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EFFECTS OF IL-2, IL-6, IL-7 AND IFN ON THE PROLIFERATION, SURVIVAL, INDUCTION AND REDUCTION OF SPONTANEOUS IN-VITRO APOPTOSIS OF B CLL CELLS

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A dissertation submitted to the Faculty of Health Sciences, Department of Medicine, and University of the Witwatersrand in fulfilment of the study for the Masters of Science Degree (Medicine).

DECLARATION

This dissertation is my own work it is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg in the Faculty of Health Sciences and Department of Medicine. No part of this dissertation has been presented for any degree or examination at any other University.

Signature:…… Date:……..

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ETHICS COMMITTEE STATEMENT

This study is approved by Human Research Ethics Committee (Medical) of the University of Witwatersrand (Ethics Ref: R14/49 Seahloli; Protocol No: M00/05/24)

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ABSTRACT

B chronic lymphocytic leukaemia (B-CLL) is a monoclonal haematopoietic disorder with expansion of small lymphocytes of B-cells. B-CLL cells accumulate in blood, bone marrow, lymph nodes and spleen, resulting in enlargement of these organs and decreased bone marrow function. B-CLL is the most common leukaemia, with an annual incidence of 1.8 to 3.0 per 100 000 population in the United States. It is characterised by the accumulation of long-lived monoclonal CD5+ B lymphocytes. In vivo normal B-lymphocytes derive growth factors through interactions with T-cells and monocytes. In culture however, survival and growth of activated B-cells depends on the availability of external factors such as interleukins.

B-CLL cells populations are unable to survive in culture long enough to respond to the addition of growth factors. Such factors are important for the proliferation and survival of many cell types and in the absence of cytokines, these cells die as a result of apoptosis. Chronic lymphocytic leukaemia cells are influenced in vitro by a number of exogenously added cytokines that include IFN- α, IFN-γ, IL-2, IL-4, IL-10, IL-13, IL-15, TGF- β and TNF- α.

The aim of this study was to investigate the effect of cytokines e.g., IFN, IL-2, IL-6, IL7 and IL-10 on the proliferation and survival of B-CLL cells and furthermore to compare the induction and reduction of spontaneous and induced apoptosis in vitro.

Patients with B-CLL were recruited from three centres. Thirty blood samples were collected, separated using Ficoll Hypaque Gradient and purified by rosetting with AET treated SRBC. The proliferation and survival of B-CLL cells were studied in vitro in response to GM-CSF, IFN, IL-2, IL-6, IL7 and IL-10,. The survival and apoptosis of B-CLL cells in cultures with or without interleukins and other growth factors were studied under microscopic examinations and DNA agarose gel electrophoresis.

It was observed in B-CLL cells cultures that IFN and IL-2 enhanced proliferation significantly. IL6, IL-7 and GM-CSF also enhanced proliferation of B-CLL cells but not to the greater extent than IL2 and IFN. IL-10 inhibited proliferation of B-CLL cells when compared to controls. In a long-term (5-day) culture, survival of B-CLL cells was greatly enhanced by IFN and followed by IL-2. Therefore it appeared that IFN and IL-2 are the two most potent growth factors tested in this study to promote B-CLL cells proliferation and survival. The combination of these mitogens did not further enhanced proliferation. IL-6 and GM-CSF enhanced proliferation and survival of B-CLL cells. IL-7 promoted proliferation but had no effect on survival of B-CLL cells in-vitro. IL-10 enhanced apoptosis and did not promote survival of B-CLL cells in-vitro.

IFN and IL2 are survival and promoting growth factors for B-CLL cells in culture. In contrast, IL-10 has demonstrated to induce apoptotic cell death of B-CLL cells.

In conclusion B-CLL cells proliferated equally well with IFN and IL-2. IL-6, IL-7 and GM-CSF had a much lower proliferation and survival effect with noticeable antiapototic activity when compared to IFN and IL-2. IL-7 was found not to promote survival of B-CLL cells and IL-10 enhanced cell death by apoptosis.

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LIST OF ABBREVIATIONS

ABS	-	Absorbance
AET	-	2-aminoethylisothiouronium bromide
Ag	-	Antigen
APC	-	Antigen presenting cells
B-CLL	-	Chronic Lymphocytic Leukaemia
ATM	-	Ataxia telangiectasia-mutated
BCR	-	B-cell antigen receptor complex
°C	-	Degrees Celsius
CD	-	Cluster of differentiation
cmu	-	Cytoplasmic immunoglobulin mu heavy chain
D	-	Maximum difference
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic Acid
EBV	-	Epstein Barr virus
EDTA	-	Ethylenediaminetetraacetic acid
FBS	-	Foetal Bovine Serum
GM-CSF	-	Granulocyte Macrophage Colony Stimulating Factor
GM-CSFP	-	GM-CSF with Pokeweed Mitogen
HBSS	-	Hanks balanced salt solution
Hr	-	Hour
ICAM	-	Intercellular adhesion molecule
Ig	-	Immunoglobulin

IgVH	-	Immunoglobulin heavy chain variable
IFN	-	Interferon 2b alpha
IL	-	Interleukin
IL-2	-	Interleukin 2
IL-2P	-	Interleukin 2 with Pokeweed Mitogen
L-LME	-	L-Leucine methyl ester
2ME	-	2-mercaptoethanol
ml	-	millilitres
min	-	minutes
MTT	-	Dimethylthiazolyl dipheny tetrazolium bromide
NaCl	-	Sodium Chloride
NK	-	Natural killer cells
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate buffered saline
PMA	-	Phorbol ester phorbol myritic acetate
PWM	-	Pokeweed mitogen
P value	-	Probability value
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
RPMI	-	Roswell Park Memorial Institute
SDS	-	Sodium Dodecyl Sulphate
SIgs	-	Surface Immunoglobulins
SRBC	-	Sheep red blood cells
TAE	-	Tris-acetate-EDTA buffer
TBE	-	Tris-borate-EDTA buffer

TBS	-	Tris-buffered saline
TCGF	-	T cell growth factors
TE buffer	-	Tris-EDTA buffer
TGF	-	Transforming Growth Factor
TNF	-	Tumour Necrosis Factor
UV	-	Ultraviolet
V	-	Heavy chain variable region gene
VCAM	-	Vascular cell adhesion molecule
ZAP-70	-	Zeta-associated protein 70

CHAPTER 1

1 INTRODUCTION

1.1 B CHRONIC LYMPHOCYTIC LEUKEMIA

B chronic lymphocytic leukaemia (B-CLL) is a monoclonal haematopoietic disorder with expansion of small lymphocytes of B-cell lineage. B-CLL cells accumulate in blood, bone marrow, lymph nodes and spleen, resulting in enlargement of these organs and decreased bone marrow function. B-CLL is the most common leukaemia, with an annual incidence of 1.8 to 3.0 per 100,000 population in the United States. Prevalence is age related, with 5.2 per 100,000 persons between 35 and 59 years of age and 30.4 per 100,000 persons between 80 and 84 years of age. It affects twice as many men as women and is less common in Japanese and other Asian populations [1 & 2].

B-CLL is the most common leukaemia in the Western world and because patients frequently survive for many years [3]. However, 10 to 20% of CLL patients carry 11q23 chromosomal deletion and often exhibit a more severe disease course [127]. Recently it was found that patients with increased CD38 expression have poor prognostic features and have short survival [121], B-CLL is characterised by highly variable clinical course. Some patients die within a few months of diagnosis, whereas others survive prolonged periods with out requiring therapy [122]. B-CLL has become the prototypical chronic B-cell malignancy and its cellular properties have been used extensively to explain the cellular properties of normal B-cells. However, by all analyses, B-

CLL is clearly different from other B-cell tumours, and its cells are so distinctive that it is an inappropriate model for normal B-cells [3].

B-CLL is characterised by the accumulation of long-lived monoclonal CD5+ B lymphocytes that expressed high levels of Bcl-2 [4 & 5]. *In vivo* normal B-lymphocytes derive their growth factors through interactions with T-cells and monocytes. In culture however, survival and growth of activated B-cells depend on the availability of external factors such as interleukins. These factors may act independently or synergistically in enhancing B-cell proliferation. *In vitro* some B-CLL cells may require signals, which are provided by more than one cytokine in order to undergo proliferation [6, 7 & 8].

The prognostic assessment of patients with B-CLL should no longer rely exclusively on the Binet or Ria stage but include biological and genetic markers at the time of diagnosis in order to make a prediction as accurate as possible [126].

1.2 THE DISTINCTIVENESS OF B-CLL

There are three major lines of evidence for the distinctiveness of B-CLL provided by epidemiology, molecular and cellular biology. Clinical investigation also underscores the distinctiveness of B-CLL. This topic was recently reviewed by Caligaris-Cappio and Hamblin [3].

Firstly, B-CLL is the only blood malignancy, which did not show an increase in frequency in atomic bomb survivors. It is also not associated with exposure to toxic drugs and chemicals [1 and 3].

There is some evidence for a genetic susceptibility and there is no evidence for retroviral initiation of the disease. There are also families in which siblings have the disease and it appears in succeeding generations at an ever-earlier age. For B-CLL, it is very rare in the Far East and in Japanese immigrants to the United States [1]. In South Africa, the presentation of CLL, as seen in the Bloemfontein academic hospitals, is similar to what is seen world-wide [132].

Secondly, B-CLL is the perfect example of a human malignancy that primarily involves defects in the induction of programmed cell death. Cellular and molecular studies have demonstrated that the gene setting of B-CLL cells is optimally organized to avoid apoptosis. The extended cell survival is further shielded by kinetic refractoriness to exogenous stimuli such as interaction with bone marrow stoma cells, cytokine that include IL-2, IL-4, IL-10, IL-13, IL-15, TNF-α, TGF-β, IFN-α, IFN-γ and B-Cell antigen receptor complex (BCR)[9 & 10].

Thirdly, patients with B-CLL have an unusually high prevalence of autoimmune phenomena. In most cases, polyclonal auto-antibodies (auto-Abs) restricted to self-antigens (self-Ags) expressed only by blood cells cause autoimmune cytopenias [11]. Paradoxically, patients also develop progressive hypogammaglobulinaemia, which deteriorates with advancing

disease and may achieve a severity not seen in other chronic lymphoid malignancies [12]. However, it was found in a case report where Mantle Cell Lymphoma was presented with aggressive features like p53 over expression, marked leukocytosis and leukostasis [130 & 131]. This kind of severity was associated with multiple myeloma with non-hyperdiploidy, often associated with translocations other than t(11;14) and chromosome 13 deletion [138]

1.3 THE NATURE OF MALIGNANT B CELLS IN B-CLL

1.3.1 Immunophenotype

Although B-CLL cells express aberrant markers not found on normal equivalent cells, as observed in most leukaemia cells, the immunophenotype it demonstrates most closely resembles that of lymphocyte follicles. They express most of the membrane antigens present on mature B cells, but their distinctive characteristic is the co-expression of CD5 with faint to virtually undetectable amounts of monoclonal surface immunoglobulins (sIgs). These sIgs are usually sIgM or sIgD and only rarely sIgG or sIgA [3 &13].

