=NS; Figure 6.17). Representative histograms are illustrated in Figure 6.18). It is worthy of note that the overall apoptosis rate of IDBL cells in this assay was higher than those illustrated in chapter 4. This is related to the use of the membrane staining dye, PKH26, which results in a modest reduction in cell viability post-staining (data not shown).

The molecular events involved in the signalling pathways of IL-2-stimulated IDBL cells were examined next. Similar to the effect that U266 cells exert on primary T cells, IDBL cells when stimulated with IL-2 in the presence of U266 cells fail to phosphorylate STAT5 (Figure 6.19). However, the phosphorylation of STAT3 was unaltered by the co-culture with tumour cells, unlike primary T cells. The co-culture with CHO-K1/25 cells in the presence of U266 cells results in failure to phosphorylates STAT3, similar to the effect that U266 cells exert on the re-stimulation of primary T cells (Figure 6.19). Thus the inter-cellular α chain presentation *in trans* to a cell line that is IL-2 dependent and lacks the α chain, induces tumour-derived TGF-β1 suppression resulting from failure to phosphorylate STAT3.

The signalling pathways which converge on the expression of *bcl-2* are numerous and include STAT3 (Shirogane et al, 1999). *Pim-1* can also be up regulated by STAT3 in addition by STAT5. The expression of *bcl-2* and *Pim-1* in IDBL cells was examined when stimulated with IL-2 in the presence and absence of U266 cells and CHO-K1/25. Using the cell tracking dye, PKH26, the expression of *bcl-2* in IDBL was analysed by flow cytometry. IDBL cells increased *bcl-2* expression in response to IL-2 stimulation, which was amplified by the co-culture with fixed CHO-K1/25 cells (Figure 6.20). The co-culture of IDBL cells with fixed CHO-K1/25 cells resulted in increased expression of *bcl-2*, though only in resting IDBL cells was this significant (p=0.05). The co-culture of IL-2-stimulated IDBL cells with U266 resulted in no change in the expression of *bcl-2* except when co-cultured with fixed CHO-K1/25 cells when a reduction in the expression was seen (not statistically significant, p=0.33). Representative histograms are illustrated in Figure 6.21.

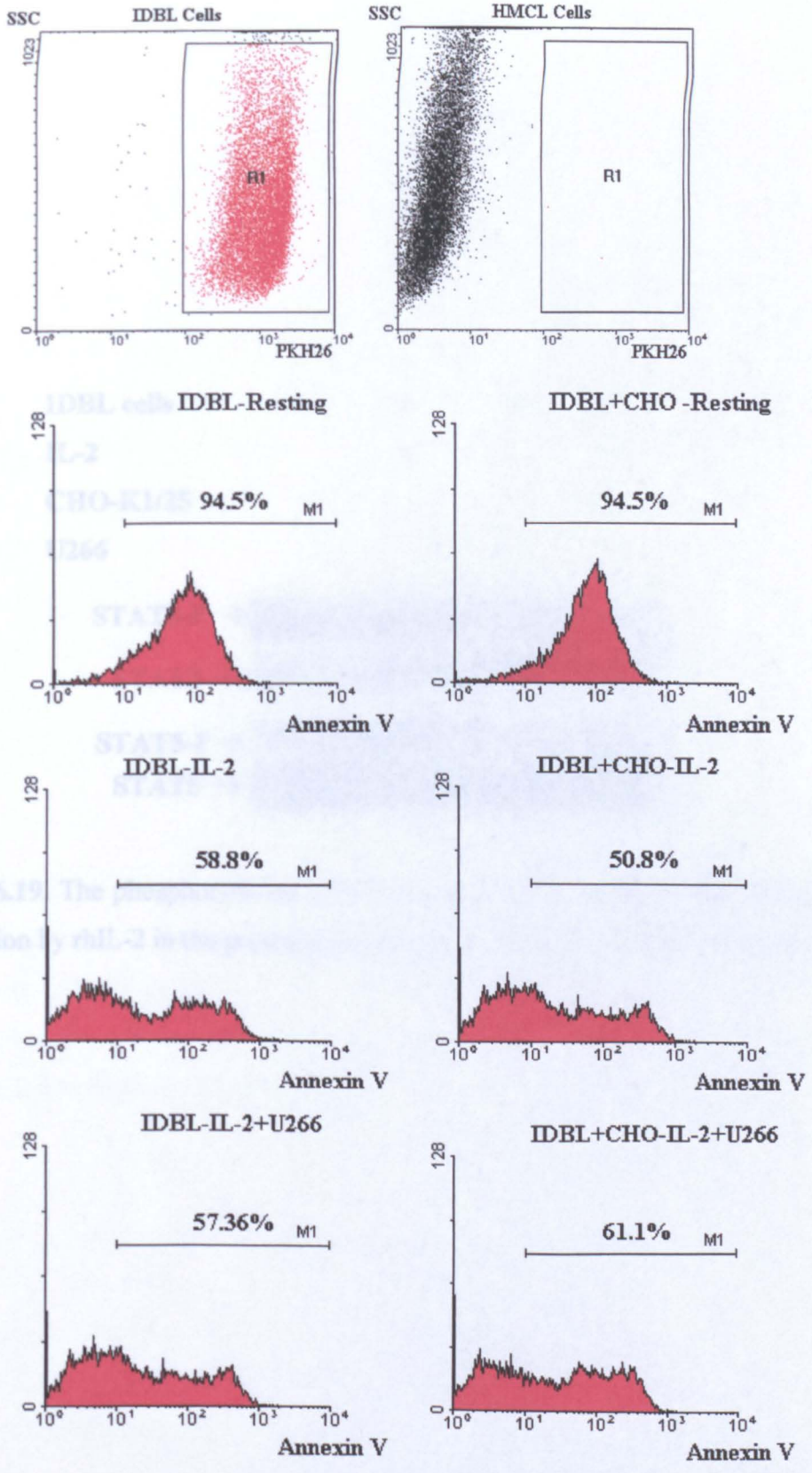


FIGURE 6.18. Representative histograms of Annexin V staining of PKH26-stained IDBL cells stimulated with IL-2 in the presence and absence of U266 cells and formalin-fixed CHO-K1/25 cells (CHO).

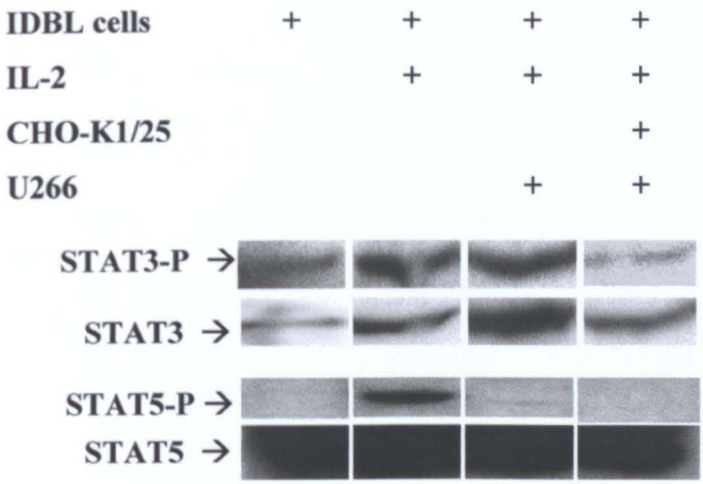


Figure 6.19. The phosphorylation of STAT3 and STAT5 in IDBL cells in response to stimulation by rhIL-2 in the presence or absence of U266 and CHO-K1/25 cells.

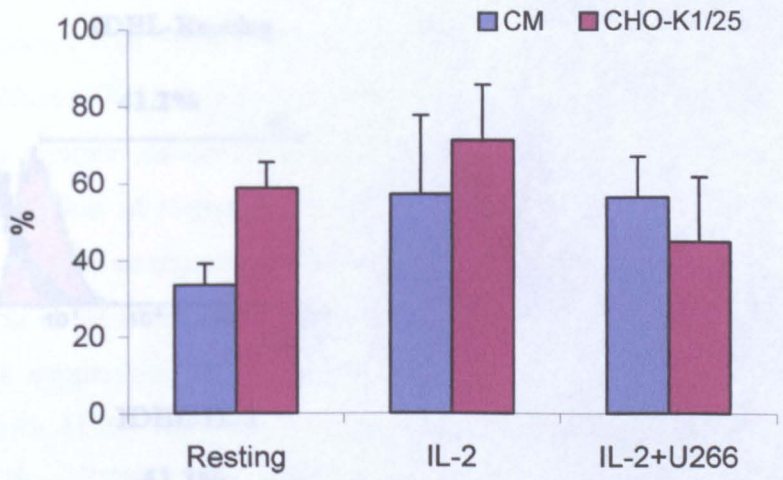


FIGURE 6.20. Effect on *bcl-2* expression by IDBL cells (n=3) when co-cultured with IL-2 and U266 cells in the presence and absence of CHO-K1/25. An increase in the expression of *bcl-2* in IDBL cells is seen when co-cultured with fixed CHO-K1/25 except when co-cultured with U266 cells, when a reduction is seen. However, these results failed to reach statistical significance.

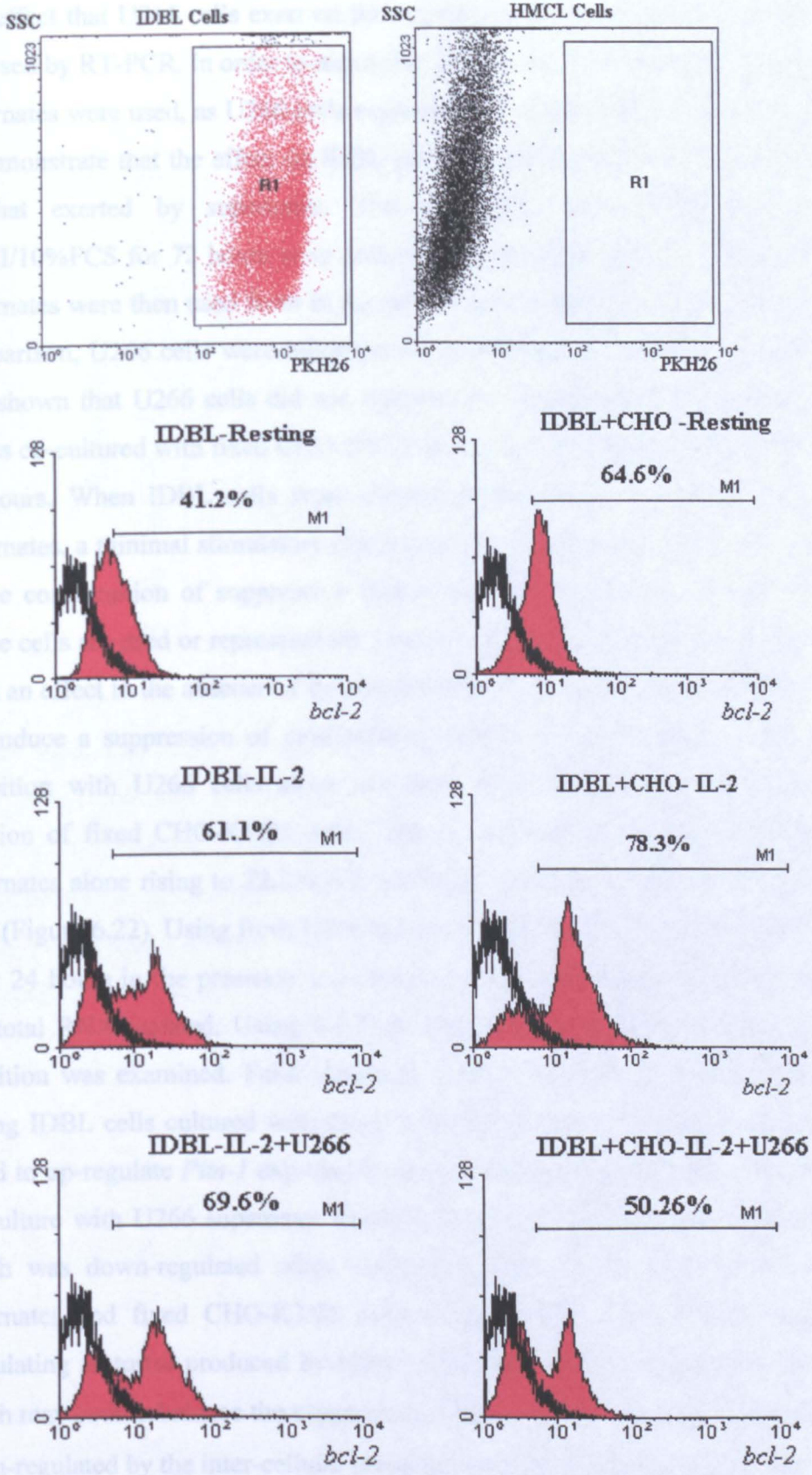


FIGURE 6.21. Representative examples of *bcl-2* expression in IDBL cells when stimulated with IL-2 in the presence of U266 cells with and without fixed CHO-K1/25 cells.

The effect that U266 cells exert on the expression of *Pim-1* mRNA in IDBL cells was analysed by RT-PCR. In order to maximise the specificity of the RT-PCR assay, U266 supernates were used, as U266 cells express *Pim-1* at the mRNA level. It was important to demonstrate that the effect on IDBL proliferation exerted by U266 cells was similar to that exerted by supernates. The supernates from U266 cells cultured in RPMI/10%FCS for 72 hours were collected by centrifugation of cell suspensions. The supernates were then used fresh in the proliferation assays at a final dilution of 1:2. For comparison, U266 cells were mitomycin-C treated and co-cultured with IDBL cells. It was shown that U266 cells did not suppress the proliferation of IDBL cells to rhIL-2 unless co-cultured with fixed CHO-K1/25 cells, as determined by ^{3}[H]dThd uptake after 24 hours. When IDBL cells were stimulated with rhIL-2 in the presence of U266 supernates, a minimal stimulatory effect could be demonstrated. This may either be due to the consumption of suppressive factors that are maintained at stable levels when whole cells are used or represents the presence of additional stimulatory factors that can exert an effect in the absence of the whole cells. The addition of fixed CHO-K1/25 cells did induce a suppression of proliferation, similar to whole tumour cells: 2.4%$\pm$6.47 inhibition with U266 cells alone compared with 44.65%$\pm$10.8 inhibition with the addition of fixed CHO-K1/25 cells. This is compared to –9.9%$\pm$3.2 inhibition with supernates alone rising to 22.2%$\pm$0.8 inhibition with the addition of fixed CHO-K1/25 cells (Figure 6.22). Using fresh U266 supernates, IDBL cells were stimulated with rhIL-2 for 24 hours in the presence and absence of supernates and fixed CHO-K1/25 cells and total RNA isolated. Using RT-PCR, the expression of *Pim-1* under each culture condition was examined. Faint transcript bands were seen in resting IDBL cells and resting IDBL cells cultured with fixed CHO-K1/25 cells. The stimulation with rhIL-2 failed to up-regulate *Pim-1* expression, even in the presence of fixed CHO-K1/25 cells. Co-culture with U266 supernates resulted in up-regulation of *Pim-1* at the RNA level which was down-regulated when stimulated with rhIL-2 in the presence of U266 supernates and fixed CHO-K1/25 cells (Figure 6.23). These results suggest that a stimulating factor(s) produced by U266 cells induces Pim-1 expression in IDBL cells which may counterbalance the suppressive effect of HMCL-derived TGF-β but which is down-regulated by the inter-cellular presentation of the α chain of the IL-2R.