The faint sIg/CD5 co-expression serves two purposes. Firstly, it distinguishes B-CLL from the small-cell lymphomas in leukaemia phase that mimic B-CLL [14]. Secondly, it provides a clue as to the cell of origin in B-CLL. Such low levels of sIgM are seen only in normal B-lymphocytes that have been energised by interaction with self-Ag [15]. Furthermore, normal CD5+ B cells, which are found at the edge of germinal centres in the mantle zone of lymphoid follicles, often produce poly-reaction, low-affinity, natural auto-Abs

[3, 16]. These auto-Abs are encoded by the same pieces (repertoire) of unmutated IgV genes that operate in many cases of B-CLL and share the same cross-reactive idiotypes, i.e., the 5Ipl-encoded idiotype, represented most frequently on the surface of malignant B-CLL cells [17].

These similarities have resulted in the generation of the hypothesis that B-CLL is a malignancy of a mantle zone-based subpopulation of anergic self-reactive CD5+ B cells that are devoted to the production of poly-reactive natural auto-Abs [18]. The evidence of auto-reactivity points essentially to 51pl, although other auto-specifities need to be authenticated. Nevertheless, the ability to produce these antibodies, which are retained after malignant transformation, may well provide a survival advantage for the leukaemia cells, because the interaction of monoclonal auto-reactive sIg with their self-Ag has been shown to prevent the apoptosis of the malignant cell [19].

B-CLL and Mantle Cell Lymphoma are mostly CD5 positive. CD5 expression can distinguish the B-CLL of the other chronic B-cell malignancies but not between B-CLL and mantle cell lymphoma. However, the presence of a strong expression of sIg and lack of CD23 in mantle cell lymphoma can distinguish mantle cell lymphoma and B-CLL [136 and 137].

A critical analysis of the immunophenotype of the chronic lymphocytic leukaemia (CLL) cell has two corollaries. Firstly, malignant CD5+B cells characteristically express markers such as CD23 [20] and the ability to form rosettes with mouse erythrocytes [21] and they share with their normal

counterparts the expression of myelomonocytic antigens [22]. The implications of these features are unknown. Secondly, it is not unusual to observe CLL cases with cells that are “atypical” on immunophenotypic grounds [3].

Recently there are two described major prognostic factors, which are immunoglobulin heavy chain variable region (IgVH) mutation status and cell membrane expression of CD38 [123 &125]. CD38 expression is associated with unmutated IgVH genes, the expression of CD38 may vary during the course of the disease and variation in the survival of patients presenting with a p53 mutations [124]. The development of newer prognostic factors, such as the mutational status of the IgVH genes, cytogenetics, CD38 expression, Zeta-associated protein 70 (ZAP-70) and some serum markers, has allowed for further discrimination of patients into risk categories [126, 133, 134 &135]. In CLL cells, functional impairment of the p53 pathway is detectable by western blotting. The type A defect is characterised by baseline p53 over expression and is associated with TP53 mutations. Type B defect is characterised by impaired Ionizing radiation induced p53 up-regulations and is associated with inactivation of the ataxia telangiectasia-mutated (ATM) gene. Both abnormalities are strongly associated with adverse clinical outcome [127,&128].

1.3.2 Kinetic Hyporesponsiveness

More than 99% of circulating B-CLL cells are in the G_o phase of the cell cycle [23]. It is not clear which of the cell characteristics are directly implicated in

the kinetic hyporesponsiveness and which are epiphenomena. A prominent role appears to involve abnormalities of the B-cell antigen receptor complex (BCR), because the mitogenic signals that induce the proliferation of normal B cells are weak stimuli for B-CLL cells [24].

The BCR is a multimeric complex formed by the slg and the IgA/IgB (CD79a/CD79b) heterodimer that translates the lg engagement into the biochemical signals that drive the B-cell response [25]. The extracellular domain of CD79b that is normally present on the malignant cell surface in B-cell lymphomas is absent in most B-CLL patients [26]. Recently it has been claimed that the diminished display of BCR on the membrane of B-CLL cells is due to the occurrence of somatic mutations, which are predicted to affect CD79b expression [27]. An alternative explanation is offered by the detection of a truncated form of CD79b that arises by alternative splicing of the CD79b gene and lacks exon 3, which encodes the extracellular Ig-like domain in a variety of human B cells and B-cell lines [28, 29]. This alternatively spliced variant has been detected in all B-CLL cases analysed, thereby suggesting a role for the alternative splicing of the CD79b gene in causing the reduced expression of BCR on the surface of B-CLL cells [3].

No matter which mechanism is responsible, the low levels of BCR on the membrane of B-CLL cells may account for the defective signal transduction via the BCR, which correlates with a reduced induction of protein tyrosine kinase activity [30] similar to that seen in anergic normal B cells [31]. A

defective Ca^{2+} response coupled with an altered pattern of protein tyrosine phosphorylation has also been observed in several cases [32].

Other membrane molecules amplify signalling via the BCR and have an accessory role in the signal transduction of B cells [33]. An example is CD22, which, when stimulated, potentiates the proliferation induced by anti-Ig. Although CD22 is present in the cytoplasm of B-CLL cells, it is absent from, or only weakly expressed on, the surface. Other B-cell malignancies express CD22 both on the cell surface and within the cytoplasm [34]. Another example is CD21, which is also the receptor for the Epstein Barr virus (EBV). B-CLL cells express the EBV receptor and have no defect in EBV receptor binding activity or in EBV uptake. Despite this, it is almost impossible to immortalize B-CLL cells with EBV [35]. In contrast, workers have used EBV to readily immortalize normal CD5+ and CD5- B cells in order to study their Ig gene repertoire (pieces) and antibody production more effectively [36].

1.4 CELL SURVIVAL

The concept that different cytokines may have a proliferative role in B-CLL is relatively old, but, to some extent, still controversial. It is possible that some B-CLL cells populations are unable to survive in culture long enough to respond to the growth factors. Such factors are important for the proliferation and survival of many cell types and, in the absence of cytokines, these cells die by apoptosis. Apoptosis is an active process which is important in haemopoiesis, not only in the selection of the T and B-lymphocytes, but also in the

generation of myeloid and erythroid cells. This process is also involved in carcinogenesis and is characterised by specific molecular events with identifiable morphological and biochemical changes [6].

1.5 APOPTOSIS

Apoptosis is a tightly regulated form of physiological cell death, which is dependent on the expression of cell-intrinsic suicide machinery, which lead to homeostasis [4, 37]. Prominent morphological changes include the nucleus condensing, cell shrinkage and cleavage of chromosomal DNA at internucleosomal sites, resulting in the generation of a characteristic ladder pattern of DNA fragments on electrophoresis. Blebbing of the cell surface results in the release of membrane-bound apoptotic bodies. Phosphatidylserine, which is normally located on the inner face of the plasma membrane, becomes exposed on the outer surface and provides a recognition signal for engulfment by phagocytes. Thus, apoptosis results in the rapid and efficient removal of superfluous or damaged cells [37].

1.6 CYTOKINES

For such an apparently anergic cell, it is surprising that the B-CLL cell produces (or at least displays the mRNA for) virtually all cytokines listed below. Interleukin (IL) 1 alpha, IL-1β, IL-6, IL-7, IL-8, IL-10, IL-13, interferon-gamma (IFN-γ), tumour necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor beta 1 (TGF- β1) have all been detected [38, 39]. Their role in the natural history of

B-CLL is still unclear, even if some are responsible for negative autocrine circuits. Although a role for IL-10 has been advocated, there is still conflicting data on its precise role [40, 41]. TGF-β [42] and IFN-γ [43] are frequently detectable at high levels in patients' serum. As a potent inhibitor of lymphocyte proliferation, TGF-β has been proposed as an endogenous growth inhibitor that might account for the slow progression of malignant growth [44]. TGF-β can also induce apoptosis in several cell systems, including human B cells [45]. Interestingly, CLL B cells are resistant to the apoptotic effects of TGF-β, which variably inhibits CLL B-cell proliferation without affecting apoptosis [46]. IFN-γ promotes the survival of leukaemic cells by preventing apoptosis [43].

Other cytokines, including TNF-α [44], soluble CD23 [20] and IL-8 [47] have been proposed as autocrine growth promoting candidates. However, no cytokine is unequivocally and consistently able to force the G_o blockade of B-CLL cells [3]. Cytokines may also derive from, and participate in, a reciprocal dialogue between B-CLL cells and the interacting T cells. Chronic lymphocytic leukaemia cells are influenced *in vitro* by a number of exogenously added cytokines including IL-2, IL-4, IL-10, IL-13, IL-15, TNF-α, TGF-β, IFN-α and IFN-γ. IL-4 may be related to the increased expression of IL-4 receptors by CLL B cells as compared to normal B cells [48].

Spontaneous apoptosis in culture of B-CLL cells is probably triggered by the absence of survival factors that are present in vivo. Candidate survival factors that prevent apoptosis of B-CLL cells in vitro are: IL-4, IFN, IL-2, IL-6, IL-8 and

IL-13 while IL-10 and IL-5 induce apoptosis in these cells [4]. The B-CLL/T cell discussion is highlighted by the observation that B-CLL cells and T cells share a number of functionally relevant molecules and their counter receptors. CD5 is associated with the BCR, is co-expressed with its natural ligand CD72 and is also present on the membrane of T cells. Likewise, the T cell-associated molecule CD27, a member of the TNF receptor family, is co-expressed with its natural ligand CD70 on the membrane of malignant B-CLL cells [49].

1.6.1 Interleukin 2

Interleukin 2 (IL-2) is a glycoprotein of relative molecular mass (Mr) 15000. It is released by T lymphocytes on stimulation by antigen or mitogen and functions as a T cell growth factor (TCGF) by inducing the proliferation of activated T cells [50]. This lymphokine was first identified by its ability to promote the long term *in vitro* proliferation of activated T cells. It also promotes the generation and proliferation of cytotoxic T cells, natural killer cells and lymphokine activated killer cells [51]. IL-2 was reported to inhibit *in vitro* apoptosis in a large percentage of B-CLL patients and, in another study, B-CLL cells seemed to survive better in the presence of IL-2 [6, 52].

Cells from B-CLL and mantle cell lymphoma (MCL) rarely express the CD95 antigen. In culture without IL-2, Bosanquet et al [53] determined that CD95 levels in B-lymphocytes from CLL and MCL could be upregulated by using clinically relevant doses of IL-2 CD95 was expressed on dying CD5+ B-lymphocytes but only on live cells in 2 out of 15 cases. In live cells from two B-CLL specimens, IL-2 caused up-regulation of CD95 and was associated with

ex vivo drug resistance. Clinically relevant doses of IL-2 had pleiotropic effects on CD95 levels in fresh CLL cells. The link between CD95-induction and *ex vivo* drug resistance may point to a clinically resistant subset of CLL patients [53].

1.6.2 Interleukin 6

Human Interleukin 6 (IL-6) is a growth factor for murine and human B-cells and it has a relative molecular mass of 21000. IL-6 induces the terminal maturation of activated B-cells into antibody producing cells. Combination of IL-6 and the soluble IL-6 receptor promote liver cell hyperplasia *in vivo*. IL-6 modulates the hepatic expression of acute-phase response genes during inflammation. Schirmacher et al [54] suggested that after hepatectomy and acute liver failure, there might be a significant production of IL-6 and the soluble IL-6 receptor from different cells in the remaining liver which could be the intrinsic mechanism of the organ to initiate liver regeneration [54]. The hepatic acute phase response is induced by IL-6. It has been shown that IL-6 and IL-3 act synergistically to support the proliferation of haematopoietic stem cells, however, IL-6 does not have antiviral activity [55]. IL-6 was originally identified as a B-cell differentiation factor [56] and is a pleiotropic cytokine which functions, not only in the immune response, but also in inflammation and haematopoiesis [56, 57].

IL-6 has been reported to induce proliferation of B-CLL cells and to enhance survival of these cells through inhibition of spontaneous apoptosis [58]. A report by Bussing *et al* [58] did not support theoretical objections of B-CLL

stimulation via induction of IL-6 in vitro [58]. It has been reported that endothelial cells inhibit apoptosis of B-cells and that human recombinant IL-6 dimer inhibits B-CLL apoptosis, whereas human recombinant IL-6 monomers do not [59]. Binding and functional competition between IL-6 dimers and monomers had similar affinity for the IL-6 receptors and also IL-6 monomers inhibited the anti-apoptotic activity of IL-6 dimers. It was suggested that IL-6 dimers derived from endothelial cells promote the survival of B-CLL cells [59].

1.6.3 Interleukin 7

IL-7 is a stromal cell derived cytokine which, to date, is the only cytokine on which the development of B- and T-cell precursors depends. It is essential for the differentiation of gamma delta T-cell receptors. Additionally, IL-7 can function as a co-factor during myelopoiesis and is also capable of activating monocytes/macrophages and natural killer cells (NK). Its receptor is a heterodimer of an alpha chain that specifically binds IL-7 and the common gamma chain that is also a component of the receptor for IL-2, IL-4, IL-9 and IL-15 [60].

Functions of IL-7 in normal lymphocyte development and activation have led to the demonstration of the ability of IL-7 to stimulate lymphopoiesis in lymphopenic mice, suggesting a possible clinical application for IL-7 in accelerating lymphoid reconstitution in lymphopenic patients [60]. IL-7 and its receptor complex are essential for the following:-

(i) Up-regulation of the expression of TdT and IL-7 Receptor alpha

(ii) Initiating the production of cytoplasmic immunoglobulin mu heavy chain (cmu).

(iii) Promoting the formation of a functional pre-B cell Ag (antigen) receptor in/on pro-B-cells.

These key events, in turn, appear to be a prerequisite both for differentiation of pro-B cells to pre-B cells and for proliferation of these cell subsets upon continued stimulation with IL-7 [61]. IL-7 is involved in protecting lymphoid progenitors from a death process that resembles apoptosis. This protection is partly mediated by IL-7 induction of Bcl-2 [62].

IL-7 induces proliferation of relatively mature tumour cells and this effect is not restricted to cells of lymphoid lineage [63]. In T-CLL cells, the IL-7 induced proliferation is not inhibited by antibodies to IL-2 receptors or the anti-IL- antibody and, therefore, IL-7 induces the proliferation of peripheral CD8+ T cells, as well as its pathological counterpart [64]

1.6.4 Interleukin 10

IL-10 is an immunoregulatory cytokine produced by TH-2 helper cells, B-cells as well as monocytes and macrophages**.** Because it is a potent inhibitor of cytokine production by Th-1 helper T cells, it was originally designated cytokine synthesis inhibitory factor [65]. It is also known to promote proliferation and differentiation of normal human B cells and is a survival factor for Epstein Barr virus infected B cells [65, 66, 67]. B cells express the IL-10 receptor the functionality of which is demonstrated by activation of STAT 1 and STAT3 proteins. Binding of IL-10 to its receptors on B-CLL diminishes

hydrocortisone (HC) induced apoptosis and provides cell survival factors for B-CLL cells [66]. On the other hand, IL-10 mRNA expression in leukaemic cells from patients with B-CLL is strongly associated with low progressive disease [68].

IL-10 has recently been found to inhibit CLL B-cell function *in vitro* by inducing apoptosis and down regulating expression of Bcl-2. Tangye *et al* [25] examined the effect of IL-10 on proliferation, RNA synthesis, IgM secretion and viability of leukaemic CD5+ B-cells induced by activation with phorbol myristate acetate (PMA) alone, or in combination with anti-Ig. IL-10 reduced PMA and PMA/anti Ig induced proliferation and RNA synthesis. Although proliferation and RNA synthesis induced by PMA/anti Ig could be enhanced by the addition of IL-2, IL-4, IL-13, IFN-gamma or TNF-alpha, the presence of these cytokines failed to abrogate the IL-10 mediated inhibition of leukaemic CD5+ B-cell activation. In contrast to the effects on proliferation and RNA synthesis, IL-10 did not inhibit IgM secretion and had only a minimal effect on the viability of activated cells [69].

It appears that IL-10 inhibits proliferation of leukaemic CD5+ B-cells by a mechanism distinct from the induction of apoptosis and may have therapeutic benefits in the treatment of B-CLL by preventing expansion of the malignant clone [69]. IL-10 has been reported to increase *in vitro* apoptotic cell numbers in stage 0 patients but not in stage I and II and, on the other hand, that it inhibits proliferation of leukaemia CD5+ B cells by mechanisms distinct from the induction of apoptosis [52, 69]. Fluckiger et al [40] found that IL-10 inhibits

the DNA synthesis and survival of B-CLL cell. The distinctiveness was on inhibiting apoptosis [40], may be through inhibition of DNA synthesis.

1.7 OTHER GROWTH FACTORS

1.7.1 Interferon alpha 2b

Interferons are a complex group of naturally occurring proteins produced by eukaryotic cells in response to various stimuli. These proteins have pleiotropic biological activity, among which are anti-proliferative, immunomodulatory, antiviral and differentiation-inducing effects [1].

IFN-α and IFN-β are acid stable interferons; they bind to the same receptor and are produced primarily by leukocytes and fibroblasts respectively [1]. Interferon alpha 2b (IFN-α2b) protects B-CLL cells from apoptotic death and promotes survival of leukaemic cells by preventing loss of expression of BCL-2 *in vitro* [37, 70]. IFN-α2b was used because it was sponsored by Schering-Plough.

1.7.2 Granulocyte macrophage colony-stimulating factor

Granulocyte macrophage colony-stimulating factor (GM-CSF) is one of a family of glycoproteins that have a potent effect on stimulating the proliferation and function of haematopoietic cells [71].

GM-CSF is produced by activated T-lymphocytes, B-lymphocytes, endothelial cells, mast cells, fibroblasts, macrophages, mesothelial cells and osteoblasts

in response to specific activating agents. The role of GM-CSF in B-cell physiology is unclear. Although B cells can respond to GM-CSF, there is controversy concerning the extent to which various resting and activated B-cell types can themselves produce these cytokines and this cytokine therefore function in an autocrine fashion. In combination with other known rescue factors, autocrine produced GM-CSF may contribute to normal and malignant B-cell survival *in vivo*. GM-CSF protects murine myeloid leukaemia cell lines from apoptotic death induced by cytotoxic drugs and has been shown to support the growth of B-CLL leukaemic cells *in vitro* [71, 72]. GM-CSF was used because it was available and shown to support growth of B-CLL [71,72 & 139].

Table: 1.7.1. Effects of cytokines and other growth factors on B-CLL cells

Cytokine	Proliferation	Survival	Apoptosis
Interleukin 2	YES	YES	NO
Interleukin 6	YES	YES	NO
Interleukin 7	YES	NO	NO
Interleukin 10	NO	NO	YES
Interferon Alpha 2b	YES	YES	NO
GM-CSF	YES	YES	NO

1.8 CYTOGENETICS AND MOLECULAR ABNORMALITIES

B-CLL is regarded as the cytogenetic Cinderella of blood malignancies for two reasons: firstly, the spontaneous mitotic rate of B-CLL cells is extremely low, secondly, the standard mitogen used in the 1960s and 1970s was phytohaemagglutinin, a T-cell, but not a B-cell, stimulator. The use of B-cell activators such as lipopolysaccharide, EBV and, especially, 12-0-tetradecanoulphorbal-13-acetate has improved the cytogenetic definition of B-CLL [73, 74]. Fluorescent *in situ* hybridisation and molecular studies progressively facilitated the detection of genetic abnormalities and even recognized microdeletions in morphologically normal chromosomes [10].

Gradually, information is accumulating on the molecular effects of the chromosomal abnormalities [3], which provided a better understanding of B-CLL cytogenetics. An abnormal karyotype is observed in approximately 50% of patients [75 &76]. However, Gozzetti et al used fluorescence in situ

hybridisation (FISH) was able to detect chromosomal abnormalities of 85% in patient with B-CLL [140].Chromosomal analysis provides prognosis information for overall survival in addition to that supplied by clinical data in patients with B-CLL cell [75]. When a normal karyotype is present, metaphase may derive from either T cells or the clonal B-cell population itself. The most frequent abnormalities are deletions or translocations of chromosome arm 13q14, trisomy 12, deletions of p53 at 17p13.3 and deletions at chromosome arms 11q23 and 6q. Approximately half the patients with an abnormal karyotype have a single chromosomal abnormality, one quarter has two abnormalities and the remainder have a complex pattern [75, 76].

Deletions or translocations at 13q are the most common abnormalities in CLL. There is no consensus on the suggestion that the breakpoint cluster region A2 (BCRA2) deletion at 13q12 is involved [77]. However, a detailed molecular delineation of a region of 13q14.3 telomeric to RB-1, which is frequently deleted, has been published [78]. With the help of molecular tools, a minimally deleted region of no more than 10 kilobases has been recognized and, from within this region, two candidate tumour suppressor genes, Leu1 and Leu2, have been cloned [79]. These genes show little sequence homology to any of those previously published genes [3].

Patients with 13q14 abnormalities characteristically have a benign disease that usually manifests as an isolated, stable or only slowly progressive lymphocytosis. The disease is monoclonal but bears none of the other

hallmarks of a malignancy. In particular, these patients survive as long as their age-and sex-matched controls [80].

In contrast, trisomy 12 is associated with progressive disease and atypical cellular morphology [81, 82]. The biological importance of trisomy 12 is still poorly understood, although it has been suggested that one or more genes may have been duplicated to lead to a more malignant phenotype [3].
A surprising difference between CLL cells with trisomy 12 and those with 13q14 deletions, is the finding that trisomy 12 is predominantly associated with unmutated VH genes, whereas CLLs with 13q14 deletions show heavy somatic mutation [83]. It is unclear whether this points to two different diseases, one deriving from a pre-germinal centre cell and the other from a postgerminal centre cell, or to a cell so disordered by genetic damage as to be unable to differentiate further [3].

Deletions and translocations at 11q fall into two distinct types. Although some cases of apparent CLL with translocations at 11q13 involving the Bcl-1 gene have been reported [84], most workers believe these to be a variant of mantle cell lymphoma [85]. Deletions at 11q23 are found in up to 1% of CLLs. Patients with these deletions are reported to have B-CLL with extensive lymph node involvement and require an aggressive clinical treatment course [86].

Mutations or deletions of p53 at 17p13.3 have been reported in up to 15% of patients with CLL p53 aberrations. They are particularly associated with atypical cellular morphology (characteristically with more than 10%

prolymphocytes), advanced disease, a high proliferation rate and shortened survival [87], i.e. the development of a high-grade lymphoma (Richter's transformation) from within the clone [89].