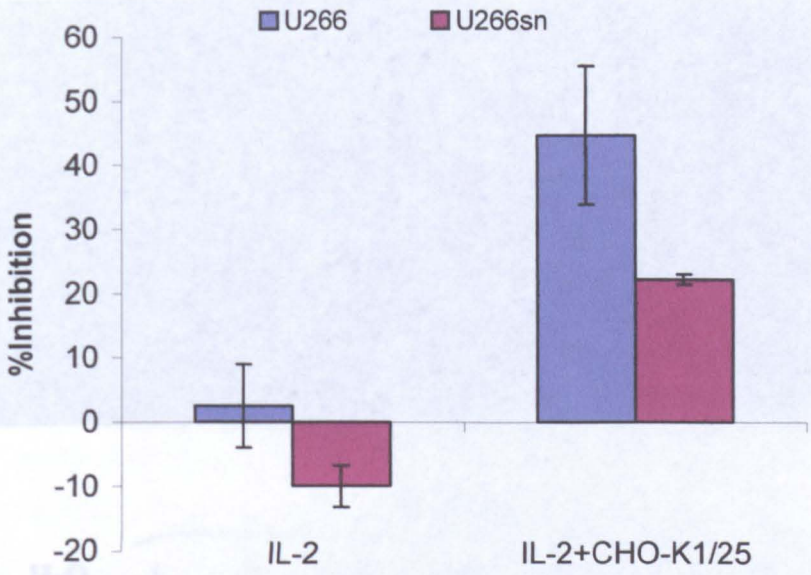


Figure 6.22. The effect on proliferation of co-culturing IDBL cells (n=3) with U266 cells and U266 cell supernates. U266 cells and supernates minimally suppress proliferation, however, when membrane bound IL-2Rα (CHO-K1/25) is present then suppression is demonstrated. Proliferation was assessed by ^{3}[H]thymidine uptake. **Key:** U266- mitomycin C-treated U266 cells, U266 sn – supernates from freshly cultured U266 cells after 72 hours.

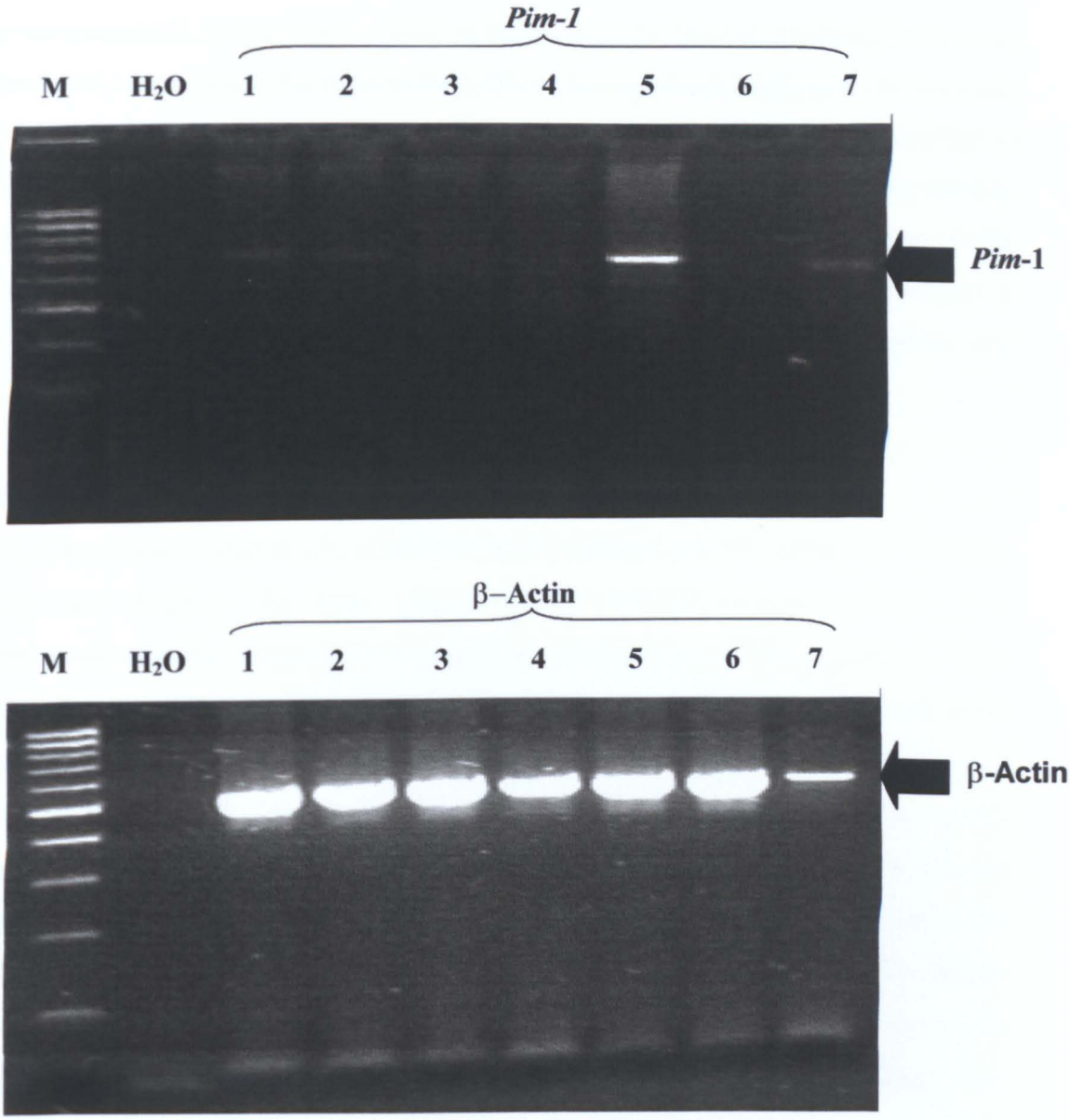


Figure 6.23. The expression of the protoncogene, *Pim-1* by IDBL cells when co-cultured with U266 cell supernates and fixed CHO-K1/25 cells, as detected by RT-PCR. Co-culture of IDBL cells with IL-2 and U266 supernates up-regulated the expression of *Pim-1*, which was abrogated by the presence of membrane-bound IL-2Rα (CHO-K1/25). **Key:** M- markers, H_2O- negative control, Lane 1- *IDBL cells*, Lane 2- IDBL + fixed CHO-K1/25 cells, Lane 3-IDBL cells + IL-2, Lane 4- IDBL, IL-2 + CHO-K1/25, Lane 5- IDBL, U266 supernates + IL-2, Lane 6- IDBL, U266 supernates, IL-2 + CHO-K1/25, Lane 7- U266 cells as a positive control.

6.4 DISCUSSION

The data presented in this chapter gives an unique insight into the mechanisms that may be involved in mediating the suppressive effects that myeloma cells exert on activating T lymphocytes. Using the complex *in vitro* model developed in chapter 4, the effect of HMCLs on activating T cells has been studied. In addition, a bone marrow plasma sample was included in this chapter to demonstrate that this effect is not unique to the transformed cell lines used in these studies. In consideration of the results presented in this chapter, the central role of the α chain of the IL-2R in the activation and proliferation of T cells warrants discussion.

Interleukin-2 Receptor (IL-2R)

The IL-2R complex consists of a combination of 3 distinct subunits or chains - IL-2Rα, IL-2Rβ and IL-2Rγ_c. The high affinity (Kd = 10^{-11}M) receptor consist of a heterotrimeric complex whereas the intermediate affinity (Kd = 10^{-9}M) receptor consists of IL-2Rβ and IL-2Rγ_c subunits only (Minami et al, 1993). The IL-2Rα subunit alone forms the low affinity (Kd = 10^{-8}M) receptor and its biochemical characterisation was greatly facilitated by the use of the monoclonal antibody directed against the antigen expressed on activated T cells (reviewed by Sugamura et al, 1996). The IL-2Rα gene is organised into 8 exons spanning more than 35 kb localised on chromosome 10p14-15 and is under the control of three distinct enhancer regions (Leonard et al, 1985; Meyer et al, 1997). The mature form of IL-2Rα, deduced from the nucleotide sequence, consists of 251 amino acid residues, sharing sequence and structural homology only with the IL-15α subunit (Giri et al, 1995). The cytoplasmic domain of the IL-2Rα conatins only 13 amino acid residues with no inherent signal transduction capability (Sugamura et al, 1996).

The surface expression of IL-2Rα is regulated mainly through gene transcription and whilst mitogenic stimulation induces a transient up-regulation of IL-2Rα, sustained up-regulation and thus surface expression requires IL-2 (Meyer et al, 1997). Transcriptional regulation is controlled by three positive regulatory regions: PRRI, PRRII and PRRIII. Mitogenic stimulation resulting in IL-2Rα up-regulation results from the interaction of NF-κB, c-Rel and SRF with PRRI together with interaction of Elf-1 and HMG-1(Y) with PRRII (John et al, 1995). PRRIII, located 1.3 kilobases upstream of PRRI and II, has three distinct binding sites that bind STAT5, GATA-like factors and Elf-1 (John et

al, 1996; Meyer et al, 1997). It has been demonstrated that the ability of mature T cells to proliferate to IL-2 correlates with IL-2Rα expression and thus IL-2Rα controls, at least in part, the IL-2 responsiveness of T cells (Lowenthal et al, 1985).

The human genomic IL-2Rβ gene is partitioned into 10 exons spanning 24 kb on chromosome 22q11.2-12 which encodes a mature protein consisting of 525 amino acid residues (Sugamura et al, 1996). Characterised as a member of the haematopoietic cytokine receptor family, the subunit has two pairs of N-terminal cysteine residues and a tryptophan rich WS motif near the transmembrane region. The cytoplasmic domain does not contain inherent signal transduction capability within the 286 amino acid residues, there are three regions which are closely involved in activating several signal transduction pathways: the serine rich region (aa 267-322) are involved in *c-myc* and Jak1 activation, the acidic region (aa 345-390) is assocaited with Src type kinases (Lck, Fyn & Lyn) and Tyr^{392} which is associated with phosphatidylinositol 3-kinase (Sugamura et al, 1996). The third subunit, IL-2Rγ_c, is also a member of the cytokine receptor family and the mature protein is 347 amino acid residues in size. The cytoplasmic domain consists of 86 amino acid residues containing two sub-domains of the Src homology region 2 (SH2) which are important for signaling. IL-2Rγ_c is a common receptor for a number of cytokines (IL-2, IL-4, IL-7, IL-9, IL-13 & IL-15) and signalling is effective through this receptor only when heterodimerized with the IL-2Rβ subunit. The IL-2-induced activation of *c-myc*, *c-fos* and *c-jun* result from interaction with the SH2 regions. In addition, the SH2 region is also involved in the activation of Jak3 which is associated with IL-2-induced cell growth and whose activation via IL-2Rγ_c is independent of interaction with other cytokine receptor subunits.

Using the *in vitro* model system described in this thesis, primary T cells activating in response to re-stimulation with exogenous rhIL-2, fail to enter the autocrine IL-2 pathway associated with failure to up-regulate the α chain of the IL-2R. This may result in failure of IL-2R sub-unit clustering which in-turn may be associated with failure of cell cycle progression and the activation of IL-2 responsive genes, such as *Pim-1*. This may explain the suppressive effect of HMCLs on primary T cells and relative insensitivity of IDBL and Jurkat cells. Jurkat cells are transformed T cells immortalised in the IL-2 autocrine pathway and as has been discussed in chapter 5, the effect of TGF-β is especially determined by the stage of activation of the T cell (Letterio & Roberts, 1998). IDBL cells have been generated beyond the IL-2 autocrine pathway and no

longer require the high affinity receptor for IL-2-mediated proliferation and viability and this may explain the insensitivity of these cells to myeloma cell-derived TGF-β.

Inter-cellular IL-2Rα presentation mediates HMCL-induced immuno-suppression.

Receptor aggregation within the immune system is a common feature. For example the interaction of CD28 with CD80/CD86 and CD4 and CD8 with MHC class II and I molecules respectively represent physiological receptor aggregation (Germain, 1997). These receptor/counter receptor interactions demonstrate the inter-cellular transmission of information however, in the case of the TCR and MHC molecule, a third molecule, namely antigenic peptides, participate in the inter-cellular signalling. It has been shown that the IL-2R components can behave in a similar fashion, in particular, the α chain on one cell can present IL-2 *in trans* to IL-2β/γ_c expressed on another cell thus augmenting IL-2-medaited signalling (Eicher & Waldmann, 1998). In this model, the IL-2 acts like the antigenic peptide in TCR/MHC interactions linking the two receptors and thus determines the specificity of the reaction. The presentation of IL-2 to IL-2Rα$^-$/ IL-2β/γ_c^+ cells by the α chain *in trans* results in amplification of the IL-2 proliferative signal and may have important physiological implications. Antigen activated T cells, by way of up-regulation of the α chain and entry into the IL-2 autocrine pathway may also induce proliferation and differentiation of by-stander cells (cytotoxic T cells, NK cells, granulocytes and monocytes) which constitutively express IL-2β/γ_c but not the α chain of the receptor (Girard et al, 1995; Espinoza-Delgado et al, 1995). This, at least in theory, could lead to an amplification of the immune response.

The data presented in this chapter demonstrates that the co-culture of cells expressing the IL-2Rα with cells that are IL-2Rα$^-$/ IL-2β/γ_c^+ neither induced cytokine-independence nor altered the cell cycle kinetics in response to IL-2 of the latter cells. However, an increase in the expression of *bcl-2* was evident whether these cells were stimulated with IL-2 or CM alone. This is further evidence to support the inter-cellular signalling model of IL-2/IL-2R proposed by Eicher and Waldmann (1998). Using an *in vitro* model system, I have shown that IL-2Rα$^-$/IL-2β/γ_c^+ cells are immune to the suppressive effects of HMCL-produced TGF-β, which is reversed when the α chain is presented in an inter-cellular fashion. This extends the model proposed by Eicher and Waldmann by suggesting that the inter-cellular clustering of the IL-2R mediated by IL-2 can mediate tumour immuno-suppression. When primary T cells were studied, the

2 can mediate tumour immuno-suppression. When primary T cells were studied, the immuno-suppressive effect of HMCLs may have been augmented by the inter-cellular α chain presentation as this population demonstrated a significant number of IL-2Rα$^+$/ IL-2β/γ_c^+ cells (60-70%). However this was not tested in these studies using agents to block α chain involvement in inter-cellular signalling e.g. anti-CD25 monoclonal antibody. The exact mechanism by which inter-cellular presentation of the α chain induces TGF-β susceptibility in IL-2Rα$^-$/IL-2β/γ_c^+ cells is not known. It may represent changes in the tertiary structure of the IL-2β/γ_c^+ receptors when engaged by its ligand that in turn may influence the use of particular signalling pathways. Further investigations in this area may help to clarify this issue.

Previous studies have examined the role of CD25$^+$ CD4$^+$ T cells in suppressing the activation of T cells in the context of tumour immunity and autoimmunity. Both in the *in vitro* model systems and *in vivo* murine models, these CD25$^+$ T cells play a pivotal role in suppressing both tumour and self-directed immune responses (Shimizu et al, 1999; Thornton & Shevach, 2000). These experimental systems demonstrated that these "professional" suppressor T cells mediate their immunosuppressive effects by specifically inhibiting IL-2 production and polyclonal T cell proliferation. This phenomenon is not mediated by cytokine production by these CD25$^+$ cells, is dependent cell-cell contact and these CD25$^+$ T cells act in an APC-independent manner. It has been shown that these CD25$^+$/CD4$^+$ T cells required to be activated by their TCR but once activated, their suppressor function is completely non-specific. (Thornton & Shevach, 2000). Such CD25$^+$/CD4$^+$ T cells account for up to 10% of CD4$^+$ T cells in lymphoid tissue in both mice and humans and exhibit potent immuno-regulatory functions *in vivo* (Suri-Payer et al, 1996). Though not confirming the role of the α chain, this evidence taken with the data presented in this chapter suggests a potential role of this molecule in cell-cell interactions mediating immuno-suppression. In the context of the myeloma-immune system conflict, the IL-2Rα chain may mediate the non-specific suppression of T cells mediated by tumour-derived TGF-β.

IL-2Rα-induced susceptibility of IDBL cells to HMCLs is related to Pim-1 expression.

Pim-1 is a protoncogene that was first identified as a common insertion site in Molony murine leukaemia virus-induced T cell lymphomas (Cuypers et al, 1984). It encodes two cytoplasmic serine/threonine protein kinases generated by alternative splicing.

remained speculative. The *Pim-1* gene expression is regulated by either STAT5 or STAT3 depending on the cytokine receptor pathway involved. For example, *Pim-1* expression induced by IL-3, IL-2, IL-5 and IL-15 is dependent on STAT5 signal transduction whereas gp130-mediated and interferon-α-induced expression is dependent on STAT3 (Matikainen et al, 1999; Shirogane et al, 1999). The *Pim-1* promoter contains a GAS element which facilitates the binding of STAT proteins (Yip-Schneider et al, 1995). *Pim-1* has been shown to promote cell cycle progression especially G1 to S phase transition, by activating Cdc25A (Mochizuki et al, 1999; Shirogane et al, 1999). Furthermore, *Pim-1* has been shown to promote factor-independence in several cell lines and inhibits apoptosis (Lilly & Kraft, 1997). The expression of *Pim-1* correlates with *bcl-2* induction in the murine myeloid cell lines studied and more recent evidence demonstrates a primary role for *Pim-1* in the expression of *bcl-2* and synergism with *c-myc* in anti-apoptosis (Shirogane et al, 1999). In the context of these studies, *Pim-1* represents a potential target gene for HMCL-induced suppression, affecting T lymphocyte cell cycle progression and proliferation.