In contrast to follicular lymphoma, the t(14;18) translocation is exceedingly rare [90]. Nevertheless, the Bcl-2 gene product is consistently over-expressed in B-CLL [91]. The mechanism involved in this over-expression is unclear, but it may depend on hypomethylation of the gene, which would lead to increased transcription [92], or it may be under the influence of apoptosis-protecting cytokines [3,5]. *In vitro* data indicate that IL-4 [93], IFN- α and IFN-γ [43] inhibition of CLL cell apoptosis is accompanied by preservation or even up-regulation of the Bcl-2 protein [3]. IL-10 leads to Bcl-2 down-regulation and bax up-regulation high Bcl-2 to bax ratios were found to protect against apoptosis [4 & 94].

Bcl-2 belongs to a rapidly expanding family of genes that have an interrelated role in the control of apoptosis [95]. Bax codes for a protein that is the partner for the Bcl-2 protein [96] and counters its repression of apoptosis; Bcl-xL synergises with Bcl-2, whereas Bcl-xS inhibits Bcl-2 function. B-CLL cells express high levels of Bcl-2, Bcl-xL, and Bax, whereas Bcl-xS is present in low to trace amounts in a smaller proportion of cases. Thus, the pattern of expression of Bcl-2 family genes in B-CLL cells favours the suppression of apoptosis. In the same vein, B-CLL cells do not express c-myc, a potent inducer of apoptosis [3, 5, 94 & 97].

Bcl-3 translocations t(14;19) (q32.3;q13.2), are even rarer than Bcl-2 translocations. In 50% of cases, they are found in association with trisomy 12. Patients are frequently younger than 40 years old and the disease is often rapidly progressive [3]. The Bcl-3 gene encodes a I kappa B-like protein that modulates the activity of NF-kappa B transcription factors [98].

1.9 MALIGNANT B-CELL / MICRO-ENVIRONMENT INTERACTIONS

Several observations indicate that the microenvironment might influence the natural history of B-CLL [3 & 72]. In vitro data demonstrates that cytokines produced by B-CLL cells modulate the environment in which they accumulate [48, 94 &141]. A striking predominance of CD4+ helper T cells is observed in involved bone marrow (BM) and lymph nodes and reflects a redistribution of T-cell subsets reminiscent of that seen in immunoregulatory disorders such as rheumatoid arthritis and sarcoidosis [99]. In vitro data indicates that the behaviour of B-CLL cells is influenced by T cell-produced cytokines. The CD4+ T cells are intrinsically normal, but their pattern of cytokine secretion is modulated by accessory cells. The tumour cells themselves act as the major accessory cells in B-CLL [100]. The co-expression of CD27 and CD70 on malignant B-cells may modify their accessory cell function and influence their ability to activate T cells [49]. Finally, it is reasonable to suppose that binding of B-CLL cells to stromal cells and extracellular matrices may account for the in vivo patterns of lymphoid infiltration and thus influence clinical presentation [3 & 141].

The migration and traffic pattern of lymphocytes is determined by the presence on the cell surface of adhesion molecules. The transmembrane heterodimers known as integrins play a central role. Integrins bind to specific ligands and have a cytoplasmic domain connected with the microfilament bundles of the cytoskeleton [101]. B-CLL cells have an unusual cytoskeletal organization that, among lympohemopoeitic cells, is shared only by cells of the monocyte-macrophage lineage [102]. They consistently express the β1 (CD29) β2 (CD18) integrins together with variable amounts of α3 (CD49c), α4 (CD49d) and α5 (CD49e). Leukocyte function-associated antigen 1(CD) 1a/CD18) and α4/ α7 are variably expressed [103]. The leukocyte function-associated antigen 1 ligands and intercellular adhesion molecules (ICAM)-1 (CD54), ICAM-2 (CD102) and ICAM-3 (CD50), are also present on the surface of CLL cells [69].

Interleukin-4 enhances homotypic adhesion of activated CLL cells by selectively up-regulating ICAM-1. The L-selection (CD62L) and the “homing receptor” CD44 have been detected in a high proportion of cases. Chronic lymphocytic leukaemia cells bind weakly to the surface of non-stimulated endothelium [103]. However, the stimulation of endothelium markedly increases the expression of vascular cell adhesion molecule 1 (VCAM-1). In this setting, the endothelial binding of CLL cells with VCAM-1 ligand, α4/β1, is enhanced [103]. The expression of α4/β1 may also allow the interaction of CLL cells with VCAM-1-expressing sites in the bone marrow and secondary lymphoid organs [104].

In vitro, malignant CLL cells have been shown to interact with bone marrow stromal cells via β1 and β2 integrins . This binding rescues CLL cells from apoptosis [105] and extends their life span. These data has suggested a potential mechanism to explain the *in vivo* accumulation and survival of malignant cells in the bone marrow. They may also account for the discrepant behaviour of cells that have a prolonged half-life *in vivo* but die rapidly of apoptosis *in vitro*. It should also be noted that in the early nodular phase of bone marrow involvement, B-CLL cells are closely associated with follicular dendritic cells, which are absent from normal bone marrow (BM) [106]. According to Caligaris-Cappio et al [3], the relationship between B-CLL and follicular dendritic cells warrants further exploration [3].

1.10 IMMUNE INCOMPETENCE AND AUTO-IMMUNITY

Immune incompetence is a cardinal feature of B-CLL. It is characterized by a progressive profound hypogammaglobulinaemia that eventually develops in all patients as well as by an impaired cell-mediated immunity to recall antigens. B-cell chronic lymphocytic leukaemia cells secrete TGF-β, which is a potent inhibitor of B-cell proliferation [42], and release high levels of circulating IL-2 thus down-regulating T helper function [107]. Furthermore, B-CLL cells (as do anergic normal B cells) fail to present soluble antigen and alloantigen whereas normal, activated B cells are very effective antigen-presenting cells (APCs) [108,109 &110]. The inability of B-CLL cells (and of anergic normal B cells) to properly act as APCs is explained, at least in part,

by the low levels of sIg and the sub-optimal expression of the co-stimulatory molecules CD80 and CD86 [110].

The defective expression of CD79 may be an additional element. It should also be considered that CLL CD4+ T cells express CD40 ligand (CD154) mRNA but fail to express the molecule on the cell surface after CD3 ligation [111]. CD40+ leukaemic cells have been shown to down-modulate CD154 on the surface of normal, donor-activated CD4+ T cells, provided that the ratio between CD4+ T cells and Leukemic B cells declines below a critical level [111]. In a disease characterized by an excess of CD40 leukaemic cells, such a receptor-mediated down-modulation of CD154 could interfere with antigen-specific cognate interaction. The conclusion drawn from all the above data is that the accumulating malignant B-cell population *per se* is obstructing the production of normal antibodies and this may lead to progressive immuno-incompetence [3].

CHAPTER 2

2 OBJECTIVES

The aim of this study was in two folds. Firstly, to investigate the effect of cytokines IL-2, IL-6, IL-7, IL-10 and growth factor IFN on the proliferation and survival of B-CLL cells. Secondly, to compare the effects of these cytokines on B-CLL cell induction and reduction of spontaneous and cytokine induced apoptosis *in vitro*.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 ISOLATION OF MONOCLONAL CELLS

Blood samples (40ml) were drawn from 30 B-CLL patients from the Chris Hani Baragwanath Hospital Haematology Clinic, ward 495 of the Johannesburg Hospital Division of Clinical Haematology and Medical Oncology and the Medical Oncology Centre of Rosebank over a period of 3 years. Monoclonal cells (MNC) from heparinised peripheral blood (PB) samples were transferred to 50ml centrifuge tubes. An equal volume of phosphate buffered saline (PBS) (Highveld Biologicals, South Africa) was added and layered on 3ml Ficoll Hypaque (Bio-Whittaker, Maryland, USA) for every 10ml of PB and PBS.

Ficoll Hypaque with PB and PBS was centrifuged in a refrigerated centrifuge (Sorvall Instruments, RC-3B) at room temperature for 30 minutes at 2000rpm. The centrifuge was allowed to slow down without braking. The plasma was removed and MNC transferred into a clean tube. MNC were washed in a three times volume of Hanks Balanced Salt Solution (HBSS) (Highveld Biologicals, South Africa) and then centrifuged for 10min at 1300rpm. The supernatant was removed and MNC were re-suspended in HBSS. MNC were manually counted and the viability was checked by the Trypan Blue Dye Exclusion test.

If MNC viability was more than 90% and these cells were taken for monocyte and macrophages depletion. Monocytes and macrophages were depleted from MNC using L-leucin methods as described below (Section 3.2).

3.2 DEPLETION OF MONOCYTES / MACROPHAGES FROM MONONUCLEAR CELLS USING L-LEUCIN

MNC were incubated in 1mM L-leucin methyl ether (L-L.M.E) (Sigma, USA) filter sterilised by 0.22µm Millex filter unit (Millipore, Bedford) in a complete serum-free Roswell Park Memorial Institute solution (RPMI) (GIBCO BRL Life Technologies, Paisley, Scotland) at a concentration of $5X10^6$ cell/ml for 40min at room temperature.

Cells were washed twice with HBSS and suspended in RPMI-10 (10% foetal calf serum in Roswell Park Memorial Institute Solution). Cells were checked microscopically for contamination.

When no contamination with mononuclear cells found lymphocytes were further separated by rosetting with AET treated SRBC.

3.3 ROSETTING WITH AET TREATED SRBC

Fresh sheep red blood cells (SRBC) (NHLS, Laboratory reagent services, South African Institute for Medical Research, South Africa) were centrifuged at 1300rpm and the 8ml 2-amino-ethyl-isothiouronium bromide (AET) (Sigma, USA) was added into 2ml packed sheep red blood cells. The mixture was incubated for 20min in a 37^0C water-bath, and then filled with cold PBS to the

top of the 50ml centrifuge tube and centrifuged for 10min at 1300 rpm at 18-20^{0}C. The supernatant was removed and cells were re-suspended in PBS. The cells were washed twice with PBS. Forty-eight millilitres (48ml) of RPMI-10 was added into 2ml of AET-SRSC and stored at 4^{0}C for not more than 3 days.

To separate T and B cells the resetting method with AET treated sheep red blood was used. The cells were rosetted twice with AET treated sheep red blood cells to get B cells (CD3 or CD14 positive cell were detected). Two millilitres (ml) of foetal calf serum (FCS) (Delta Bio products, South Africa) and 4ml of AET-SRBC were added to 1ml of PBMC and centrifuged for 5min at 800rpm at 4^{0}C. The solution was then incubated in ice for 1hr. Ficoll (~3ml) was placed in a centrifuge tube for every seven mls of PBMC/FCS/SRBC at ratio of 1:2:4. The mixture was gently re-suspended by tilting the centrifuge tube to re-suspend the pellet solid layer over Ficoll and was then centrifuged for a further 35min at 2000rpm at 4^{0}C. Approximately three quarters (3/4) of the upper layer (culture medium) was removed and the intermediate layer removed and transferred into a clean centrifuge tube. The tube was filled with Hanks Balanced Salt Solution (HBSS) and centrifuged for 10min at 1300rpm at 20^{0}C. The washing was repeated and the B-CLL cells re-suspended in HbSS. T-cells bound to SRBC were discarded.