In the *in vitro* model of T cell activation presented in this thesis, the effect of co-culture with HMCL on *Pim-1* expression was studied. In human primary T cells, *Pim-1* was induced, at the mRNA level, by re-stimulation with rhIL-2. This up-regulation was abolished by the co-culture with HMCLs. When the expression of *Pim-1* was studied in the Human T cell line, IDBL, only a faint trace of the protoncogene was seen in both resting and IL-2 stimulated cells, suggesting that *Pim-1* does not play a significant role in the proliferation and survival of IDBL cells. When the supernates from U266 cells were added to the culture system, marked up-regulation of *Pim-1* was seen which was abolished by IL-2 stimulation in the presence of intercellular IL-2Rα presentation *in trans*. This effect, as discussed, was associated with a reduction in the proliferative capacity of IDBL cells but not associated with any significant alteration in the apoptosis rate. This is particularly interesting as the expression of *Pim-1* in IDBL cells is not necessarily IL-2-dependent, unlike in primary T cells. However, several cytokines can stimulate *Pim-1* expression, including cytokines that signal through the gp130 receptor (Yip-Schneider et al, 1995). HMCLs produce many cytokines and may in fact secrete soluble gp130 that may potentially augment the signalling through this pathway. Indeed, the addition of U266 supernates to IDBL cells stimulated with rhIL-2 demonstrates a modest stimulatory effect. The exact factor(s) which are responsible for this remain to

be identified and are beyond the scope of this thesis but will form the basis of subsequent investigations.

In addition to the effect of *Pim-1* on cell cycle progression, *Pim-1* expression has been associated with *bcl-2* expression and protection from apoptosis (Shirogane et al, 1999). Despite increased expression of *bcl-2* in IDBL cells, no anti-apoptosis effect was demonstrated. Furthermore, the intercellular presentation of IL-2Rα reduced the *bcl-2* expression when IDBL cells were cultured with U266 cells though no effect on apoptosis was evident. The expression of *bcl-2* is associated with phosphorylation of STAT3 and I have shown that HMCLs do not prevent phosphorylation of STAT3 in IDBL cells, unlike the effect they exert on primary T cells. However the intercellular presentation of IL-2Rα in the presence of HMCLs resulted in failure of STAT3 phosphorylation, in addition to STAT5 which may account for the effect on *bcl-2* expression.

It is clear from the data presented in this chapter that HMCL-derived TGF-β1 mediates its effect through the failure to phosphorylate key signalling molecules. In primary T cells this is associated with failure to express the high affinity IL-2R and up-regulation of the proto-oncogene, *Pim-1*. The effect on IDBL cells is different and susceptibility to HMCL-derived TGF-β1 can be induced by inter-cellular presentation of the α subunit resulting in similar inhibition of phosphorylation of STAT3. The nature of the contribution of IL-2Rα presentation *in trans* has on TGF-β susceptibility is unknown but may represent a conformational change in the β/γ_c chains which alters the signalling pathway emphasis. However, clear molecular differences exist in these two cell groups and this may reflect inherent signalling pathway aberrations in IDBL because it is a cell line. It may also represent differing emphasis placed on alternative signalling pathways used by cells at the cytokine withdrawal stage of activation, as IDBL cells represent. The extension of the signal transduction studies and consequent molecular events presented here will help to clarify these differences and lead to a greater understanding of what potentially could be a differential suppressive effect that myeloma tumour cells may exert on T cells at varying stages of activation.

The molecular studies presented here provide an insight into how myeloma cells may influence the activation of T cells. In addition, the data suggests that the tumours mediate this not only through the production of TGF-β but by utilising the cell-cell interactions which occur naturally within the immune system, perhaps via inter-cellular

interactions which occur naturally within the immune system, perhaps via inter-cellular IL-2Rα presentation. This understanding will greatly assist in the design of immunotherapy strategies to maximise the perceived clinical efficacy.

CHAPTER 7

Summary and Conclusions

Although there is little doubt that tumour-specific antigens exist for most human cancers, it is still unclear why these antigens do not cause tumour rejection. A successful immune response against a tumour is dependent on the detection of that tumour and the ability of the host immune system to mount an effective response. Clearly malignant cells have evolved to evade the natural host defence mechanisms resulting in growth and dissemination at the expense of the host. It is essential that the mechanisms employed by tumour cells to avoid immune regulation be elucidated so that immunotherapeutic strategies can be designed which will realise their true potential for eradicating the malignant clone.

Multiple Myeloma (MM), a mature peripheral B cell malignancy remains essentially incurable despite massive escalation in treatment intensity. In addition to the associated paraproteinaemia, bone marrow failure, destructive bone disease and renal impairment, this disease is also associated with many defects in the host immune system. Such defects include quantitative and qualitative abnormalities of cell mediated immunity and secondary hypogammaglobulinaemia (Mellstedt et al, 1982). However, as yet, the mechanism that induces these abnormalities of the immune system remain to be determined.

Experimental animal model systems have provided an insight into the role of T cells in the development of B cells and the orchestration of the normal immune response. The role of such mechanisms in human malignant disease of B lymphocytes is uncertain. However, the question that remains fundamental to innovative treatment strategies in MM is: if the immune response can be restored to recognise the "aberrant" B cells, will this induce control over the malignant clone? Circumstantial evidence is available to demonstrate that immune effectors can be directed towards the malignant cells of myeloma. Allogeneic bone marrow transplantation (BMT) does cure some patients due to the combination of intensive therapy plus a graft-versus-tumour effect mediated in part through minor histocompatibility antigen mismatching but also through tumour-specific antigen expression (Gahrton et al, 1995; Bensinger et al, 1996; Bjorkstrand et al, 1996; Corradini et al, 1999). Certainly, donor lymphocyte infusions can re-instate remission after relapse following allogeneic BMT in the absence of GvHD (Bertz et al, 1997). In the autologous setting, peripheral blood lymphocytes from patients with myeloma have been shown to be capable of proliferative responses to autologous tumour cells and, with high effector:target cell ratios, cytotoxicity can be demonstrated (MacKenzie et al, 1977; Paglieroni et al, 1979). Such responses could explain the

disease stability, even without therapy, seen during plateau phase. Furthermore, it has been shown that a higher serum level of IL-2 is associated with earlier stage disease and a better prognosis suggesting a potential role for immuno-regulatory control of the malignant clone (Cimino et al, 1990). These features suggest that the immune system, either the host's immune effector cells or adoptively transferred donor immune effector cells, can be utilised to regulate and even eradicate the malignant clone of multiple myeloma.

Tumour immunotherapy remains the "Holy Grail" of tumour immunologists and clinical oncologists alike. However, before such treatments can be applied on an entirely rational basis and achieve their optimum efficiency in eradicating the tumour clone, more knowledge is required as to why the immune system fails to identify multiple myeloma plasma cells as foreign. With this understanding, it is envisaged that such tumour defence mechanisms could be by-passed or over-come not only in the allogeneic but especially the autologous setting thus targeting a greater number of patients with this disease. The experimental data presented in this thesis has addressed the issue of the interaction of myeloma tumour cells with T lymphocytes, indicating a potential mechanism that may propagate the immune privilege of the tumour clone and thus highlight areas that might be targeted when designing new therapeutic strategies.

Myeloma Tumour Cells Inhibit T cell Activation via TGF-β1

The data presented in this thesis demonstrate that the malignant plasma cells of multiple myeloma are capable of suppressing the activation of T lymphocytes. The myeloma cells prevent activation of T cells from healthy donors by allo-antigen, mitogen and IL-2, mediated by the production of a soluble, immuno-suppressive factor. This soluble factor was responsible for inducing cell cycle arrest and failure of the T cells to progress into the IL-2 autocrine pathway, which is of critical importance in the activation of T cells. The myeloma cells constitutively expressed high levels of TGF-β1 mRNA transcripts as detected by RT-PCR, which were translated into latent protein detected by immunohistochemistry and secreted as detected by ELISA. The identification of a potential mediator of the suppressive effects alone is insufficient proof. However, the reversal of the immuno-suppression induced by the myeloma cells using the specific

using the specific TGF-β antagonist, LAP, confirms that TGF-β1 is a major factor in myeloma-associated suppression of T lymphocyte activation. The use of a bone marrow serum sample (BM6) in chapter 6 sought to demonstrate similar effects to that induced by the HMCLs. Clearly, the BM6 serum was capable of suppressing the expression of the α subunit of the IL-2R and as such this indicates that the suppressive effects induced by the HMCLs is not a function of being a cell line. However, demonstrating the suppression of primary T cells using one sample is by no means conclusive and work is on-going to demonstrate such effects with a large cohort of BM sera and to determine whether these suppressive effects are altered in severity with varying tumour burdens as indicated by clinical staging.

The studies presented in this thesis strongly implicate TGF-β1 in the suppression of T cell activation. Others have proposed a role of FasL and the underglycosylated surface mucin, muc-1, in myeloma-associated immune dysfunction (Villunger et al, 1997; Treon et al, 1999). The evidence presented here however opposes these potential mediators for the following reasons. Firstly, in previous reports of the role of FasL, the authors used a Fas-sensitive target (CEM-C7H2 cell line) in their model which at least demonstrates that HMCLs express (and even secrete) functional FasL. In my *in vitro* model system, I used another Fas-sensitive T cell line, Jurkat E6.1, with no resulting decrease in proliferation and increase in apoptosis seen when co-cultured with HMCLs. Secondly, FasL has previously been shown to induce cell cycle arrest in primary T cells and whilst this was demonstrated in my *in vitro* model, the reversal by the TGF-β-specific antagonist LAP demonstrates that the major mediator of the suppressive effects seen in my studies is in fact TGF-β. The issue of muc-1 remains to be clarified. Other investigators have shown that it induces cell cycle arrest and apoptosis in primary T cells. However, this effect can be overcome by increasing concentrations of IL-2. In the studies presented in this thesis, not only did apoptosis not play a significant role in mediating the HMCL-induced suppressive effects, increasing concentrations of IL-2 could not over-come these effects. This suggests that, at least in my in vitro model, muc-1 does not play a significant role. However, the *in vivo* role of muc-1 remains to be clarified in on-going studies with clinical material.

T lymphocytes utilise many different intra-cellular signalling cascades when activated which vary depending on the stimulus and the receptor complex involved, i.e. IL-2/IL-2R *cf.* mitogen-TCR/CD3 activation (Kuo & Leidin, 1999). Data presented in this thesis

demonstrates that the myeloma cells prevent the T cells, upon activation, from up-regulating the surface expression of the α-chain of the IL-2R thus preventing the formation of the high-affinity receptor. This results from failure to up-regulate the transcription of the α-chain gene in response to re-stimulation of primary T cells with IL-2. The transcriptional regulation of the α-chain gene is complicated and requires an orchestration of several gene transducers. Of these, STAT5 has been shown to be important in mediating up-regulation of genes involved in activation and cell division. The evidence presented here demonstrate that primary T cells, when responding to re-stimulation with IL-2, fail to phosphorylate both STAT3 and STAT5, mediated by myeloma-derived TGF-β. However, using a novel IL-2-dependent T cell line, IDBL, it was shown that these cells are insensitive to the myeloma-derived TGF-β, in terms of DNA synthesis and proliferation, despite demonstrating failure of phosphorylation of STAT5. It was demonstrated that phosphorylation of STAT3 was unchanged when IDBL cells were co-cultured with myeloma cell lines. However when the α-chain of the IL-2R was presented inter-cellularly *in trans*, this induced a susceptibility of the IDBL cells to HMCLs. This resulted from failure of STAT3 phosphorylation associated with down-regulation of *bcl-2* and *pim-1* genes. As a consequence, the IDBL cells demonstrated reduced DNA synthesis and proliferation, similar to that seen with primary PBL, when IL-2Rα was presented in an inter-cellular fashion. Taken together, these features demonstrate that the presence of the α-chain of the IL-2R presented inter-cellularly *in trans*, from a transfectant in the case of IDBL cells assists the susceptibility of activating T cells to myeloma-derived TGF-β1 resulting in growth arrest and failure to enter the IL-2 autocrine pathway. The contribution of $CD25^+$ T cells in the heterogeneous population of primary T cells used in these studies, remains speculative. This data supports the role of the α-chain of the IL-2R in regulating the immune responses, here potentiating the T cell suppressive effects of myeloma-derived TGF-β1.

Implications for Immunotherapy

The improved disease control associated with allogeneic HSC transplantation is in part related to the direct effect of the graft-versus-myeloma effect that has been reported by several investigators (Tricot et al, 1996; Verdonk et al, 1996). A close relationship

between Graft-versus-host disease (GvHD) and GVM in these studies suggests that the latter effect is mediated by donor allo-reactive T cells directed against minor histocompatibility antigens present on both normal and myeloma cells. Some investigators have demonstrated the successful transfer of myeloma idiotype-specific immunity from an actively immunised bone marrow donor and showed the recovery of a $CD4^+$ T-cell line generated from the peripheral blood of the recipient which demonstrated specific idiotype sensitivity (Hornung et al, 1995; Kwak et al, 1995). These immunotherapy strategies have been tested in the relapsed or resistant setting and the role of such strategies in the setting of minimal residual disease following conventional chemotherapy or HCT offers an increased potential for tumour cell control by adoptively transferred immune effectors.

However, these attempts at immunotherapy are empirical and the true effector cells are not known. The role of adoptive immunotherapy in MM may be developed more appropriately in the setting of directed immune effectors. Many investigators have turned to generating professional antigen presenting cells such as dendritic cells which have been pulsed with tumour derived peptides or idiotype (Reichardt et al, 1999; Dabadghao et al, 1998; Wen et al, 1998; Zeis et al, 1998; Hart et al, 1999). These studies demonstrate that idiotype-reactive T cells can be generated using such systems, although the results of pre-clinical studies have failed so far to be translated into meaningful improvements in overall and event-free survival of patients with MM (MacKenzie et al, 1998).

Early pre-clinical experience using adenovirus to transfect plasma cells with immuno-stimulatory genes (e.g. IL-2, IL-12 and CD80; Wendtner et al, 1997) has lead a phase I clinical trial which injected an IL-2 gene directly into the tumours of two patients with multiple cutaneous plasmacytomas with resultant evidence of successful tumour cell transfection using PCR and work has now started on IL-2 gene transfected autologous plasma cell vaccines. Other investigators have utilised similar gene transfectant systems to transfer suicide genes e.g. herpes simplex thymidine kinase gene, which may have therapeutic potential (Stewart et al, 1998). These advances in the field of immunotherapy hold great potential and it is hoped that the success in the pre-clinical models can be translated into efficacious treatment modalities.

All these immunotherapeutic strategies have focussed on providing appropriate stimulation for the immune effectors cells but have not taken into consideration the fundamental defect in the host immune system. As has been shown in this thesis,

myeloma cells impair the response of T cells to allo-antigen, mitogen and re-stimulation with IL-2, mediated by TGF-β1. This may account for the peripheral tolerance induced by the MM tumour-bearing host. As TGF-β is a pleiotrpic cytokine with many functions, blocking its *in vivo* effect may be both difficult and may be associated with numerous unwanted side-effects. However, as shown in this thesis, the effect of TGF-β is particularly pronounced when T cells either require the assembly of the high affinity receptor for activation or are presented with the α-chain *in trans*, which results in failure of phosphorylation of both STAT3 and STAT5. This induces the pronounced T cell suppressive effects through targeting the entry of cells into the IL-2 autocrine pathway. As such, activation of T cells outside this IL-2 autocrine pathway may offer a means to by-pass the suppressive effect of the tumour cells on the activating T cells and potentially result in anti-tumour immune responses. For example, Interleukin-15 (IL-15) is a monocyte/macrophage-derived T cell stimulatory cytokine that can enhance T cell proliferation and up-regulation of IL-2Rα expression (reviewed by Ma et al, 2000). Experimental studies conducted in the research laboratory of the ATMU has suggested that IL-15 can render T cells resistant to suppression by tumour-derived TGF-β, mediated, at least in part, by phosphorylation of STAT3 and STAT5. This may provide a useful mechanism that may be exploited in the design of immunotherapy for MM. Whatever the mode of activation of the T cells in the afferent phase of an evolving anti-tumour response, one thing is clear. In light of the evidence presented in this thesis and published by other investigators, the micro-environment in patients with MM is hostile to activation of T cells and any immunotherapy strategy needs to take this into account if the true efficacy of such treatment is to be realised. Therefore, it would seem rational to concentrate on *ex vivo* induction of the afferent anti-tumour immune response with the expectation that this will allow for the generation of a more effective anti-tumour effect *in vivo*.

It is worthy of note that if the wild type tumour is capable of an array of defence systems which can prevent the generation of an immune response or switch off an established response, then the design of immunotherapy intervention will need to overcome these. It is evident from the data available that multiple myeloma tumour cells adopt numerous mechanisms to avoid regulation by the host immune system. The tumour cells evade detection through alterations in their phenotype whilst at the same time switch off any immune effector cells directed against them using

immunosuppressive cytokines and other molecules (Figure 7.1). It is likely that more than one of these pathways is responsible for the failure of the host immune system to regulate the growth of the tumour clone. As more research in this field unfolds and more evidence of the established pathways emerges, or newer mechanisms of immunosuppression are discovered, physicians can use this information to design new immunotherapy strategies that may maximise such treatment modalities, especially in patients with minimal residual disease.

The Role of TGF-β1 in the Pathology of Myeloma

The data presented in this thesis strongly supports TGF-β as a major immune modulating cytokine produced by the malignant clone in myeloma. However, what role, if any, does this pleiotropic cytokine play in the pathogenesis of the disease? Whilst functioning as an immunosuppressive factor thus permitting the malignant clone to escape regulation and thus rejection in a potential hostile environment, it is probable that TGF-β has a more central role in maintaining clonal growth and survival. For example, multiple myeloma is frequently associated with a normochromic/normocytic anaemia which has been presumed to result from bone marrow infiltration, especially as it is more prominent in advance disease (Tricot, 2000). This may not simply be a physical phenomenon as TGF-β1 has been shown to suppress haematopoiesis mediate through inhibition of the expression for the gene products for steel factor and its receptor (*c-kit*) and has been suggested to cause the bone marrow failure associated with B cell chronic lymphocytic leukaemia (Heinrich et al, 1995). Similarly, secondary or acquired hypogammaglobulinaemia associated with a non-specific polyclonal B cell defect results in poor primary immune responses to infection and significant morbidity and mortality (Tricot, 2000). This was originally thought to relate to tumour burden. It has been demonstrated that TGF-β1 suppresses the proliferation of normal B cells and immunoglobulin production though it has been shown that fresh myeloma cells and patient-derived cell lines are not inhibited by TGF-β1, either in terms of DNA synthesis or immunoglobulin production (Kerhl et al, 1986; Urashima et al, 1996). Also, TGF-β1 has been shown to inhibit IL-2-dependent T cell proliferation and cytotoxic T cell

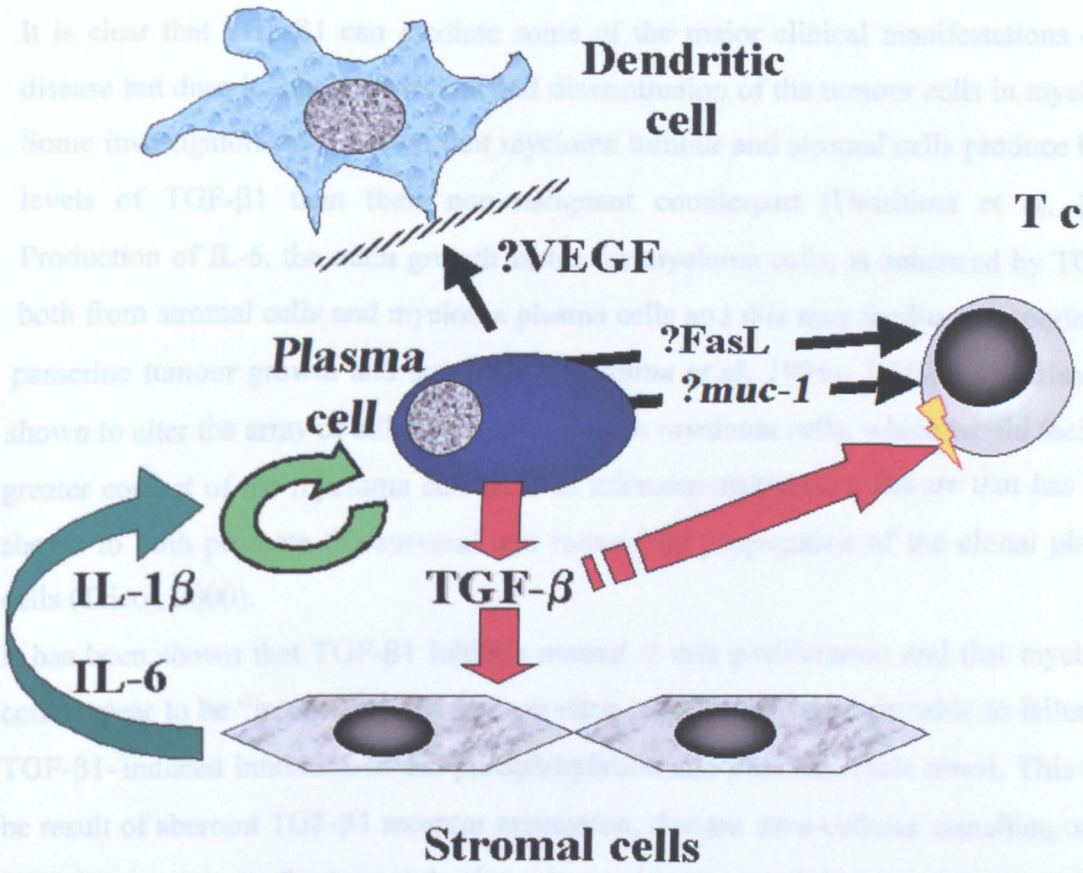


FIGURE 7.1. Multiplicity of interactions between *MM plasma cells* and their environment. In addition to defects in co-stimulatory molecules and antigen presentation, MM tumour cells may utilise numerous "counter attack" mechanisms to suppression the host immune system. **Key:** TGFβ: transforming growth factor beta; VEGF: vascular endothelial growth factor; IL1β: interleukin 1 beta; IL6: interleukin 6.

generation related to the fact that activated T cells increase their surface expression of TGF-β receptors (Kerhl et al, *1986; Letterio & Roberts, 1998).*

It is clear that TGF-β1 can mediate some of the major clinical manifestations of the disease but does it enhance survival and dissemination of the tumour cells in myeloma? Some investigators have shown that myeloma tumour and stromal cells produce higher levels of TGF-β1 than their non-malignant counterpart (Urashima et al, 1996). Production of IL-6, the main growth factor for myeloma cells, is enhanced by TGF-β1 both from stromal cells and myeloma plasma cells and this may facilitate autocrine and paracrine tumour growth and survival (Urashima et al, 1996). TGF-β1 has also been shown to alter the array of adhesion molecules on myeloma cells, which would facilitate greater contact of the myeloma cell with its microenvironment, a feature that has been shown to both promote the survival and induce the propagation of the clonal plasma cells (Tricot, 2000).

It has been shown that TGF-β1 inhibits normal B cell proliferation and that myeloma cells appear to be "insensitive", at least *in vitro*, which may be attributable to failure of TGF-β1–induced inhibition of Rb phosphorylation and thus cell cycle arrest. This may be result of aberrant TGF-β1 receptor expression, deviant intra-cellular signalling of the TGF-β1 receptors or fundamental cell cycle regulatory gene defects associated with the malignant transformation of the B cell in multiple myeloma. Whilst this is circumstantial evidence that indicates TGF-β1 is a major cytokine involved in the maintenance and survival of the malignant B cell clone, the exact role of TGF-β1 in the pathogenesis of multiple myeloma remains to be clarified.

CHAPTER 8

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APPENDIX

CM	RPMI1640, 10% heat-inactivated foetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.
Cryopreservation medium	Equal volumes of 20% DMSO in heat-inactivated FCS and 50% FCS in RPMI1640.
dNTP	20μl of 100mM dATP solution, 20μl of 100mM dGTP solution, 20μl of 100mM dCTP solution, 20μl of 100mM dTTP solution and 120 μl RNAse-free H_20 = 10Mm of each base.
DNA gel	1.4g agarose, 70 mls x1 TBE and 1 μl of ethidium bromide (10 mg/ml).
10X MOPS	0.2M MOPS(3-(*N*-morpholino)propansulphonic acid), 50 mM sodium acetate, 10mM EDTA (all Sigma) pH7 , autoclaved and stored in the dark.
Northern Loading Buffer	0.75 ml deionised formamide, 0.15ml 10xMOPS, 0.24 37% formaldehyde, 0.15ml DEPC-treated H_2O, 0.1ML glycerol, 50μl Bromophenol Blue/XC 10% dye mix and stored at -20^0C.
PBS	0.9% NaCl, 0.107% Na_2HPO_4 and 0.051%NaH_2PO_4 (all Fisons) w/v aqueous solution.
Propidium iodide	50μg/ml aqueous solution of propidium iodide (Sigma) with 0.04% RNAse (Sigma).
RIPA buffer	50 mM Tris-HCL (pH 8), 150 Mm NaCl, 1% NP_4O, 1mM EDTA, 0.05% SDS and dH_2O.
RNA gel	1.2g agarose (Sigma), 10ml 10xMOPS, 85ml ultrapure dH_2O and 5ml 37% formaldehyde .
10% Acrylamide gel	12.5mls Acrylamide/bis 40%, 20.8mls H_2O, 16.7mls 1.5M Tris HCL (pH 8.8), 0.5mls SDS, 0.5ml Ammonium Persulphate & 40μl TEMED.
5% Acrylamide gel	3.125mls Acrylamide/bis 40%, 18.375mls H_2O, 3.5mls 0.5M Tris HCL (pH 6.8), 0.25mls SDS, 0.25μl Ammonium Persulphate & 20μl TEMED.
10X TBE	108g Tris, 55g boric acid (both Sigma), 40ml 0.5M EDTA (pH8) in 1 litre of dH_2O. Equivalent to 0.045Tris borate, 0.1mM EDTA when diluted to 1X TBE.
TBS	0.9% NaCl buffered with 50 mM Trizma base (Sigma), adjusted to pH 7.6 with 1 N HCL.
TTBS	TBS with 0.05% Tween
TE	10mM Tris-HCL pH 7.6 and 1mM EDTA in dH_2O.
WB Blocking Buffer	1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
WB Loading Buffer	62.5Mm Tris-HCL (pH 6.8) 2% w/v SDS, 10% glycerol, 50Mm dtt, 0.1% w/v bromophenol blue.
10XWB Running Buffer	15.15g Tris, 72g glycine, 5g SDS, 500 ml H_2O
WB Transfer Buffer	3.03g *Tris, 14.4g glycine, 200 ml* methanol and 800 ml H_2O

Key: WB – Western Blot,

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Immune regulation in multiple myeloma: the host–tumour conflict

G. Cook, J. D. M. Campbell

Multiple myeloma (MM) remains essentially incurable by conventional anti-tumour therapy. This has led to increased interest in the possibility that forms of immune therapy might be effective. The successful use of donor lymphocyte infusions (DLI) in a few cases of MM relapse following allogeneic bone marrow transplantation have added weight to this view. MM is characterized by several defects in the host's immune system. The influence of the malignant clone on the function of the immune effector cells results from both passive and active suppression. Despite an array of functional adhesion molecules and HLA class I and II molecules on their surface and the secretion of a tumour-specific peptide, they fail to express adequate levels of co-stimulatory molecules thus inducing anergy in potentially tumour-specific T cells. In addition to this passive evasion of immune regulation, MM tumour cells are capable of producing a number of immunologically active agents which can induce immunosuppression such as transforming growth factor-beta, Fas ligand (FasL), vascular endothelial growth factor and Muc-1. It is postulated that these agents may be produced by the tumour cell to influence the microenvironment to support growth and differentiation of the clone but may have the additional benefit of altering the function of the host immune effector cells and thus preventing tumour rejection. This duality of function is important if clinicians are to design immunotherapy strategies which will achieve the true potential and result in improved survival in MM. © 1999 Harcourt Publishers Ltd

INTRODUCTION

Although there is little doubt that tumour-specific antigens exist for most human cancers, it is still unclear why these antigens do not cause tumour rejection. A successful immune response against a tumour is dependent on the detection of that tumour and the ability of the host immune system to mount an effective response. Clearly, malignant cells have evolved to evade the natural host defence mechanisms resulting in growth and dissemination at the expense of the host. It is essential that the mechanisms employed by tumour cells to avoid immune regulation are elucidated so that immunotherapies can be designed which will realise their promise.

Multiple myeloma (MM) is a mature, peripheral B cell malignancy and remains essentially incurable despite massive escalation in treatment intensity. Allogeneic bone marrow transplantation (BMT) does cure some patients owing to the combination of intensive therapy plus a graft-versus-tumour effect. In addition to the associated paraproteinaemia, bone marrow failure, destructive bone disease and renal impairment, this disease is also associated with many defects in the host immune system. In patients with MM, evidence exists to suggest that phenotypic and functional changes occur in T-cells, B-cells, macrophages and NK/LAK cells.[1–4] While this evidence suggests an

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attempt at immunoregulation of tumour cell growth and differentiation by the host, their in vivo and thus clinical relevance has yet to be proven. However, it has been shown that a higher serum level of interleukin-2 is associated with earlier stage disease and a better prognosis suggesting a potential role for immunoregulatory control of the malignant clone.[5]

Destruction of immune targets requires the activation of efficient, immune competent T cells. Such an induction is postulated to require a two-signal system for efficient activation.[6,7] Following the interaction of cellular adhesion molecules and their ligands, the initial signal is generated by T cell recognition of antigenic peptides (Ag) presented with major histocompatibility complex molecules (MHC) via T cell receptors.[8] Following this Ag/MHC-restricted signal, a second signal (non-MHC-restricted) is generated via co-stimulatory pathways which determines whether the TCR complex engagement results in functional activation or clonal anergy.[9] Several co-stimulatory pathways may exist but one important pathway is the interaction of B7-1 and B7-2 (CD80 and CD86 respectively) expressed on B cells and antigen presenting cells (APC) with their ligands CD28 and CTLA-4, expressed on T cells.[10–12] Providing that this second or co-stimulatory signal is received, then signalling from the TCR-CD3 complex (particularly via CD3ζ chains) is transmitted from the cell membrane to protein tyrosine kinases (especially p56 Lck and ZAP70, reviewed by ref 13). These act on many intra-cellular pathways culminating in the upregulation of new genes required by T cells to become activated, including proto-oncogenes (Fos, Myc) and interleukins (IL) involved in the immune response e.g. IL-2 and its receptor complex (IL-2Rαβγ$_c$), IL-3, IL-4, IL-5, IL-6.[14] This early activation is followed by DNA synthesis, proliferation and the up-regulation of genes expressed in late T cell activation, e.g. RANTES, Granzymes, Perforin, 519/granulysin.[15]

Clearly tumour cells can interfere with this activation process at any stage either by a single method or an orchestrated manipulation of the recognition and effector mechanisms (Fig. 1.). This review aims to discuss the current understanding of how myeloma tumour cells interact with the host immune system, highlighting areas which might be useful in designing new therapeutic strategies.

EVADING DETECTION

T-lymphocytes: ineffective policemen?

Patients with MM have altered distribution of CD4 and CD8 lymphoid subsets.[3] The imbalance of the CD4:CD8 ratio relates predominantly to a reduction, both in percentage and absolute numbers of CD4$^+$ cells particularly the naive CD4$^+$/CD45RA$^+$ cells.[16,17] These reports demonstrate that the observed decrease in CD4$^+$ T cells is more pronounced in patients with advanced disease and decreased numbers of CD4$^+$ T cells ($<700\times10^6$/l) are associated with a significantly lower survival rate and increased probability of relapse.[16,17] With regards to CD8$^+$ T cells, although increased numbers of cytotoxic T cells have been reported in patients escaping from plateau phase this increase is only in percentage and not absolute numbers.[18]

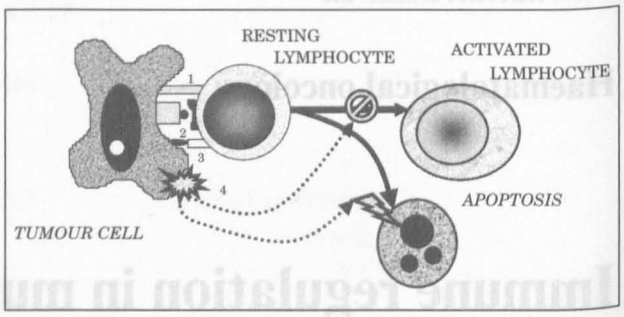

Fig 1 Interaction between tumour cells and the host immune system. Key: 1. Defects in inter-cellular adhesion; 2. Lack of MHC molecules, defective antigen processing/presentation, absent/silent antigens; 3. Absence of co-stimulatory molecules; and 4. Mediation of apoptosis (+) or prevention of activation (–) by soluble factors or cytokines.