3.4 PROLIFERATION ASSAY

B-CLL cells were prepared at a concentration of 2X10^6/ml and were cultured with or without pokeweed mitogen (PWM) (Sigma UK) at a concentration of

8µg/ml on microtitre plates in a total volume of 0.2ml RPMI 1640 culture medium supplemented with 0.3g/ml L-glutamine (Glaxo, Greenfold, UK), 2-mercaptoethanol ($5X10^6$/ml Sigma), 10% FCS and penicillin streptomycin (100U/ml & 100ug/ml) (Sigma, USA) or Gentamycin 20 to 50 µg/ml (Schering Plough, ZA). Recombinant interleukins (ILs) (Boehringer Mannheim, Germany) were added at a concentration of 100ug/ml, interferon 2b alpha (Schering-Plough ZA) at 5 MIU/ml and granulocyte-macrophage-stimulating factor (GM-CSF) at 100ug/ml.

Three wells containing 0.1ml of cells were filled with 0.1ml RPMI to make total of 0.2ml per well. These cells were treated with different interleukins (IL-2, IL-6, IL7, IL10 (Schering-Plough, New Jersey, USA), IFN2b alpha, GM-CSF & Pokeweed mitogen) and these growth factors were used as a single agent and also in combination. The cells were cultured in triplicate at 37^0C for 3 to 5 days in a humidified atmosphere of 5% CO_2. Then 20µl of 5mg/ml Dimethylthiazolyl-diphenyl-tetrazolium-bromide (MTT) (Sigma, USA) was added in 200µl of cells and the cells were incubated for further 1 to 4 hours before a solubiliser (dimethyl sulfoxide DMSO) (Sigma USA) was added and then incubated again for one hour or overnight. The absorbance was measured at 570nm using a Titrek Multiskan MC microtitre plate reader and the results were expressed as median counts/culture [6].

The combinations of cytokines and growth factors used are presented in table 3.4.1. below.

Table: 3.4.1. Cytokines, growth factors and combinations used in this study.

Cytokines	Growth Factors	Combinations of cytokine and growth factors used		
IL-2	PWM	IL-2 + PWM	IL-2 + IL7	IL-2 +IL-10
IL-6	GM-CSF	IL-6 + PWM	IL-2 + IL-6	IL-6 + IL-10
IL-7	IFN	IL-7 + PWM	INF + PWM	IL-7 +IL-10
IL-10		IL-10 + PWM	GM-CSF+PWM	IL-6 + IL-7

These combinations were taken at random to test for the proliferation effect since no combinations had been tested before except in case of IL-10 where it was compared to IL-2, IL-4 and IFN [40].

Background (i.e. medium) was subtracted from the results. The absorbance reading of the culture medium was subtracted from all absorbance readings of the cells and controls tested. The results with background are presented in appendix I.

These results were expressed in medians and standard deviation was calculated and recorded in table: 4.1.1.

3.5 EFFECT OF CYTOKINES ON CELL SURVIVAL

Twenty six (26) blood samples of the 30 blood samples collected were used for survival tests and the other four samples the volume drawn was not enough to be used for the survival tests.

B-CLL cells were prepared at a concentration of 1 to 2 X 10^{6} cells/ml and cultured either at 4°C (control) and 37°C (experimental) for five days with and without cytokines. These cells were treated with different cytokines (IL-2, IL-6, IL7, IL10, IFN, GM-CSF & PWM).

After day 1, day 3 and day 5, two drops of cells were taken from B-CLL cells cultured with cytokines and growth factors. Slides of B-CLL cells were prepared for the staining and counting of cells. These slides of B-CLL cells were stained with 5µg/ml Acridine Orange (Sigma, USA) for five minutes and the stained slides of B-CLL cells were counted using flourescent microscopy.

The total number of stained B-CLL cells and B-CLL cells with visibly condensed chromatin (apoptotic) were counted. At least 400 cells were counted from different randomly selected fields and the results are resented in appendix ii and means results in table: 4.2.1.

B-CLL survival was expressed in percentages. This was calculated from number of apoptotic cells versus the number of viable cells.

The survival score was presented as percentage, depending on the survival factors observed under the microscope and calculated. Cells with less condensed chromatin were expressed with higher percentage score than cells with more condensed chromatin. The results are presented in table: 4.2.1. All scores were recorded and the median of all patients for a particular cytokine or growth factor tested were recorded and presented in figure: 4.2.1.

Formula used to calculate the % survival of B-CLL cells

$$= \frac{\text{Total No of survived cells}}{\text{Total No of cells counted}} \times 100$$

The ANOVA procedure and Bonferroni (Dunn) t Test was used to compare the difference in survival between day 1 and day 5 on treatment with different mitogens comparing day1 versus day 5. Furthermore, comparing difference in survival between day1, day3 and day5 between the mitogens tested in B-CLL cells culture.

3.6 DNA EXTRACTION AND AGAROSE GEL ELECTROPHORESIS

Cells treated with IL-2, IL-6, IL-7, IL-10, IFN, and GM-CSF with or without pokeweed mitogen and cells without cytokines from the culture and the controls (cells stored at 4ºC) were transferred into 10ml centrifuge tubes, washed with PBS, and centrifuged at 2300rpm at 4°C. The supernatant was discarded and 3ml of Tris-EDTA buffer (20mM Tris; 5mM EDTA, pH 8.0 (T20E5)) (Sigma, USA), 200µl 10% sodium dodecyl sulphate (SDS) (Sigma, USA) and 500µl of 2mg/ml proteinase K (Promega, Madison, WI USA) was added and mixed in each centrifuge tube containing cells. The mixtures were incubated at 42-50°C in a water-bath overnight. Approximately 1ml of saturated sodium chloride (NaCl) (SAARCHEM, ZA) was added to each tube, mixed well and placed on ice for 5min.

The tubes were then centrifuged at 2300rpm for 30min and the supernatant decanted into a clean centrifuge tube. Twenty ml of absolute ethanol (Merck Lab Supplies, ZA) was added to the supernatant at room temperature. DNA was usually observed as fine white particles in the mixture. If the DNA threads were not visible, the sample was stored at -20°C overnight or at -70°C for 30min and then re-centrifuged. All DNA samples were centrifuged at 2300rpm for 30min and the supernatant was discarded.

Ice cold 70% ethanol was added up to the 10ml mark and the samples centrifuged for a further 30min at 2300rpm. The supernatant was decanted

carefully and the DNA pellet left to dry at room temperature. The DNA pellet was dissolved in 1ml Tris-borate EDTA (TBE) buffer (Sigma, USA) and placed in the incubator at 37°C for 1 to 2 hours to ensure complete dissolution.

The DNA was then analysed on 1.2% agarose gels (BDH Chemicals, England) with a 1Kb DNA marker (Promega, Madison, WI USA) Electrophoresis was performed in TBE buffer containing 50µl 10mg/ml ethidium bromide per litre using a Midi cell Primo EC330 electrophoretic gel system (Sigma USA) at a voltage of 100 volts. The gels were photographed under ultraviolet illumination.

3.7 STATISTICAL ANALYSIS

The optical density data generated by the B-CLL proliferation assay did not meet the assumptions of normality or of equal variances required for parametric statistical analysis. The Kolmogorov-Smirnov two-sample test, a nonparametric analogue of the Student two-sample t-test, was therefore used to compare the optical densities produced by culture of B-CLL cells in control conditions and with different cytokines and growth factors. ANOVA procedure, Bonferroni (Dunn) t test and student t test was used to analyse the survival of B-CLL cells at Day 5 treated with different mitogens and to present the significance of these differences.

CHAPTER 4

4 RESULTS

4.1 PROLIFERATION ASSAY

The effect of adding cytokines to the cultures of B-CLL cells is shown in Table 4.1.1 and summarized in figure 4.1.1. The results are expressed in terms of absorbance at 570nm, which increases with increased growth.

Table: 4.1.1: Proliferation of B-CLL cells in response to cytokines and growth factors, added either individually and in combination. Growth of B-CLL cells is expressed as absorbance of the culture at 570nm (see Methods 3.4 section) Medians and 95% Confidence Intervals (CI) for each combination are shown at the bottom of each column