T cells within bone marrow mononuclear cell fractions from patients with myeloma showed increased proliferative responses to CD3 monoclonal antibody stimulation compared with healthy, age-matched controls with demonstrable anti-plasma cell activity. This was thought to imply an immunoregulatory role for such T cells.[19] Certainly, peripheral blood lymphocytes from patients with myeloma have been shown to be capable of proliferative responses to autologous tumour cells[20] and with high effector:target cell ratios cytotoxicity can be demonstrated.[21] Peripheral blood T cells with idiotype specificity capable of down-regulating growth and differentiation of the myeloma clone have been demonstrated.[22]

The idiotypic immunoglobulin may be regarded as a tumour specific antigen and as such, proliferation and differentiation of idiotype-expressing malignant B cells may accordingly be under regulation by idiotypic reactive T cells. In animal plasmacytoma models, T cells can be shown to regulate the growth of the plasma cells by specific recognition of the idiotype antigen.[22] Furthermore, evidence of T cell tumour recognition is suggested by the growth of T cell clones by stimulation with IL-2 and F(ab')$_2$ fragments derived from autologous idiotype, and the production

of cytokines such as interferon-γ, IL-2 and IL-4 by peripheral blood T cells after stimulation with autologous idiotype.[23,24] However, when TCR variable region repertoires were analysed in peripheral blood T cells of myeloma patients with low tumour burden, oligoclonal $CD8^+$ cell expansions with Vβ3 and Vβ5.2 restricted usage did not demonstrate idiotypic reactivity but non-expanded Vβ22 restricted $CD8^+$ cells did.[25]

The antigen specificity of the clonal $CD8^+$ cells remain uncharacterized but may represent chronic antigenic stimulation by other tumour associated antigens. TCR beta gene rearrangements can be detected in 32% of patients more commonly found in disease progression.[26] The presence of these oligoclonal T cells was identified as an independent prognostic factor and correlated with the presence of peripheral blood T cells which are reactive to the tumour idiotype detected by autologous $F(ab_2)$ fragment binding to $CD3^+$ cells.[27] The ability to generate 'tumour reactive' T cells in vitro whilst clinical evidence fails to demonstrate that these cells have an ability to keep the disease under control in vivo serves to suggest that tumour cells are often antigenic but not immunogenic. The ability to recognize and eliminate tumour cells may be compromised in cancer patients by many factors including inadequate antigen presentation and tumour-derived immunosuppressive factors. T cells of patients with myeloma have abnormalities in signalling molecules particularly PKC-α and NFκB and enhanced susceptibility to apoptosis resulting from increased surface Fas expression and reduced intracellular bcl-2 levels.[28–30] Taken together, these features represent a fundamental defect in the T cells. In order to achieve the maximal effect from immunotherapy strategies, a fuller understanding of the biological interplay between myeloma tumour cells, which cause these defects, and immune effectors is mandatory.

Altered co-stimulation molecules: the unseen enemy

T cell activation is accomplished with specific antigen presentation associated with co-stimulatory cell–cell interaction.[6,7] Tumour cells may evade detection by disrupting this three – stage process; cell–cell interaction, antigen processing/presentation and co-stimulation. Myeloma tumour cells express an array of surface adhesion molecules involved in both cell–stroma and cell–cell interaction (reviewed in 31). Using a panel of human myeloma cell lines we have detected surface expression of both HLA-ABC and HLA-DR (Table 1) which reflect studies of MHC surface expression on freshly isolated cells from patients with multiple myeloma.[32,33] However, we did not detect significant surface expression of the co-stimulatory molecules, CD80 and CD86 nor did we detect expression of CD80 mRNA by RT-PCR (Table 1). These results are similar to that reported by other groups though variable expression has been reported between human myeloma cell lines (HMCL) and fresh tumour cells from patients which might represent the level of maturation or the state of activation.[34–36] In addition it has been demonstrated that surface expression of CD80 can be up-regulated on myeloma cell lines by co-stimulation through CD40L.[37,38]

Interestingly, plasma cells from myeloma patients, plasmacytomas and HMCL demonstrate surface expression of CD28 which is constitutively expressed on the majority of CD4 T cells and approximately half of CD8 T cells.[35,39] Myeloma cells expressed a similar density of CD28 antigen to that of normal T cells and it has been demonstrated to be able to bind B7-Ig molecules.[40] Blockade of CD28 signalling failed to alter the proliferation, survival, differentiation or surface expression of antigens and cytokine receptors. However, the CD28 molecule was demonstrated to be functionally active by binding of the p85 sub-unit of PI_3 to CD28 being triggered by CD80-transfected cells. Whether CD28 has a role in autostimulation of the tumour clone or contributes to a down-regulation of the immune control of the malignant plasma cells in vivo remains to be elucidated. However, fresh tumour cells from patients with early and plateau disease are $CD28^-$ compared with fresh tumour cells from patients with aggressive disease which were $CD28^+$.[35] The relevance of these findings in vivo and their effect on the hosts ability to generate competent immune responses remains unclear.

Can myeloma cells present antigen?

The myeloma-derived idiotype (Id) is a tumour-specific antigen that can be recognized by antigen-specific T cells in the form of peptides bound to MHC molecules (class I and II). Professional antigen presenting cells such as dendritic cells can efficiently present the Id.[41] However, despite the high production of Id by the clonal plasma cells, a clinically significant naturally occurring immune response has rarely been demonstrated. In addition to the variable surface expression of co-stimulatory molecules and the potential for an immunologically hostile microenvironment (see below), MM tumour cells may function as poor antigen presenting cells. Several studies have shown that fresh MM tumour cells can induce an allogeneic MLR which was augmented by anti-CD28 MoAb or pre-treating the tumour cells with CD40L or TNFα and interferon-γ[42,43] with the T cell proliferative responses seen being predominantly from the

Table 1 Expression of surface molecules involved in secondary costimulation of T lymphocytes in human myeloma cell lines using monoclonal antibodies. The presence of CD80 and CD86 mRNA by RT-PCR

			Surface antigen expression					
Cell Line	CD80 mRNA	CD86 mRNA	CD80	CD86	CD28	CD40	HLA-ABC	HLA-DR
JIM-3	Neg	Neg	N	N	++	++	+++	N
JIM-1	Neg	Neg	N	N	+++	++	+++	++
JJN	Neg	Neg	N	N	++	++	+++	+++
U226	Neg	Neg	N	N	+++	++	+++	+++
RPMI-8226	NT	NT	N	N	++	++	+++	+++
EJN	NT	NT	N	N	++	++	+++	++

Key: N <20% cells positive, + 20–40% cells positive, ++ 40–70% cells positive, +++ 75–100% cells positive, NT not tested.

CD8+ lymphocyte compartment. However, we have shown that HMCL when used in a one-way MLR do not induce an allogeneic response which may reflect the fact that the HMCL are derived from patients with aggressive, often terminal phase disease and thus the tumour cells may have adopted additional evasive measures.[44]

The ability of malignant plasma cells to process and present recall antigens (purified protein derivative and tetanus toxoid) to autologous T cells with the generation of proliferative responses and IFN-γ secretion has been demonstrated.[42] However, when MM cells alone were used to stimulate autologous T cells, no proliferative response directed towards the tumour cells was demonstrated despite the addition of anti-CD28 MoAb.[43] This unresponsiveness was partly reversed when the tumour cells were pre-activated with CD40L then used to stimulate an autologous reaction. These features suggest that myeloma tumour cells have the capacity to process and present antigens that can be recognized by T cells, at least in vitro. This implies that T cells, especially tumour-specific T cells, may play a role in the in vivo tumour regulation of growth and differentiation. However, the fact that large number of tumour cells are not destroyed in patients with MM suggests that in vivo, this attempt at regulation may indeed be hampered either by insufficient co-stimulatory molecules in vivo or that specific peripheral tolerance has been induced, as has been observed in other human B cell malignancies.[45]

Antigen silencing

Assuming there is a clonal origin and a series of genetic modifications involved in the development of neoplastic cells it is reasonable to expect that tumour cells express multiple cellular proteins that are distinctive from the tissues from which they are derived. However, cytotoxic T lymphocytes (CTL) are not active in vivo against most of these epitopes despite the association of these antigens with MHC molecules.[46] This results in limited T cell responses to a reduced number of antigens out of a larger available pool – a phenomenon known as 'immunodominance'. Silent tumour antigens are natural peptides presented in association with MHC molecules that are not immunogenic during the clonal expansion of the tumour cells. Despite these antigens failing to be immunogenic in vivo, they are fully competent targets for CTL lysis.[47]

Many factors contribute to antigen silencing including altered TCR ligand peptide affinity, TCR occupancy thresholds, levels of co-stimulatory and adhesive molecules and the status of the T cells (naïve *vs.* memory). When taken together, a 'compensation model' of CTL activation is evident: weak antigenic signals may require a larger degree of adhesion/co-stimulation than stronger antigenic signals. This coupled with a potentially immunologically hostile environment may provide a route for evading immunological surveillance. In respect to multiple myeloma, little if any data exist regarding the existence of silent antigens. If present then these might provide a useful source of antigenic material to stimulate CTL responses in vitro particularly if the amino acid structure can be subtly modified to improve their immunogenicity and dendritic cells are employed as professional antigen presenting cells.[48,49] This is particularly important as silent antigens work in the induction phase, not in the effector phase and thus pre-activated CTLs against these antigens may operate with improved efficiency as has been shown in a murine lymphoma model.[50] Further research in myeloma is needed to identify silent antigens, if such exist, which might provide the tumour immunotherapist with an additional weapon in their armoury.

Table 2. The effects of cytokines on immune effector cells

Effect	TGF-β	IL-10	VEGF	Ref.
Inhibition of T-cell growth	+	–	+	65,107
Inhibition of CTL differentiation	+	+	+	54,59,107
Inhibition of T cell cytokine production	+	+	–	53
Induction of T-cell anergy	+	–	–	65,73,74
Shift in the Th1–Th2 balance towards Th2	+	+	–	54,70
Down-regulation of adhesion/co-stimulatory molecules	+	+	–	60,65

Key: IL, interleukin; TGFβ, transforming growth factor beta; Th, T helper lymphocyte; VEGF, vascular endothelial growth factor.

ATTACK IS THE BEST FORM OF DEFENCE

Tumour-induced cytokines and immunosuppression

An array of cytokines and immune-modulating agents have been reported by many investigators in the field of tumour immunobiology.[51] In particular, B cells can produce a large number of cytokines both in disease and health (reviewed in 52). These cytokines, plus a range of immune-modulatory molecules including interleukin 10 (IL-10), transforming growth factor beta (TGFβ), Fas and FasL, vascular endothelial growth factors (VEGF), and MUC-1 warrant particular attention. The effects of the cytokines are summarised in Table 2.

IL-10

This is a pleiotropic cytokine produced by B cells, monocytes, macrophages and keratinocytes which inhibits synthesis of the pro-inflammatory cytokines IL-1, IL-6, IL-8, IL-12, TNF-α and GM-CSF.[53] Originally identified in mouse T helper cells, murine IL-10 was referred to as CSIF (cytokine synthesis inhibitory factor) and was shown to be a product of Th_2 T helper cells which inhibited cytokine production by Th_1 cells.[54] Murine and human IL-10 share 73% amino acid homology including hydrophobic leader sequences and although huIL-10 is active on murine T cells, muIL-10 appears not to be significantly active in human cells.[55] It is interesting to note that IL-10 proteins and cDNA reveals a strong predicted amino acid sequence to BCRFI (84% amino acid homology), an uncharacterized open reading frame in the Epstein–Barr viral genome. It has been proposed that that the BCRFI gene represents a cytokine gene that assists the virus to evade or attenuate the host defences.[56] Multiple roles have been suggested for IL-10 including inhibition of monocytes/macrophages and T cell effector function. However, IL-10's effects are not all inhibitory since it can enhance the viability and proliferation of B cells and differentiation.[53] IL-10 is the most potent inducer of immunoglobulin secretion in various B cell activation systems in vitro.[57]

IL-10 is spontaneously secreted by a variety of human tumours and local secretion by tumour cells can render themselves insensitive to CTL lysis.[59,60] In multiple myeloma the role of IL-10 is less clear. Normal B cells differentiating to Ig-secreting B cells lose IL-10 mRNA transcripts, though terminally differentiated plasma cells retain expression of the IL-10R.[61,62] We have not found evidence of IL-10 mRNA in five human MM cell lines assayed. Other investigators, whilst confirming these observations, demonstrated that IL-10 is a growth factor for malignant plasmablastic cell lines and that IL-10-dependent MM cell lines can be obtained.[63] This growth promoting activity is independent of IL-6, though these IL-10 dependent MM cell lines demonstrated a plasmablastic morphology and low Ig secretion. This suggests that these cell lines were more primitive than the mature malignant plasma cells seen in the disease in vivo and the addition of IL-10 to the culture system did not induce terminal maturation of these cell lines. These same investigators, using a sensitive ELISA assay, demonstrated a low level of serum IL-10 in patients with stable disease (3.3%) compared to a higher level of detection in patients with terminal phase disease and plasma cell leukaemia (60%). Similarly, other investigators have demonstrated that serum IL-10 levels are higher in patients with multiple myeloma compared with control patients and that serum IL-10 levels correlate with the paraprotein level.[64] Thus the role of IL-10 in the pathogenesis and immune dysfunction in MM remains uncertain. However, this cytokine may be involved in the later phases of the disease associated with widespread dissemination of tumour cells and deterioration of clinical status.

Transforming growth factor beta

Transforming growth factor beta (TGFβ) was originally purified from platelets and found to mediate anchorage-independent growth of fibroblasts and play

a key role in the growth and differentiation of epithelial cells (reviewed in 65). TGFβ is a pleiotropic cytokine that can affect a wide variety of tissues and cells. There are five isoforms of TGFβ (TGFβ1–5) which signal through the same serine–threonine kinase receptors (TGFβR I and II). TGFβ has effects on all of the mediators of the immune response – T cells, B cells, monocytes/macrophages and dendritic cells. TGFβ is a potent immunosuppressive cytokine and influences all stages of T lymphocyte development and differentiation through to their activation and proliferation, the exact effect being determined by the stage of maturation of the T cell. A subset of T helper cells (Th_3) produce TGFβ[66–70] upon activation, in addition to IL-4 and IL-10, and these may represent a subgroup of cells capable of suppressing the inflammatory response to limit tissue damage, though their exact function is not known. Exogenous TGFβ inhibits T cell proliferation by down-regulating IL-2 mediated-signals mediated through reduced tyrosine phosphorylation of proteins of 120, 100, 85, 75 and 50 kDa and inhibition of IL-2 mediated phosphorylation of the retinoblastoma susceptibility gene product, which is pivotal in the progression of cell cycle.[59,71–73] However, in contrast to these inhibitory functions, a growing body of evidence is being produced to suggest that TGFβ can also enhance T cell growth, especially those with naïve phenotypes and promote effector function through enhancement of cytokines and inhibition of apoptosis.[74–76] TGFβ suppresses normal B cell proliferation and immunoglobulin production, induces apoptosis of pre-B cells mediated through interactions with stromal cells and suppresses haemopoiesis through antagonism of stem cell factor.[65,77–79] TGFβ has been reported to account for the profound suppressive effects on normal haemopoietic stem cells and B cell function associated with malignancy.[80,81]

In multiple myeloma, several studies have shown TGFβ mRNA in tumour cells and derived cell lines, and we have shown this correlates with secretion of TGFβ.[61,82–85] Some investigators have shown that the level of TGFβ secreted by MM cells is greater than normal B cells and CD40L-activated B cells and that BM stromal cells from MM patients secrete more TGFβ than BM stromal cells from normal control subjects.[85] Other groups have demonstrated high serum levels of TGFβ in patients with MM using ELSIA assays and there is a suggestion that serum levels correlate with the extent of myeloma bone disease.[86,87] In contrast to its effects on normal B cells, TGFβ does not decrease the proliferation of MM cells and may even augment IL-6 secretion and related proliferation.[85] We have shown that MM tumour cells can inhibit T cell activation and induce a G_1 cell cycle arrest of T cells mediated by TGFβ.[84] However, the exact role of TGFβ in the pathogenesis is unclear, although data are emerging that indicate TGFβ to be a major cytokine involved in the maintenance and survival of the malignant clone in myeloma, perhaps mediated through autocrine/paracrine IL-6 and other, as yet poorly understood, interactions with the stromal elements in the bone marrow micro-environment. It is also possible that TGFβ contributes to both the identified cellular and humoral immune deficiencies associated with this disease.