	Control	PWM	GMCSF	GMCSF +P	IFN	IFN +P	IL2	IL2 +P	IL2 +6	IL2 +7	IL2 +10	IL6	IL6 +P	IL6 +7	IL6 +10	IL7	IL7 +P	IL7 +10	IL10	IL10 +P
PT 001	0.370	0.709	0.687	0.703	1.069	0.727	0.726	0.742	0.854	0.657	0.508	0.793	0.780	0.678	0.518	0.723	0.677	0.535	0.494	0.448
PT 002	0.038	0.131	0.086	0.078	0.181	0.068	0.131	0.117	0.129	0.050	0.028	0.131	0.099	0.149	0.096	0.082	0.072	0.033	0.019	0.015
PT 003	0.195	0.203	0.303	0.226	0.440	0.230	0.246	0.223	0.226	0.255	0.233	0.303	0.208	0.279	0.221	0.233	0.156	0.137	0.070	0.144
PT 004	0.193	0.291	0.217	0.181	0.257	0.310	0.290	0.320	0.185	0.259	0.153	0.213	0.277	0.197	0.209	0.261	0.361	0.065	0.126	0.215
PT 005	0.391	0.423	0.494	0.503	0.661	0.708	0.618	0.725	0.576	0.504	0.652	0.475	0.772	0.462	0.235	0.556	0.562	0.339	0.260	0.354
PT 006	0.429	0.449	0.298	0.486	0.599	0.599	0.472	0.602	0.429	0.472	0.394	0.595	0.676	0.521	0.397	0.472	0.56	0.404	0.526	0.509
PT 007	0.557	0.475	0.636	0.436	0.700	0.500	0.607	0.541	0.523	0.580	0.593	0.613	0.571	0.615	0.458	0.505	0.519	0.462	0.357	0.484
PT 008	0.542	0.484	0.686	0.534	0.742	0.616	0.664	0.589	0.579	0.544	0.556	0.708	0.607	0.647	0.545	0.638	0.591	0.564	0.580	0.525
PT 009	0.444	0.600	0.637	0.486	0.675	0.612	0.636	0.577	0.543	0.524	0.561	0.511	0.518	0.602	0.436	0.529	0.494	0.403	0.317	0.454
PT 010	0.470	0.468	0.495	0.493	0.604	0.555	0.601	0.608	0.533	0.551	0.452	0.467	0.561	0.526	0.419	0.469	0.522	0.357	0.373	0.419
PT 011	0.241	0.223	0.123	0.152	0.268	0.265	0.235	0.260	0.277	0.210	0.233	0.238	0.238	0.276	0.189	0.203	0.267	0.148	0.128	0.205
PT 012	0.145	0.257	0.354	0.345	0.385	0.273	0.407	0.336	0.298	0.340	0.235	0.341	0.251	0.382	0.192	0.284	0.219	0.155	0.079	0.173
PT 013	0.139	0.135	0.235	0.182	0.247	0.254	0.275	0.193	0.190	0.167	0.114	0.187	0.105	0.205	0.070	0.191	0.145	0.056	0.091	0.081
PT 014	0.042	0.161	0.162	0.135	0.206	0.201	0.205	0.189	0.177	0.208	0.047	0.137	0.125	0.170	0.043	0.113	0.151	0.043	0.025	0.033
PT 015	0.043	0.132	0.198	0.198	0.255	0.266	0.244	0.284	0.177	0.134	0.101	0.147	0.187	0.172	0.030	0.117	0.187	0.014	0.035	0.017
PT 016	0.046	0.117	0.114	0.164	0.282	0.269	0.222	0.286	0.179	0.218	0.130	0.104	0.228	0.121	0.048	0.123	0.184	0.085	0.051	0.023
PT 017	0.069	0.099	0.187	0.185	0.150	0.203	0.185	0.218	0.000	0.193	0.067	0.145	0.193	0.169	0.063	0.082	0.139	0.077	0.050	0.065
PT 018	0.121	0.176	0.152	0.168	0.211	0.187	0.190	0.260	0.152	0.204	0.133	0.113	0.119	0.173	0.120	0.132	0.205	0.095	0.095	0.126
PT 019	0.275	0.377	0.378	0.308	0.397	0.561	0.553	0.545	0.337	0.357	0.210	0.506	0.473	0.331	0.227	0.423	0.375	0.235	0.182	0.277
PT 020	0.365	0.426	0.332	0.369	0.503	0.630	0.397	0.660	0.381	0.426	0.118	0.346	0.560	0.337	0.320	0.418	0.408	0.302	0.254	0.359
PT 021	0.118	0.191	0.130	0.130	0.221	0.198	0.356	0.267	0.193	0.314	0.110	0.271	0.207	0.236	0.196	0.191	0.109	0.192	0.085	0.102
PT 022	0.181	0.373	0.347	0.212	0.499	0.293	0.320	0.372	0.323	0.353	0.178	0.284	0.305	0.487	0.260	0.320	0.294	0.269	0.146	0.220
PT 023	0.122	0.370	0.369	0.372	0.454	0.397	0.581	0.490	0.344	0.552	0.246	0.510	0.413	0.367	0.345	0.446	0.265	0.228	0.061	0.072
PT 024	0.314	0.553	0.501	0.624	0.755	0.688	0.620	0.891	0.556	0.645	0.454	0.520	0.730	0.576	0.214	0.548	0.516	0.330	0.355	0.335
PT 025	0.113	0.213	0.329	0.320	0.467	0.301	0.468	0.400	0.263	0.376	0.236	0.149	0.259	0.250	0.299	0.163	0.187	0.136	0.032	0.065
PT 026	0.118	0.200	0.217	0.216	0.251	0.246	0.203	0.284	0.231	0.230	0.223	0.134	0.139	0.260	0.083	0.141	0.194	0.104	0.056	0.206
PT 027	0.125	0.162	0.193	0.189	0.318	0.361	0.305	0.359	0.246	0.202	0.125	0.204	0.272	0.178	0.189	0.309	0.250	0.091	0.158	0.155
PT 028	0.042	0.221	0.171	0.237	0.345	0.419	0.358	0.502	0.272	0.341	0.104	0.205	0.239	0.202	0.103	0.213	0.312	0.113	0.017	0.029
PT 029	0.034	0.069	0.091	0.139	0.149	0.124	0.084	0.244	0.092	0.127	0.042	0.052	0.210	0.088	0.095	0.108	0.167	0.103	0.023	0.031
PT 030	0.063	0.262	0.296	0.195	0.305	0.335	0.434	0.293	0.209	0.255	0.139	0.191	0.292	0.294	0.205	0.250	0.185	0.170	0.068	0.132
Median	**0.142**	**0.240**	**0.297**	**0.221**	**0.365**	**0.306**	**0.357**	**0.348**	**0.268**	**0.327**	**0.194**	**0.254**	**0.266**	**0.278**	**0.207**	**0.256**	**0.258**	**0.151**	**0.093**	**0.164**
95% CI	0.039	0.107	0.101	0.132	0.164	0.152	0.155	0.191	0.108	0.130	0.044	0.108	0.111	0.133	0.045	0.094	0.122	0.037	0.021	0.019
	0.509	0.579	0.664	0.584	0.749	0.699	0.651	0.734	0.578	0.616	0.578	0.665	0.753	0.632	0.491	0.601	0.578	0.502	0.511	0.498

Figure: 4.1.1: Proliferation of B-CLL cells in response to cytokines and growth factors, added either individually or in combination. Growth of B-CLL cells is expressed as Means absorbance of the culture at 570nm (see Methods 3.4 section and Table 4.1.1)

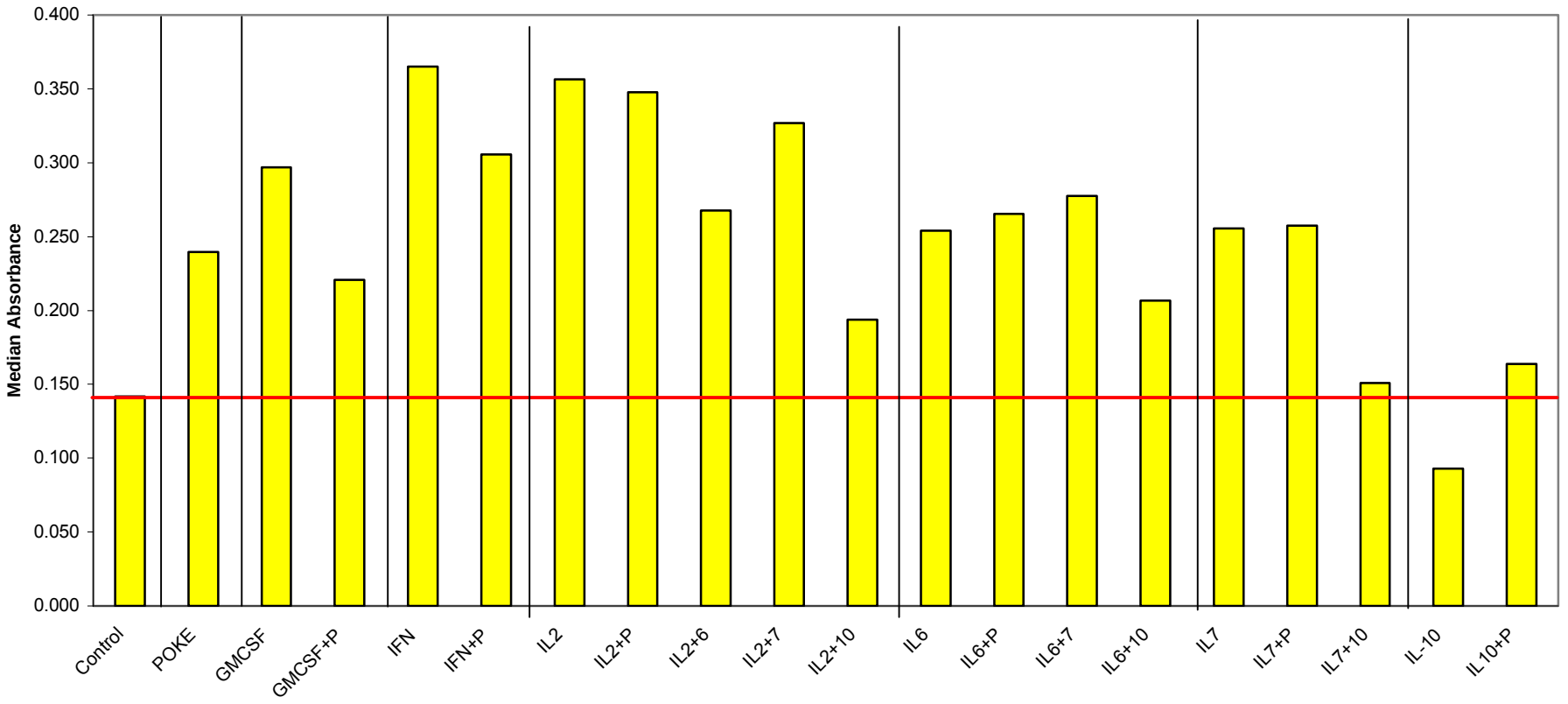


Table No: 4.1.2: Kolmogorov-Smirnov Two Sample test results of B-CLL, Maximum differences between groups.

	Control	POKE	GMCSF	GMCSF+P	IFN	IFN+P	IL2	IL2+P	IL2+6	IL2+7	IL2+10	IL6	IL6+P	IL6+7	IL6+10	IL7	IL7+P	IL7+10	IL10
Control	0.000																		
POKE	0.367	0.000																	
GMCSF	0.367	0.133	0.000																
GMCSF+P	0.433	0.133	0.133	0.000															
IFN	0.533	0.300	0.267	0.367	0.000														
IFN+P	0.533	0.300	0.233	0.333	0.100	0.000													
IL2	0.500	0.267	0.233	0.300	0.133	0.100	0.000												
IL2+P	0.533	0.367	0.333	0.400	0.133	0.133	0.133	0.000											
IL2+6	0.433	0.167	0.133	0.133	0.267	0.233	0.233	0.267	0.000										
IL2+7	0.467	0.200	0.200	0.267	0.200	0.200	0.200	0.270	0.167	0.000									
IL2+10	0.133	0.267	0.267	0.333	0.533	0.500	0.433	0.533	0.333	0.367	0.000								
IL6	0.367	0.170	0.133	0.167	0.130	0.270	0.267	0.367	0.200	0.230	0.267	0.000							
IL6+P	0.400	0.200	0.167	0.200	0.230	0.200	0.200	0.300	0.167	0.170	0.333	0.200	0.000						
IL6+7	0.467	0.170	0.133	0.167	0.270	0.200	0.200	0.267	0.100	0.130	0.367	0.200	0.130	0.000					
IL6+10	0.233	0.230	0.233	0.300	0.470	0.430	0.400	0.500	0.267	0.300	0.167	0.270	0.300	0.300	0.000				
IL7	0.267	0.100	0.133	0.167	0.300	0.270	0.233	0.333	0.167	0.230	0.267	0.100	0.200	0.200	0.230	0.000			
IL7+P	0.433	0.167	0.133	0.133	0.333	0.300	0.267	0.333	0.100	0.230	0.333	0.170	0.200	0.130	0.270	0.170	0.000		
IL7+10	0.100	0.330	0.333	0.367	0.500	0.500	0.500	0.533	0.433	0.470	0.200	0.330	0.400	0.430	0.230	0.300	0.400	0.000	
IL10	0.267	0.500	0.467	0.567	0.622	0.633	0.633	0.667	0.533	0.567	0.400	0.500	0.533	0.567	0.367	0.467	0.533	0.267	0.000
IL10+P	0.189	0.300	0.322	0.367	0.500	0.467	0.467	0.533	0.400	0.400	0.200	0.333	0.367	0.400	0.200	0.322	0.367	0.133	0.200

Table No: 4.1.3: Kolmogorov-Smirnov two-sided probability results for B-CLL cells proliferation assay in response to cytokines and growth factors. The significant ones are in bold.

	Control	PWM	GMCSF	GMCSF+P	IFN	IFN+P	IL2	IL2+P	IL2+6	IL2+7	IL2+10	IL6	IL6+P	IL6+7	IL6+10	IL7	IL7+P	IL7+10	IL10
Control	0																		
POKE	**0.026**	0																	
GMCSF	**0.026**	0.921	0																
GMCSF+P	**0.004**	0.921	0.921	0															
IFN	**0.000**	0.108	0.197	**0.026**	0														
IFN+P	**0.000**	0.108	0.334	0.055	0.995	0													
IL2	**0.001**	0.197	0.334	0.108	0.921	0.995	0												
IL2+P	**0.000**	**0.026**	0.055	**0.011**	0.921	0.921	0.921	0											
IL2+6	**0.004**	0.740	0.921	0.921	0.197	0.334	0.334	0.197	0										
IL2+7	**0.002**	0.522	0.522	0.197	0.522	0.552	0.522	0.197	0.740	0									
IL2+10	0.921	0.197	0.197	0.055	**0.000**	**0.001**	**0.004**	**0.000**	0.055	**0.026**	0								
IL6	**0.026**	0.740	0.921	0.740	0.055	0.197	0.197	**0.026**	0.522	0.334	0.197	0							
IL6+P	**0.011**	0.522	0.740	0.522	0.334	0.522	0.522	0.108	0.740	0.740	0.055	0.522	0						
IL6+7	**0.002**	0.740	0.921	0.740	0.197	0.522	0.522	0.197	0.995	0.921	**0.026**	0.522	0.921	0					
IL6+10	0.334	0.334	0.334	0.108	**0.002**	**0.004**	**0.011**	**0.001**	0.197	0.108	0.740	0.197	0.108	0.108	0				
IL7	0.197	0.995	0.921	0.740	0.108	0.197	0.334	0.055	0.740	0.334	0.197	0.995	0.522	0.522	0.334	0			
IL7+P	**0.004**	0.740	0.921	0.921	0.055	0.108	0.197	0.055	0.995	0.334	0.055	0.740	0.522	0.921	0.197	0.740	0		
IL7+10	0.995	0.055	0.055	**0.026**	**0.001**	**0.001**	**0.001**	**0.000**	**0.004**	**0.002**	0.522	0.055	**0.011**	**0.004**	0.108	0.108	0.011	0	
IL10	0.197	**0.001**	**0.004**	**0.000**	**0.000**	**0.000**	**2.32E-06**	**0.000**	**0.000**	**0.000**	**0.011**	**0.001**	**0.000**	**0.000**	**0.026**	**0.002**	**0.000**	0.197	0
IL10+P	0.593	0.108	0.069	**0.026**	**0.001**	**0.002**	**0.002**	**0.000**	**0.011**	**0.011**	0.522	0.055	0.026	**0.011**	0.522	0.069	**0.026**	0.921	0.522

The addition of GM-CSF, IL-2, IL-6, IL-7, INF and PWM individually to cultures of B-CLL cells all showed significant increase in proliferation when compared with negative control cultures (Figure 4.1.1; Tables 4.1.2 and 4.1.3). The greatest effect was shown by INF ($d=0.533$; $p<0.001$) and IL-2 ($d=0.500$; $p>0.01$) while PWM and GM-CSF (positive controls) produced the smallest increase ($d=0.367$; $p<0.05$). Proliferation in the presence of IL-10 was less than that seen in negative control cultures but the difference was not significant ($d=0.267$; $p>0.1$). The difference between the effect of IL-10 and the other individual factors tested was striking (IL-2: $d=0.633$ $p<0.001$; PWM: $d=0.500$ $p< 0.001$).

Incubation with a combination of cytokines produced some interesting results. Although incubation with IL-2, IL-6 or IL-7, each on their own, increased proliferation significantly, in combination the effect was not enhanced further. In fact, growth appeared to be less although the differences were not significant (Table 4.1.3; Figure 4.1.1). However, where IL-10 was included in the combination of cytokines, proliferation was suppressed, although this only reached significance with the combination of IL-2 and IL-10 ($d=0.433$; $p<0.01$). In none of the cultures in which IL-10 was included was proliferation better than that seen in control cultures ($d<0.24$; $p>0.1$). With the exception of IL-7 ($d=0.197$;$p>0.1$), proliferation with IL-10 in combination with other cytokines was still statistically greater than with IL-10 alone ($d>0.37$; $p<0.05$). Similarly, the addition poke weed mitogen (PWM) did not enhance proliferation and,

although the levels were lower than when the individual cytokines were used, they were not significantly different ($d<0.2$; $p>0.1$).

In summary, with the exception of IL-10, all of the factors tested increased proliferation when compared to control cultures. IL-10, however, failed to enhance growth and, in combination with the stimulatory factors, appeared to inhibit their proliferative effect. This was particularly striking when IL-10 was added to IL-2.

4.2 CELL SURVIVAL

In this study B-CLL survival was expressed as a percentage calculated from number of apoptotic cells versus viable cells. Total numbers of 400 cells were counted and percentage survival was calculated from number of survived cells divided by total number of cells counted and multiplied by 100. A table of the results is presented in appendix II. B-cells cultured at 37°C without cytokines were considered as negative controls and B-cells cultures at 4°C without cytokines and those cultured with GM-CSF were considered as positive controls. The ANOVA and Bonferroni (Dunn) t Test statistical procedure was used to compare the survival results.

In a four to five day liquid culture, B-CLL cell morphology was observed under the fluorescent microscope. The results of these experiments are presented in table 4.2.1, figure 4.2.1 and appendix II and III. B-CLL cells treated with IL-2

were analysed and a number of morphological changes were observed. At Day 1 B-CLL cells showed survival rate of 72%. At Day 3 the survival rate decreased to 62% and morphological apoptotic changes increased. At Day 5, the results were slightly less than at day 3 with 58% survival. At day 5, survival of cells in IL-2 cultures was significantly better than IL-6, positive controls (GM-CSF and PWM), IL-7, controls (negative) and IL-10 (Bonferroni t $p<0.05$) but was significantly less than with INF (Bonferroni t, $p <0.05$) A similar trend was seen at days1 and 3. (Figure 4.2.1 and appendix III)

B-CLL cells were also experimentally treated using IL-6, with 59% survival rate at day 1 and 50% at day 5. Compared to other mitogens at day 5 survival of cells cultured with IL-6 was similar to GM-CSF (positive control) but was significantly greater than PWM (positive control), IL-7, controls (negative) and IL-10 and less than INF and IL-2 (Bonferroni t, $p <0.05$). A similar trend was seen at days1 and 3. (Figure 4.2.1 and appendix III)

IL-7 treated B-CLL cells, at Day 1 had survival rate of 48%, which dropped to 40% at Day 3 and 37% at Day 5. The survival at day 5 compared to other B-CLL cells treated with other mitogens was similar to control and PWM but was significantly better than IL-10 (Bonferroni t, $p < 0.05$). When comparing the survival of IL-7 was significantly less than IFN, IL-2, IL-6 and GM-CSF.

B-CLL cells cultured with IFN appeared to survive better than any other mitogens tested in this study. At Day 1, B-CLL cell survival is 81% and dropped to 73% at Day 3. At Day 5, survival dropped slightly to 67%, which

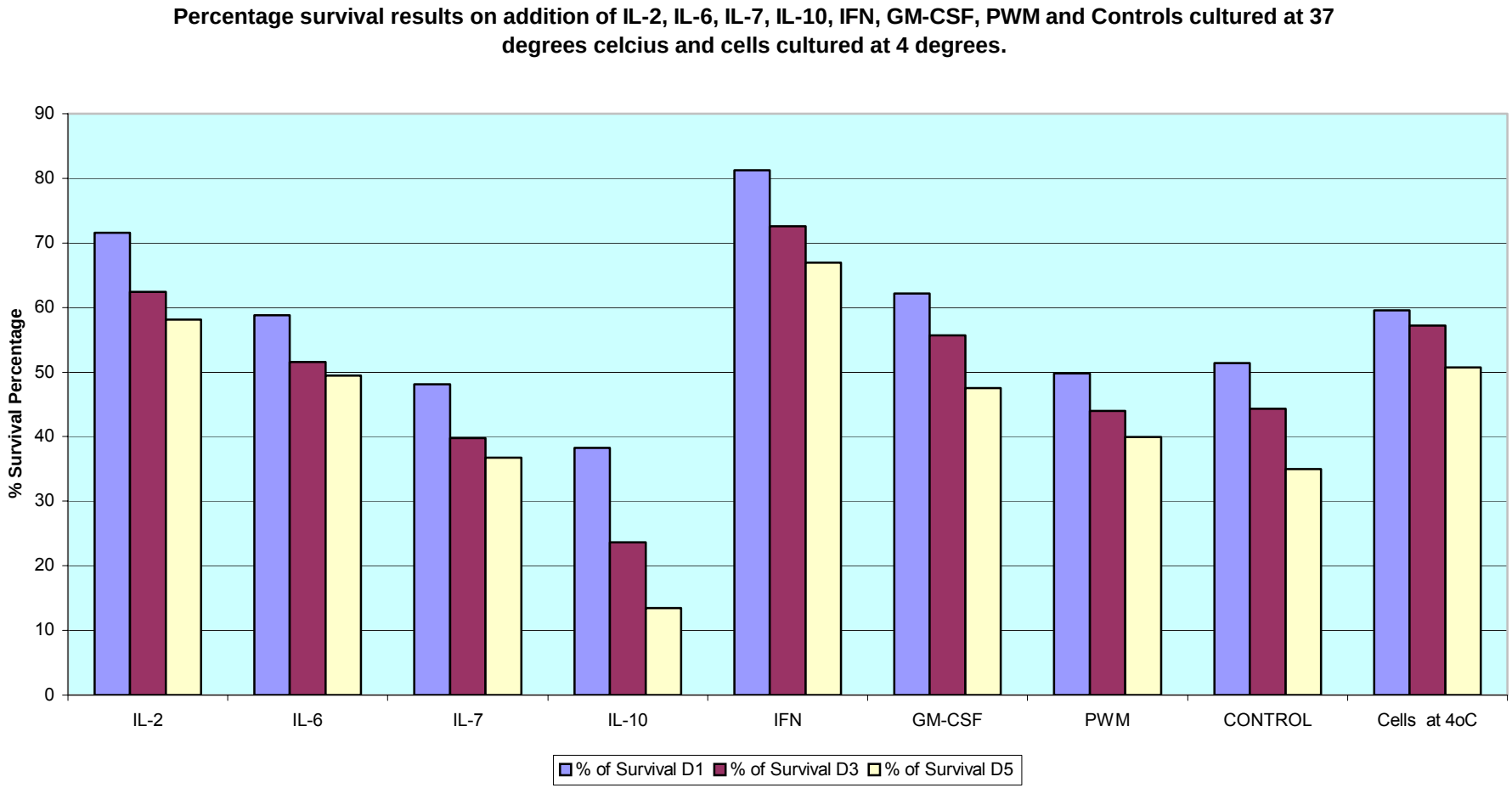
was found to have the highest survival at Day 5 compared to all mitogens and controls (Bonferroni t, $p < 0.05$).

In this study morphological apoptotic changes were more prominent in IL-10 treated cells than all other growth factors and cytokines examined. As demonstrated in figure 4.2.1., a significant decrease of viable cell numbers was usually detected after Day 3 and Day 5. IL-10 treated B-CLL cells results from Day 1, there were significant number of apoptotic changes in the cells counted with a survival rate of 38% at day 1, 24% at Day 3 and 14% Day 5. . The survival was significantly less than all mitogens and controls tested (Bonferroni t, $p < 0.05$). A similar trend was seen at days1 and 3. (Figure 4.2.1 and appendix III)

Table 4.2.1: B-CLL Cell Survival results based on the morphological changes. Percentage survival results on addition of IL-2, IL-6, IL-7, IL-10, IFN, GM-CSF, PWM and Controls cultured at 37 degrees Celsius and cells cultured at 4 degrees.