Fas/Fas ligand

Fas (Apo-1/CD95) is a member of the TNF receptor/nerve growth factor receptor family and it regulates apoptosis through interaction with its ligand, FasL, which is also a member of the TNFR/NGFR family and can act both in the membrane bound and soluble forms.[88–92] Fas is present on the cell surface of a variety of cells including activated T cells. FasL is constitutively expressed in sites of immune privilege such as the eye, the spleen and at a low level in the thymus and more recently it has been described on the surface of tumour cells.[93,94] The recognized role of Fas/FasL in the immune system lies in the process of acquisition of self tolerance through clonal deletion of thymocytes and T cell mediated cell killing as part of the host defence against virally infected or transformed cells.[95–97] Recently it has been shown that FasL engagement inhibited CD4+ T-cell proliferation, cell-cycle progression and IL-2 secretion in vitro and prevented superantigen-mediated CD4+ T-cell expansion in a murine model.[98] Thus with the acquisition by tumour cells of FasL, the active suppression of tumour-specific Fas^+ T cells might be an active mechanism of escape from immune surveillance. However, this 'counter attack' or immune privilege mediated by Fas/FasL interactions in immune evasion by human cancers is more complicated than first thought and is rapidly becoming an established area of tumour immunology research. Disarming this counter-attack might offer a potential for therapeutic intervention (reviewed in ref. 99).

In myeloma, some investigators have demonstrated the expression of both Fas (CD95/Apo-1) and its ligand, FasL on the surface of myeloma cell lines.[100] The FasL was shown to be functionally active in inducing apoptosis in Fas-sensitive T-cell acute lymphoblastic leukaemia cell line. One question is raised by these and other similar findings: why do the tumour cells which are $Fas^+/FasL^+$ not commit autocrine 'suicide' or juxtacrine 'fractricide'? They do not because of an intrinsic resistance to Fas-mediated apoptosis. Many mechanisms have been proposed to account for this, e.g. expression of antagonistic soluble Fas, failure to

establish death-inducing signalling complexes (DISCs), altered regulation and function of the caspases, the exact pathways involved remain uncertain.[95,99,101] The understanding of Fas resistance and counterattack is fundamental to designing interventional strategies to disarming this system for effective cancer management and we await the developments in this field with anticipation.

Vascular endothelial growth factors

Vascular endothelial growth factor (VEGF) is a 34–42 kDa cytokine produced in large amounts by most tumours and previously recognized mainly for its angiogenic properties.[102] It stimulates the proliferation of endothelial cells and thus has a pivotal role in tumour neovascularisation. More recently it has been shown to have a profound inhibitory effect on haemopoiesis.[103,104] In particular, VEGF has been shown to responsible for defective dendritic cell (DC) maturation in vivo. VEGF binds to haemopoietic progenitor cells through specific binding to its receptor, Flt-1 which blocks activation of the transcription factor NF-κB.[105] NF-κB is composed of 50–65 kDa subunits which bind to a 10 bp motif in the promoter sequence in responsive genes and include p50, p52, p65 (RelA), cRel and RelB. It has been shown that reduced DC generation results from targeted disruption of RelB.[106] Similarly, disruption of NF-κB expression has been shown to result in dysregulation of T cell growth and development.[107]

VEGF mRNA transcripts have been detected in both fresh MM tumour cells and HMCL.[108] Interestingly, the investigators demonstrated that when HMCL were cultured with excess rhIL-6, there was an increase in the level of VEGF mRNA, and when VEGF was added to cultures of human bone marrow stromal cells there was an increase in IL-6 production. Thus MM tumour cells may produce VEGF to stimulate the stromal cells to produce the growth and differentiating agent, IL-6, for the malignant clone and in addition, this cytokine may serve to 'disarm' the potent antigen-presenting DC from stimulating a host reaction. Despite this in vivo effect on progenitor cells, DCs can be generated from the peripheral blood DC-progenitor cells of patients with MM and that these DCs are both functionally and phenotypically similar to those generated from healthy donors.[109,110] This is an area of the biology of multiple myeloma that warrants further investigative research.

Muc-1

Cell surface mucins play a significant role in cell–cell communication.[111] The muc-1 mucin is a high molecular weight glycoprotein consisting of a core protein with highly branched carbohydrate side chains and is expressed on the apical surface of a number of epithelial cells, including malignant cells.[112–114] A high level of muc-1 expression is associated with high metatstatic potential and poor prognostic index in epithelial malignancies.[115–117] Muc-1 is a ligand for ICAM-1 and high levels of expression may induce immunosuppression or anergy by interaction with its ligand on T cells.[118] Furthermore, muc-1 expressed and shed by breast cancer cells has been shown to induce apoptosis in T cells which may be rescued by IL-2.[119,120]

Myeloma tumour cells, both fresh cells and HMCL, express muc-1 which can be upregulated by dexamethasone and produce a soluble variant which is capable of suppressing an alloantigen T cell response.[121–123] It has been shown that HLA-unrestricted CTL can be generated from PBMNC of some MM patients which directly recognise the under-glycosylated form of muc-1 and demonstrated direct cytotoxic capability against muc-1$^+$ MM and breast cancer cell lines.[124] However, it is worthy of note that this phenomenon was demonstrated in only 2 of the 6 patients tested which may suggest that muc-1 may limit the generation of adequate populations of immune effector cells. Clearly the role of muc-1 as an immunosuppressive agent warrants further attention, particularly its potential as a tumour-specific target.

IMMUNOTHERAPY IN MM

The use of the immune system to treat human malignancies has attracted much interest by cancer physicians. Evidence exists demonstrating the potential of the immune system to eradicate haematopoietic malignancies, particularly in relapse post-BMT and in the setting of minimal residual disease.[125] In myeloma, the use of allogeneic haemopoietic cell transplantation (HCT) has resulted in lower relapse rates and disease progression with higher molecular remission rates.[126–129] However, these clinical effects are achieved with higher treatment-related morbidity/mortality in the first year than is seen in the autologous setting. In those patients who survive one year there is significantly improved disease-free survival. The improved disease control is partly related to the intensive chemo-radiotherapy but is also a direct effect of the graft-versus-myeloma effect which has been reported by several investigators.[130,131] A close relationship between graft-versus-host disease and GVM in these studies suggests that the latter effect is mediated by donor allo-reactive T cells directed against minor histocompatibility antigens present on both normal and myeloma cells. Further evidence for the

graft-versus-myeloma can be deduced from the use of donor lymphocyte infusions to induce similar effects to that achieved after allogeneic HST in conjunction with high dose therapy or following relapse in the post-transplant period.[132–134] Some investigators have demonstrated the successful transfer of myeloma idiotype-specific immunity from an actively immunised bone marrow donor and showed the recovery of a $CD4^+$ T-cell line generated from the peripheral blood of the recipient which demonstrated specific idiotype sensitivity.[135,136] Other groups have studied the effect of immunizing the donor using the tumour-specific idiotype and GM-CSF.[137] All patients demonstrated evidence of de novo anti-idiotype T cells and a transient rise in IgM anti-idiotype antibodies which is suggestive of successful primary immunisation. These immunotherapy strategies have been tested in the relapsed or resistant setting and the role of such strategies in the setting of minimal residual disease following conventional chemotherapy or haemopoietic cell transplantation offers an increased potential for tumour cell control by adoptively transferred immune effectors.

However, these attempts at immunotherapy are empirical and the true effector cells are not known. The role of adoptive immunotherapy in MM may be developed more appropriately in the setting of directed immune effector. Many investigators have turned to generating professional antigen presenting cells such as dendritic cells which have been pulsed with tumour derived peptides or idiotype.[138–143] These studies demonstrate that idiotype-reactive T cells can be generated using such systems, although the results of pre-clinical studies have failed so far to be translocated into meaningful improvements in overall and event-free survival of patients with MM.[144,145] An alternative approach in immunotherapy is the use of adenovirus-infected autologous plasma cells to transfect immunostimulatory genes for IL-2, IL-12 and B7-1.[146] HMCL and primary plasma cells can be infected by adenoviral vectors containing marker genes or genes with therapeutic potential.[147] A recent phase I clinical trial injected an IL-2 gene directly into the tumours of two patients with multiple cutaneous plasmacytomas with resultant evidence of successful tumour cell transfection using PCR and restriction enzyme digestion.[148] Work has now started on IL-2 gene transfected autologous plasma cell vaccines. Other investigators have utilized similar gene transfectant systems to transfer suicide genes, e.g. herpes simplex thymidine kinase gene, which may have therapeutic potential.[149,150] These advances in the field of immunotherapy hold great potential and it is hoped that the success in the preclinical models can be translated into efficacious treatment modalities. It is worthy of note that if the wild type tumour is capable of an array of defence systems which can prevent the generation of an immune response or switch off an established response, then the design of immunotherapy intervention will need to overcome these if the true potential of targeted immune effector cells is to be maximised.

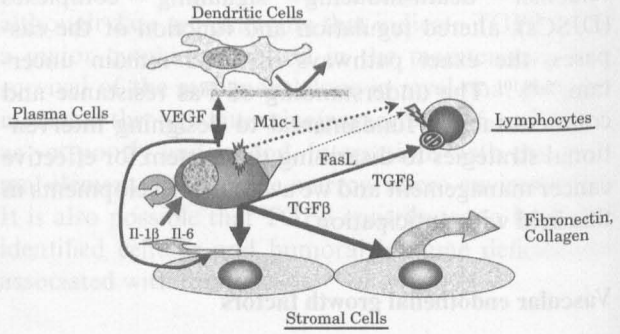

Fig 2 Multiplicity of interactions between MM plasma cells and their environment. In addition to defects in co-stimulatory molecules and antigen presentation, MM tumour cells have numerous 'counter attack mechanisms' available to suppress the host immune system. Key: TGFβ: transforming growth factor beta; VEGF: vascular endothelial growth factor; IL1β: interleukin 1 beta; IL6: interleukin 6.

CONCLUSIONS

It is evident from the data available that MM tumour cells adopt numerous mechanisms to avoid regulation by the host immune system. The tumour cells evade detection through alterations in their phenotype whilst at the same time switch off any immune effector cells directed against them using immunosuppressive cytokines and other molecules (Fig. 2). It is likely that more than one of these pathways is responsible for the failure of the host immune system to regulate the growth of the tumour clone. As more research in this field unfolds and more evidence of the established pathways emerges, or newer mechanisms of immunosuppression are discovered, physicians can use this information to design new immunotherapy strategies that may maximise such treatment modalities, especially in patients with minimal residual disease.

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Transforming growth factor β from multiple myeloma cells inhibits proliferation and IL-2 responsiveness in T lymphocytes

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Abstract: Multiple myeloma (MM) is a cancer of plasma cells, characterized by profound suppression of host immune responses. Here we show that MM cell lines significantly suppress the proliferation, blasting, response to interleukin-2 (IL-2), and expression of CD25 by concanavalin A (Con A)-activated or allostimulated peripheral blood T lymphocytes. T cells arrest in the G1 stage of the cell cycle, and do not enter the IL-2 autocrine growth pathway. T cell inhibition was mediated by a soluble factor. MM cell lines did not produce IL-10 but did produce large amounts of transforming growth factor β1 (TGF-β1). T cells were assessed for their ability to respond to IL-2 when co-cultured with MM cells in the presence or absence of the TGF-β inhibitor, TGF-β latency-associated peptide (LAP). MM cells suppressed IL-2 responses but this inhibition was completely reversed by TGF-β LAP. A CD25⁻, IL-2-dependent blast cell line was not inhibited by MM cells or rhTGF-β, confirming the specificity of the inhibition mechanism for the IL-2 autocrine growth pathway. We conclude that MM cells suppress T cells in their entry into the autocrine IL-2/CD25 pathway and in response to IL-2, and that TGF-β has a significant role to play. *J. Leukoc. Biol.* 66: 981–988; 1999.

Key Words: *tumor · immunity · cytokines*

INTRODUCTION

Multiple myeloma (MM) is a clonal malignant disorder of terminally differentiated B cells (plasma cells) characterized by bone marrow plasmacytosis, lytic bone lesions, the production of a paraprotein, and secondary hypogammaglobulinemia [1]. In addition to these altered antibody responses, patients demonstrate quantitative and qualitative defects in their cellular immune responses. T cells from patients have impaired responses to mitogens [2] and significantly lowered ability to generate LAK cells in response to interleukin-2 (IL-2) [3]. T cells from patients with MM have increased surface expression of Fas (APO-1/CD95), lower *Bcl-2* expression, and are susceptible to apoptosis [4].

Suppressing T cell responses is of great potential benefit to the tumor. It has been unequivocally demonstrated that T cells can recognize the tumor paraprotein antigen. Expanded $CD4^+$ and $CD8^+$ T cell [5, 6] subsets with restricted usage of Vα and Vβ segments [7, 8], which produce high levels of IL-2 and interferon-γ in response to idiotype, have been isolated. High serum IL-2 levels in patients with MM correlated with other prognostic indices and were associated with improved survival [9]. Also, donor lymphocytes infused after chemotherapy and allo BMT can demonstrate anti-myeloma effects [10], and transfer of immunity with idiotype-specific T cells has been demonstrated [11]. There is therefore no doubt that the immune system has the capacity to reject this tumor. However, despite these attempts at host immune regulation of the tumor clone, ultimately all patients progress to relapse. Escape from immune surveillance is therefore an essential strategy for survival of the tumor.

There are a number of possible mechanisms that tumor cells may adopt to evade immune detection. Although myeloma cells express an array of adhesion molecules [12] they generally lack the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on their surface, which are essential for the induction of T cell responses [13]. Myeloma tumor cells have been shown to constitutively express FasL on their surface and produce the MUC-1 antigen, both of which may mediate apoptosis of T cells [14, 15]. Myeloma tumor cells have also been reported to produce potentially immunomodulatory cytokines, including IL-6 and transforming growth factor β (TGF-β) [16].

Thus MM tumor cells could adopt various defense mechanisms against rejection by the host immune system, allowing continued growth and dominance in a potentially hostile microenvironment. As yet, there is no clear evidence to indicate which of these mechanisms is used by the tumor cells. In this study we have investigated the interaction between human MM cell lines and normal peripheral blood T cells to determine how MM cells can interfere with T cell responses, independently of any acquired defect in patient's T cells. The study was designed to examine the effect that exposure to MM tumor cells has on the initiation and maintenance of T cell activation. Specifically, do MM tumor cells suppress T cell responses or induce programmed cell death?

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MATERIALS AND METHODS

Myeloma cell lines

The MM cell lines (MMC) U266, JJN3, and JIM 1 were used in these experiments. Cell lines were maintained in complete tissue culture medium: RPMI-1640 medium, 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin.

Peripheral blood mononuclear cells (PBMC)

Venous blood was collected from healthy volunteers with the use of sodium heparin as anticoagulant. PBMC were separated over Ficoll/Hypaque (Lymphoprep, Nycomed, Oslo, Norway) and washed four times in phosphate-buffered saline (PBS).

Mitomycin C treatment

MMC or PBM to be used as stimulators in mixed lymphocyte cultures were treated with mitomycin C (Sigma). PBM (2×10^7/mL) were treated with 25 μg/mL mitomycin C in complete medium for 30 min at 37°C, followed by two washes in RPMI-1640 medium. MMC were treated as above, except that 50 μg/mL mitomycin C was used.

Cytokines

Recombinant human IL-2 (rhIL-2) and TGF-β latency-associated peptide (TGF-β-LAP) were purchased from Sigma. rhTGF-β1 was purchased from R & D Systems.

IL-2-dependent Con A blasts

Long-term IL-2-dependent Con A blasts were generated as follows: 2.5×10^6 PBMC/mL were cultured for 7 days with 5 μg/mL concanavalin A (Con A; Sigma), followed by stimulation with 20 U/mL rhIL-2 for 7 days. After purification over Ficoll/Hypaque, blasts were re-seeded at 10^5 cells/mL with an equal number of mitomycin C-treated autologous PBMC and 2.5 μg/mL Con A for 7 days. Blasts were then separated over Ficoll/Hypaque and re-stimulated with 20 U/mL rhIL-2. Growing cells were maintained in medium containing 20 U/mL IL-2 twice weekly. Dead cells were removed from the culture by centrifugation over Ficoll/Hypaque before use in any assays.

Mixed lymphocyte assays

PBMC (4×10^6/mL) were incubated with mitomycin C-treated MMC and/or allogeneic PBMC in serial dilutions to give PBM/stimulator ratios of 1:5 to 1:160. Samples were also prepared as above, but with the addition of 2.5 μg/mL Con A or 20 U/mL rhIL-2. Cultures were incubated for 1–5 days. Samples for flow cytometry and cell cycle/apoptosis assays were cultured in 24-well (2 mL/well) plates. Samples for proliferation assays were cultured in quadruplicate in 96-well microtiter plates, and proliferation assessed by the addition of 1 μCi [^{3}H]thymidine in the last 6 h of incubation. Incorporated radiolabel was assessed in a Packard Matrix 96 counter.

Assays were also performed with the PBMC separated from the stimulator by a 0.2-μm-pore membrane (Life Technologies, Paisley, UK). Because membrane inserts were too large to use in 96-well plates, cells were grown in membranes for the desired period of time, plated in 96-well plates at the same dilution as normal assays, pulsed, and counted.

IL-2 re-stimulation assay

The ability of normal T cells to respond to IL-2 was assessed essentially as described elsewhere [17]. Briefly, PBMC (4×10^6/mL) were stimulated with 5 μg Con A for 5 days. The cells were then washed twice in complete medium followed by 24-h culture in complete medium alone. The cells were then purified over Ficoll-Hypaque, washed twice in complete medium (resulting cells were >98% CD3$^+$, results not shown), and re-stimulated in complete medium containing 20 U/mL rhIL-2 for 24 h. Proliferation was assessed by [^{3}H]dThd counting. These cultures were also performed with the addition of mitomycin C-treated MMC as above. TGF-β LAP was also added to these cultures at 80 ng/mL [an approximate ED_{75} for neutralization of the anticipated TGF-β production by the MM cell lines (see below)].

Flow cytometry

Cells (5×10^5/sample) were stained by direct immunofluorescence for two-color flow cytometry using isotype-matched controls. mAb used were as follows: anti CD3, CD4, CD8, CD25, CD69, CD95, and HLA-DR (Becton Dickinson, Oxford, UK). Data were analyzed using Cell Quest software (Becton Dickinson). The relative numbers of blasting cells in the different cultures were assessed by gating on the FSC vs. SSC parameters of the cells.

Apoptosis and cell cycle assays

Apoptosis was assessed by Annexin V-affinity assay and cells were stained according to the manufacturer's protocol (R & D Systems). Cells were washed in cold PBS and then incubated with 10 μL of fluorescein isothiocyanate (FITC)-conjugated Annexin V (An) and 10 μL of 50 μg/mL propidium iodide (PI; Sigma) solution for 15 min at 20°C. This was followed by the addition of 400 μL of binding buffer (buffered HEPES and 25 mM $CaCl_2$) without washing. Cells were analyzed within 1 h by flow cytometry revealing three cell populations: live cells (An$^-$/PI$^-$), necrotic cells (An$^+$/PI$^+$), and apoptotic cells (An$^+$/PI$^-$). PBMC were analyzed for apoptosis after 72-h culture with Con A or Con A + MMC. IDB were analyzed after 36 h culture with no cytokine; IL-2; IL-2 and U266 cells; and IL-2 and rhTGF-β1, 10 μg/mL.

For cell cycle analysis, cells were harvested, washed in cold PBS, and fixed in 2% formaldehyde at 4°C for 15 min and permeabilized in 70% ethanol at −20°C for 1 h. Cells were washed in cold PBS and incubated with RNase (100 μg/mL) at 37°C for 30 min followed by PI (50 μg/mL) in PBS at 20°C for 30 min. The DNA content of cells was determined by flow cytometry and the percentage of cells at different cell cycle stages determined using "Modfit" software (Verity Software House, Inc.).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of cytokine and cytokine receptor mRNA was detected by RT-PCR. Total RNA was isolated using the Quiagen RNEasy system according to the manufacturer's protocol. Five micrograms RNA was synthesized to cDNA using the "Superscript" system (Life Technologies) and oligo dT_{12-18} primer. Two microliters (equivalent to 0.5 μg total RNA) of cDNA were used in RT-PCR reactions (50-μL reaction volume). Primers specific for human cytokine sequences were used to amplify cDNA during a 30-cycle PCR program with the use of AGS Gold Taq polymerase (Hybaid, UK). Primers for IL-1α, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α [18], IL-1β, and TGF-β1 (Clontech Inc.) were used. Primers for CD122 (IL-2 receptor β-chain) and CD132 (common γ-chain cytokine receptor) were run in a 35-cycle PCR program [19]. β-Actin was used as an amplification and loading control. All primers were run at an annealing temperature of 60°C (except IL-6 and IL-10, run at 65°C). Twenty microliters of PCR product was visualized on a 2% agarose gel under ultraviolet illumination.

TGF-β1 enzyme-linked immunosorbent assay (ELISA)

Cell culture supernates were assayed for the presence of TGF-β1 using a Promega TGF-β1 ELISA system. MM cells (10^6) were cultured for 72 h in complete tissue culture medium. Samples were acid activated and diluted up to 1/100 in PBS to obtain results within the linear working range of the system (16–1000 pg/mL). The background levels of TGF-β1 in normal tissue culture medium were also assayed, and the results from the cell lines expressed minus the levels in normal medium.

Statistical analysis

Statistical analysis was performed using the Student's t test. Means are expressed ± SEM.

RESULTS

MMC suppress MLR responses

When PBMC from normal, healthy, volunteers were co-cultured with mitomycin C-treated allogeneic PBMC, brisk proliferation was observed at day 5 of culture (**Fig. 1**). In contrast, PBMC failed to show any proliferation in one-way MLR with the MMC U266, JIM1, and JJN3 (data not shown). When MMC were co-cultured in MLR reactions between normal allogeneic PBMC, tritiated thymidine uptake was greatly suppressed, with the degree of suppression correlated to the numbers of MMC added to the MLR (Fig. 1). MLR responses were completely suppressed at ratios of 1 MMC:5 PBMC. Full responses were only regained at the lowest concentration of MMC (1 MMC:80 PBMC). MMC therefore are capable of suppressing MLR reactions.

MMC cells suppress mitogen responses

When PBMC were activated with Con A, maximal proliferation was seen 72 h post-stimulation (data not shown). Co-culture of Con A-activated PBMC with MMC lines (U266, JIM 1, and JJN3) strongly suppressed Con A-induced proliferation at MMC/PBM ratios of 1:5 and 1:10 at 72 h (**Fig. 2**). The suppression of responses was still present after 5 days of culture (results not shown). Using a panel of normal donors, the mean inhibition of Con A-induced proliferation after co-culture with U266 was 59.7 ± 4.9% at a ratio of 1:5 (six individual donors, illustrated in **Fig. 3A**) dropping to <10% at 1:80 (results not shown). The addition of 20 U/mL rhIL-2 (the standard T cell stimulation dose, see above) did not reverse the suppression of Con A responses by U266 or JJN3 (Fig. 3B). When PBMC were separated from MM cells by a 0.2-μm pore membrane during

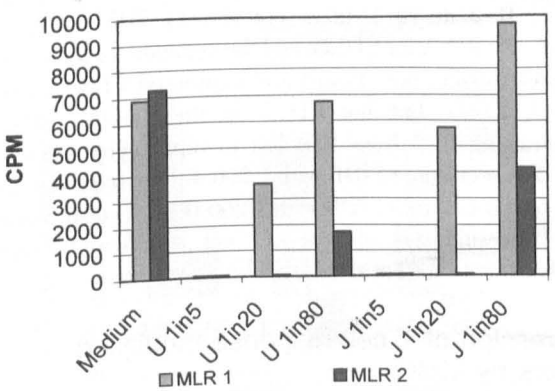

Fig. 1. Representative data of MLR responses of two different donor's PBMC against allogeneic PBMC at 5 days. MLR responses are completely inhibited by the addition of U266 and JJN3 MMC at ratio of 1 MMC:5 responder PBMC. Responses are partially restored at lowering MMC concentrations. Tritiated thymidine uptake measured as counts per second. U, U266; J, JJN3.

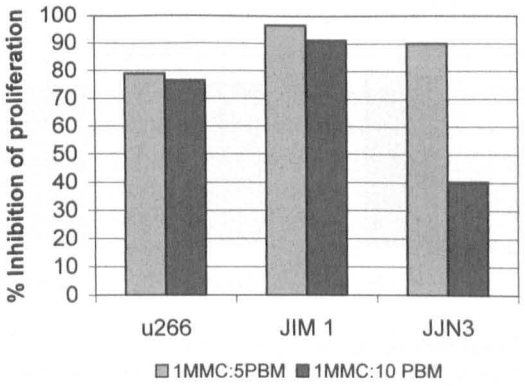

Fig. 2. Inhibition of Con A-induced thymidine uptake of normal PBMC from the same donor by different MMC at 72 h.

Con A stimulation, equal levels of inhibition to those co-cultured in contact with U266 and JJN3 cells was seen. (Fig. 3B). This indicates that inhibition of Con A-induced proliferation by MMC was mediated by soluble factors.

MMC induce cell cycle arrest rather than apoptosis

The lack of proliferation in Con A/MMC cultures was due to cell cycle arrest rather than apoptosis. The percentages of Annex-

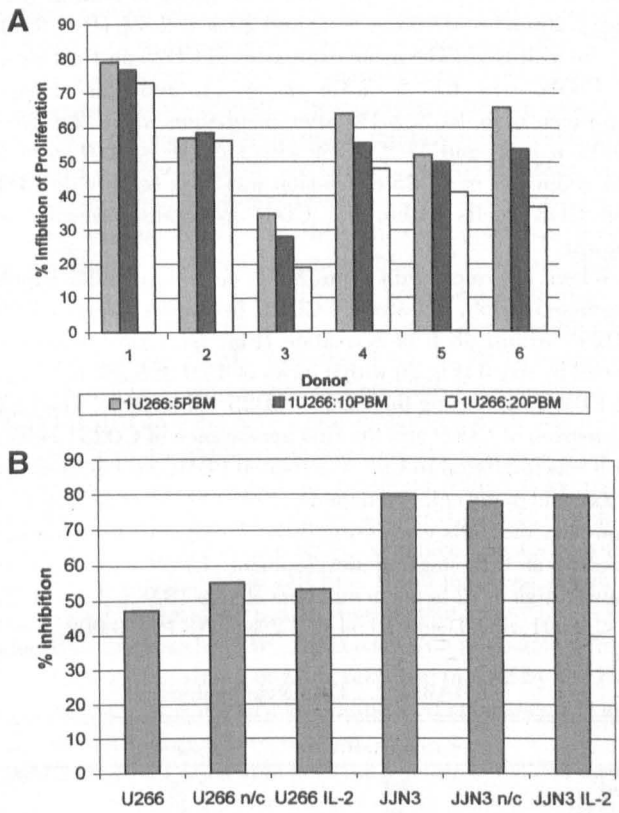

Fig. 3. (A) Inhibition of Con A-induced proliferation (thymidine uptake) of PBMC by the U266 line at different MMC/PBM ratios in six normal donors (72 h). (B) The ability of U266 or JJN3 to reduce thymidine uptake by Con A-activated PBMC is not reversed by either the addition of IL-2 or separation of MMC from the PBMC (72 h activation, single normal donor, 1 MMC:5 PBM; n/c, no contact.

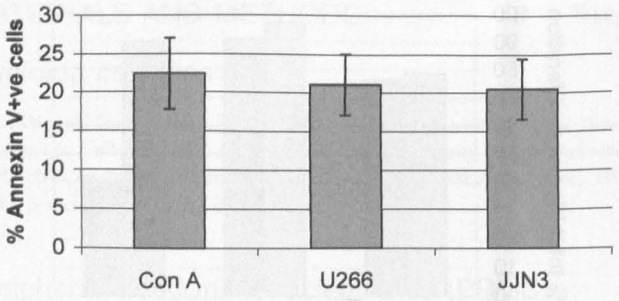

Fig. 4. The percentages of Annexin V$^+$ (AnV$^+$) cells are not significantly different between Con A-activated PBMC and Con A-activated PBMC + MMC 1:5 at 72 h. ($n = 7$).

inV$^+$ cells in Con A/MMC cultures were not significantly different from PBMC cultured with Con A alone (**Fig. 4**). Cell cycle analysis showed that, although large numbers of Con A-treated PBM had left the G0/G1 phase of the cell cycle, cells in the Con A/MMC cultures remained largely in the G0/G1 phase (**Table 1**).

MMC inhibit blasting and the expression of activation markers on T cells

Con A activation of PBMC led to an increase in the numbers of blasting cells to 56.5 ± 5.8% ($n = 7$). This was significantly inhibited by incubation with MMC, dropping to 28.2 ± 5.1% with U266 ($P < 0.005$, $n = 7$) and 20.4 ± 2.3% ($P < 0.002$, $n = 6$) with JJN3. The mean expression of CD25 post-activation of PBMC was 67 ± 5.3% ($n = 7$), and this dropped significantly to 40 ± 5.1% after incubation with U266 ($P < 0.005$, $n = 7$) and 37 ± 2.7% with JJN3 ($P < 0.01$, $n = 5$). The reduction in CD25 expression was seen equally on CD4$^+$ and CD8$^+$ cells within the CD3$^+$ population (results not shown).

When activated with Con A, T cells in PBMC rapidly expressed CD69, followed by CD25, becoming largely CD69$^+$ CD25$^+$ within 48 h of activation (**Fig. 5**). CD69 expression waned by day 3 (Fig. 5) with a mean of 45.9 ± 5.3% ($n = 7$) of all PBMC expressing the CD69$^-$/CD25$^+$ phenotype. The rapid expression of CD69 and the first appearance of CD25$^+$ cells at 24 h was unaltered in Con A-activated PBMC co-cultured with U266, and many cells remained CD69$^+$/CD25$^+$ at 48 h (Fig. 5). However, the cells expressing these T cell activation markers arrested at this stage of development. CD69 expression remained high at 72 h, mean numbers of only 18.9 ± 3.5% (U266 $P < 0.001$, $n = 7$) and 10.54 ± 2.2% (JJN3 $P < 0.002$, $n = 5$)

TABLE 1. Cell Cycle Analysis

	% Cells in G0/G1	SEM	P*
Med	82	7.55	
Con A	48	8.2	<0.02
U2	77	8.2	>0.5

Mean numbers of PBMC remaining in G0/G1 of the cell cycle after culture with medium alone, Con A, or Con A + U266 cells at a ratio of 1 MMC:5 PBMC. U2–U266.

* Compared to medium control, $n = 5$.

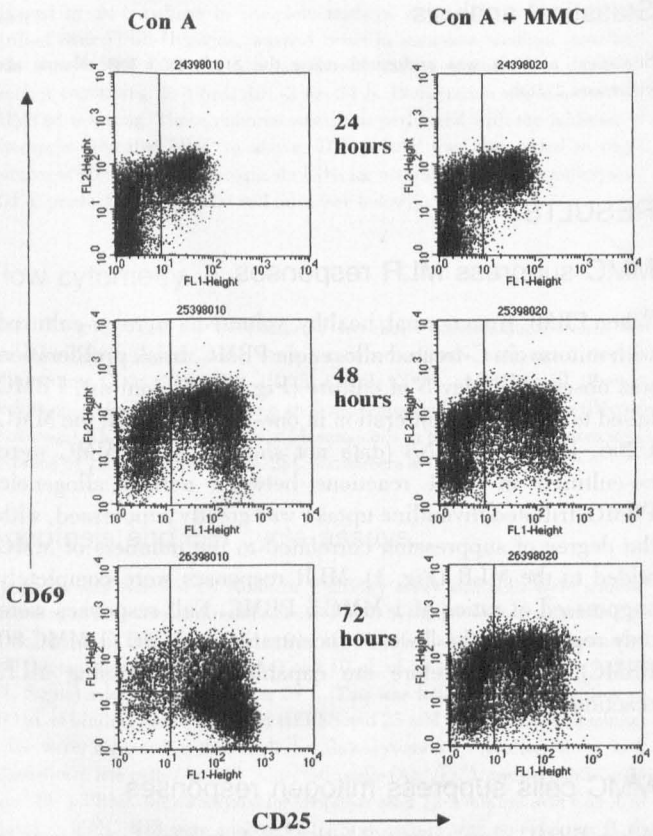

Fig. 5. The percentages of CD69$^+$/CD25$^-$ and CD69$^+$/CD25$^+$ PBMC after culture with Con A or Con A and MMC are identical at 24 h. By 48 h the Con A-treated cells are largely double positive, and more cells have become CD25$^+$ than in the Con A/MMC culture. By 72 h the vast majority of normal Con A-activated cells are CD69$^-$/CD25$^+$, whereas Con A/MMC cells remain at the CD69$^+$/CD25$^-$ or CD69$^+$/CD25$^+$ stage. CD69, *y*-axis; CD25, *x*-axis. U266 1:5. Quadrants are set on appropriate negative control samples.

of PBMC progressed to the fully activated CD69$^-$ CD25$^+$ phenotype (Fig. 5).

IL-2-dependent blasts are not inhibited by MMC or recombinant TGF-β

The IL-2-dependent blast line (IDB) showed maximal proliferation to IL-2 at 72 h (data not shown). IDB constitutively expressed mRNA for CD122 and the common γ-chain cytokine receptor (results not shown) and expressed CD3 (results not shown), CD95, but not CD25 on their surface (**Fig. 6**). Withdrawing IL-2 from IDB led to rapid apoptosis/necrosis (Fig. 6). Co-culture of IDB with U266 did not induce apoptosis (Fig. 6). Co-culture of IDB with U266 (**Fig. 7**) or recombinant TGF-β (results not shown) did not significantly suppress proliferation to IL-2 at any of the dilutions tested.

Expression of T cell response modifying factors by MMC

Strong transcripts for TGF-β1 were detected in all MMC lines assayed (**Fig. 8**). IL-6 was also detected, although the intensity of amplified products was variable (results not shown). No other cytokine transcripts were routinely detected, although very

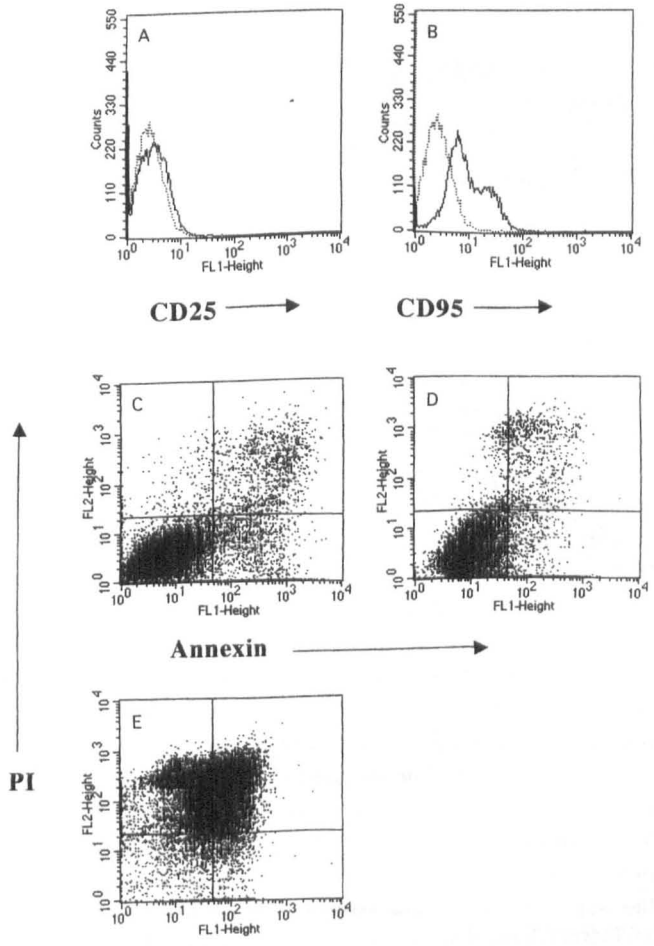

Fig. 6. (A, B) The IL-2-dependent blast cell line (IDB) does not show surface expression of CD25, but is $CD95^+$. Isotype negative control, dotted line. (C–E) Co-culture of IDB with (C) IL-2 or (D) IL-2 + U266 does not lead to measurable differences in the percentages of $Annexin^+$ (apoptotic) or $annexin/PI^+$ (apoptotic/necrotic) cells at 36 h. Withdrawing IL-2 from the cultures leads to large numbers of cells dying (E).

faint products for IL-1α, IL-2, and TNF-α were detected, especially from the U266 line (results not shown).

MMC supernates contained high net levels of total TFG-β1 protein as follows: U266, 8.62 ng/mL; JJN3, 11.02 ng/mL; JIM 1, 6.72 ng/mL.

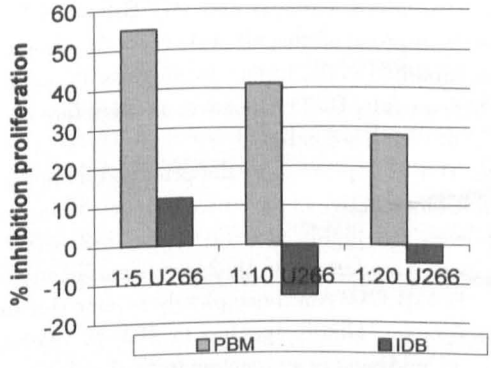

Fig. 7. Comparative inhibition of thymidine uptake in IL-2-treated IDB and Con A-activated normal PBMC by U266. Negative values indicate that proliferation was higher with tumor cells added than with IL-2 or Con A alone.

T cell IL-2 responses are inhibited by MMC and restored by LAP

T cells re-stimulated with IL-2 proliferated briskly, and this was again significantly inhibited by co-culture with MM cells (**Fig. 9A**). The ability of T cells to respond to IL-2 when co-cultured with MM cells was completely restored by the addition of TGF-β LAP (Fig. 9B).

DISCUSSION

Although there is good evidence that T cells have at least initially responded to the disease [4–8], these responses ultimately do not control MM. Failure to express costimulatory molecules by MM cells, and the induction of apoptosis in T cells by MM cells have been put forward as possible mechanisms for tumor cells to avoid or interfere with T cell responses. In this study we have shown for the first time that MM cell lines directly disrupt the activation and proliferation of T cells.

MM cells did not stimulate a primary MLR when used at the same effector/target ratios as non-transformed allogeneic cells. Thus, even with the high alloreactive CD8 precursor frequency in the periphery, MM cells do not readily induce activation of T cells. This is likely to be due to active suppression rather than a lack of immunogenicity (MM cells express HLA-ABC and HLA-DR molecules [20]) because MM cells can also fully suppress normal MLR reactions, mitogen activation, and response to IL-2 as bystanders. Because separation of the MMC from the responding T cells by a membrane did not affect the suppressive capacity of MMC, the suppressive factors appear to be soluble. The increasing levels of inhibition of T cells with increasing numbers of MMC are likely to be due to intensifying levels of suppressive soluble factor.

There was no significant difference in apoptosis levels between normal activated peripheral blood T cells and MMC-inhibited cells. This is not surprising because as activation of the T cells was blocked they should not be susceptible to activation-induced death. Although FasL or MUC-1-induced apoptosis has been put forward as a possible T cell inhibitor in MM [14, 15], it does not have a role to play in the inhibition of T cells seen here. In addition, Fas/FasL interactions may cause T cells to cell cycle arrest/fail to proliferate (rather than apoptose), but this can be overcome by adding IL-2 [21], which is not the case here. U266 also failed to induce apoptosis in the Fas^+ IL-2-dependent blast cell line. U266 has been shown to express FasL [14] and our results showing that a Fas^+ line is not induced to apoptose by MMC are at odds with previous findings [14]. However, the strong ongoing response to IL-2 by the IDB may contribute to resistance to Fas-induced apoptosis [22] and this area merits further investigation.

MMC stopped T cells from entering S-phase of the cell cycle. The failure to enter S-phase was not due to a complete lack of activation because T cells expressed similar levels of CD69 when cultured in the presence or absence of MMC. CD69 is the earliest cell surface activation Ag to be expressed after T cell activation [23]. Normally the expression of CD69 at 24 h post mitogen activation correlates with maximal thymidine incorpo-

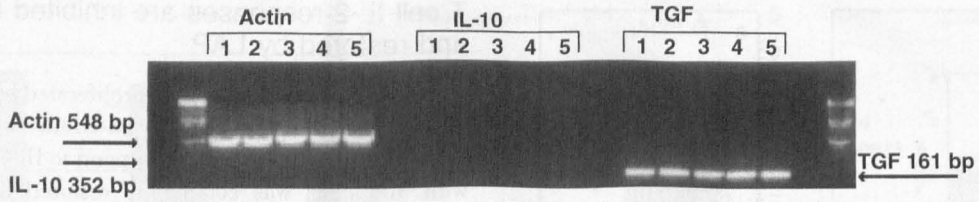

Fig. 8. RT-PCR analysis of different MMC for IL-10 and TGF-β. Strong transcripts for TGF-β1 are detected in all cell lines, whereas no product for IL-10 is found. Cell lines (reading left to right for each primer set): lane 1, JIM 1; lane 2, JJN3; lanes 3–5, U266.

ration at 72 h [24]. The exact role of CD69 in T cell activation is unclear, but it is thought to synergize with signaling through TCR/CD3 [25]. Signaling through CD69 results in the synthesis of IL-2 and its receptors and cell proliferation [26–28]. Thus the T cells that are inhibited by MMC have received the signal to proliferate and produce IL-2, evidenced by the normal expression of CD69, but have failed to progress beyond this point because they have low thymidine incorporation and reduced S-phase by DNA content.

Normal T cells in these experiments progressed through from a $CD69^+/CD25^-$ state to being largely $CD69^-/CD25^+$ by 72 h of activation. The large increase in $CD69^-/CD25^+$ cells indicates that the T cells have entered the IL-2 autocrine pathway of growth. CD69 is largely lost on these cells by 72 h, as progression into activation and proliferation pathways leads to the rapid degradation of CD69 mRNA [29]. The expression of significantly lower levels of CD25 by MMC-inhibited cells, coupled with their persistent expression of CD69 and lack of thymidine incorporation, strongly suggests that the majority of these cells have not entered the IL-2 autocrine growth pathway. The failure of MMC-inhibited T cells to respond to exogenous standard doses of IL-2 either during mitogen stimulation or re-stimulation post-activation strongly suggests disruption of the IL-2 autocrine pathway.

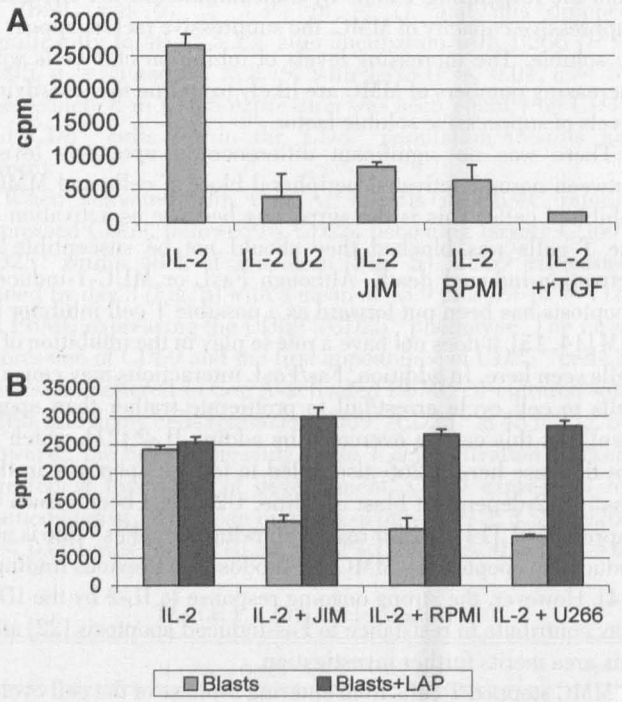

Fig. 9. (A) T cells re-stimulated with IL-2 are significantly inhibited by MMC (means of three normal donors). (B) Addition of TGF-β LAP completely reverses MMC-induced inhibition of IL-2 responses in T cells ($n = 4$). Tritiated thymidine uptake 24 h, 20 U/mL IL-2, 1 T cell:5 MMC. CPM, counts per minute. Note that LAP elevates responses slightly higher than IL-2 alone; LAP neutralizes the small amounts of active TGF-β present in FCS.

MMC or rhTGF-β failed to inhibit proliferation of the IL-2-dependent blast cell line (IDB) or induce apoptosis. IDB did not express IL-2 or CD25, but used the β and γ chains (CD122 and CD132) of the IL-2 receptor. Thus the IDB were independent of the CD25/IL-2 autocrine pathway and could only respond to exogenous IL-2. Removing IL-2 from IDB resulted in cell death. Clearly MMC or rhTGF-β (even at the $10\times$ ED_{50} dose used here [17]) did not reduce the ability of IL-2 to signal through β and γ chains because no apoptosis or loss of proliferation was seen. Because IL-2-induced-proliferation of IDB was completely unaffected by MMC, it appears that the suppression of IL-2 pathways by MMC (and similarly TGF-β) is dependent on signaling through the CD25 molecule.

The principal cytokine produced by the MMC was TGF-β1. No other cytokines, such as IL-10, that can be instrumental in suppression of T cell responses [30] were detected. The TGF-β family of cytokines is thought to play an important down-regulatory role in the control of both Th1 and Th2 T cell responses [31]. TGF-β is produced by many different types of tumor, including myeloma cell lines [16], a result confirmed here, and has been reported to be elevated in MM patients [32]. TGF-β mediates suppression of IL-2 responses in T cells [33] and prevents T cell proliferation by inhibiting the phosphorylation of Jak-1 kinase and activation of STAT 5 transcription factor [17]. These factors control the entry of the cell into the IL-2 autocrine growth pathway and TGF-β-treated T cells fail to progress to S-phase of the cell cycle [17]. The characteristics of TGF-β-inhibited cells, failure to progress through the cell cycle or become fully IL-2 responsive, are therefore identical to those of MMC-inhibited cells.

Indeed, TGF-β appears to be the principal immunosuppressive soluble factor active in our assay systems. Suppression of T cell responses was highly specific to the IL-2 pathway: pure $CD3^+$ cells re-stimulated with IL-2 were significantly inhibited by MMC. TGF-β LAP could completely reverse this inhibition of IL-2 responses. Highly specific to TGF-β, binding active cytokine and holding it in an inactive form, the ability of LAP to reverse immunosuppression strongly suggests that TGF-β is mediating the inhibition of IL-2 responses assayed here. This also indicates that the large amounts of TGF-β protein detected

in the supernates of MMC contain biologically significant levels of active cytokine: LAP is only effective against active TGF-β.

This study has shown that myeloma cells, in sufficient concentrations, can very efficiently shut off T cell responses, and that the IL-2 autocrine loop is inhibited. Low levels of tumor cells had little effect on the proliferation of T cells. High IL-2 serum concentration correlates with long-term survival of MM patients, whereas patients who progress to bulk disease show a marked decrease in IL-2 serum levels [9]. Conversely, high serum levels of immunosuppressive TGF-β1 correspond to a poor prognosis [32, 33]. When the tumor mass is reduced in conjunction with autologous BMT, infusion of donor lymphocytes leads to strong anti-myeloma responses [10]. Although patients remain disease free for some time, the vast majority of cases eventually relapse. Drawing a crude analogy to the experiments described here, a low tumor burden (represented by low MMC/PBMC ratios) is unlikely to greatly inhibit T cell activation and IL-2 production. Increased tumor numbers will ultimately lead to a profound suppression of T cell activation and IL-2 production. Thus, while low levels of tumor are present, T cells are able to expand and develop anti-tumor activity. Although these expanded T cell populations are still detectable from patients with advanced disease [5, 6], it is likely that their lack of function is due to the large-scale production of immunosuppressive cytokines. Our results show that TGF-β has a major role to play in this immunosuppression; the role of other factors such as MUC-1 and Fas/FasL has still to be fully elucidated.

ACKNOWLEDGMENTS

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