	Number of S cells			Number of A cells			Total			% of Survival		
	D1	D3	D5	D1	D3	D5	D1	D3	D5	D1	D3	D5
IL-2	286	250	233	114	150	167	400	400	400	72	62	58
IL-6	235	206	198	165	194	202	400	400	400	59	52	50
IL-7	192	159	147	208	241	253	400	400	400	48	40	37
IL-10	153	95	54	247	305	346	400	400	400	38	24	14
IFN	325	290	268	75	110	132	400	400	400	81	73	67
GM-CSF	249	223	190	151	177	210	400	400	400	62	56	47
PWM	199	176	160	201	224	240	400	400	400	50	44	40
CONTROL	206	177	140	194	223	260	400	400	400	51	44	35
Cells at 4°C	238	229	203	162	171	197	400	400	400	60	57	51

Figure: 4.2.1: B-CLL Cell Survival expressed in percentage



4.3 GEL ELECTROPHORESIS

The results of DNA fragmentation in B-CLL cells shown by agarose gel electrophoresis are presented in figure: 4.3.1. In these experiments, untreated B-CLL cells cultured at 37ºC were used as positive controls and untreated B-CLL cells cultured at 4ºC were used as negative controls. Experiments were done on IL-2, IL-6, IL-7, IL-10, GMCSF and IFN. All these cells were cultured for 4 - 5 days before extraction of DNA took place.

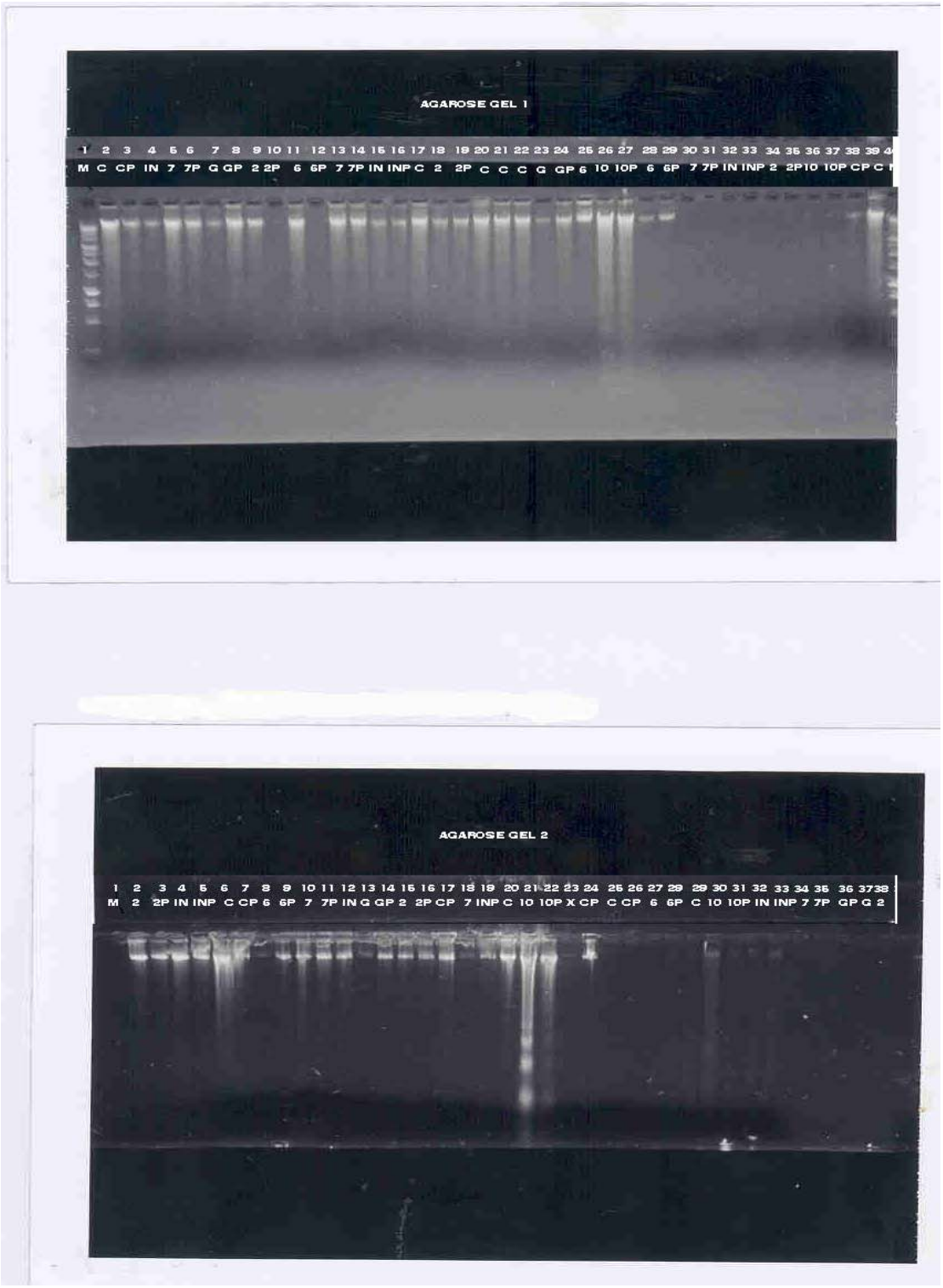
DNA analysis by agarose gel electrophoresis revealed that DNA of the B-CLL cells treated with IL-2, IL-6, IL-7, IFN and GM-CSF did not show a typical ladder pattern of internucleosomal DNA cleavage and degradation of DNA into fragments. Internucleosomal DNA cleavage and degradation of DNA was not observed in the negative controls.

Cell necrosis was noted in these experiments. This made the results difficult to analyse as the typical DNA ladder pattern of apoptosis could have been hidden by necrosis. The only typical ladder patterns observed were with the B-CLL cells treated with IL-10 and positive controls (B-CLL cells without cytokines or growth factors). The ladder pattern of the positive control was not as prominent as that of IL-10. B-CLL cells stored at 4ºC (positive controls) did not show a typical ladder pattern. The results of DNA fragmentation in B-CLL cells shown by agarose gel electrophoresis are presented in figure: 4.3.1. In these experiments, untreated B-CLL cells cultured at 37ºC were used as

positive controls and untreated B-CLL cells cultured at 4ºC were used as negative controls.

Figure No 4.3.1: DNA Assay and Gel electrophoresis of B-CLL cells treated with different interleukins and growth factors

IL-2 = 2, IL-6=6, IL-7=7, IL-10 = 10, Interferon alpha 2b=IN, GM-CSF=G, Cells with out cytokines = C, Pokeweed Mitogen = P, M = marker



CHAPTER 5

5 DISCUSSION

5.1 PROLIFERATION ASSAY

The studies included cell cultures with three mitogens (PWM, GM-CSF and INF), which have previously been shown to enhance lymphocyte proliferation [10,37,70,71,72], as positive controls (Figure 4.1.1). The inclusion of each of these mitogens in the B-CLL cell cultures resulted in significant enhancement of proliferation when compared to control cultures containing no mitogen ($p< 0.05$ (PWM and GMCSF); $p<0.01$ (INF)). The addition of the non-specific mitogen (PWM) to either GMCSF or INF appeared to reduce proliferation slightly although not significantly. The enhancement of proliferation using these combinations was still significantly greater than control cultures ($p<0.05$). Although there was an apparent gradation of effect (INF > GMCSF > PWM) the differences were not significant. The fact that these mitogens had a significant proliferative effect in these experiments indicates that the methodology used in the proliferation assays is valid.

It has been well established that a number of interleukins enhance B-CLL cell proliferation. This study has shown that IFN and IL-2 are potent enhancers of B-CLL cell proliferation in culture, probably through inhibition of apoptosis. These findings are in agreement with published data [6, 37, 52, 70, 72]. IFN was presented differently compared to other positive control mitogens.

Because it its exceptional proliferation results over IL-2. It was not expected for IFN to show a better proliferation effect (not significant) and interesting to see the difference in efficacy compared to IL-2.

In contrast, IL-10 promotes apoptosis and inhibits DNA synthesis in B-CLL cells, thus inhibiting proliferation [40, 52 & 69].

Several studies indicated that levels of Bcl-2 and Bax proteins regulate the mechanism of proliferation of B-CLL cells and apoptosis. A high level of Bcl-2 protein leads to both the proliferation of B-CLL cells and inhibition of apoptosis. High level of Bax protein leads to apoptosis of B-CLL cells. Thus, these effects depend on the ratio of Bcl-2 and Bax proteins [3, 37, 69, 94 & 112]. The Bcl-2 to Bax ratio in B-CLL cells is up regulated by IFN and IL-2 and down regulated by IL-10 [3, 37 & 70]. In this study Bcl and Bax proteins were not tested.

5.1.1 Enhancers of proliferation

5.1.1.1 Interleukin 2

In the studies conducted on the effects of IL-2 on B-CLL cells, IL-2 demonstrated better proliferation effect on B-CLL cells compared to control cultures ($p<0.001$). The proliferation was similar to that seen with positive controls. These findings are in agreement with previously reported

observations [6, 52 & 53]. Mainou-Fowler et al [6] found that B-CLL cell proliferated after adding IL-2 ($p<0.001$)[6] and the magnitude of proliferation was comparable to the one in this study. Recent literature has shown that IL-2 is associated with increase levels of Bcl-2 and that over expression of Bcl-2, commonly found in B-CLL, is believed to protect the cells from apoptosis in vitro and in vivo [40]. However, the addition of other cytokines or PWM, each of which was shown in this study to enhance proliferation in their own right, failed to enhance growth further. There have been not enough reports previously on the effect of such combinations and it is interesting to speculate on how these combinations might effect B-CLL cell proliferation in culture.

When IL6 and IL7 were added to IL-2 B-CLL cultures, neither enhanced the proliferation effect but rather decreased it slightly. Reduction in this proliferation effect was not significant. None of the previous investigators reported having tried these combinations. It is not clear why the effect was not increased when IL-2 was combined with IL-6 or IL-7. It is speculated that they might be competing for the same receptors and if IL-6 or IL-7 occupied a receptor, there will be a reduction in proliferation effect since IL-6 and IL-7 have less proliferation effect compared to IL-2. Reittie et al [113] found that IL-6 does inhibit TNF induced DNA synthesis [113] and it may affect efficacy of IL-2 by inhibiting the IL-2 induce DNA synthesis.

The addition of IL-10 to cultures with IL-2 reversed the proliferative effect of IL-2 leading to IL-2+10 proliferation results not significantly better than the

controls. However, proliferation in the presence of IL-2+10 was significantly greater than the effect of IL-10 alone ($p<0.05$). Therefore IL-2 can reverse the down regulatory effect of IL-10 on B-CLL cells and these results are supported by previous findings [40]. The combination of GM-CSF and IFN were not studied since were used as controls.

5.1.1.2 Interleukin 6

In this study B-CLL cells cultured with IL-6 demonstrated a significant increase in proliferative effect when compared to B-CLL cells without any interleukins (controls) and was as effective as the mitogens used as positive controls ($p<0.05$). Although IL-6 appeared to be less potent than the other interleukins, the differences were not significant ($p>0.1$).

When IL-10 was added to IL-6 B-CLL cultures, proliferation was reduced (Figure 4.1.1). Although this reduction was not significant when compared to controls ($p>0.3$) or to that seen with IL-6 alone ($p<0.2$), proliferation was significantly less than the proliferation seen with either IL-2 or INF ($p<0.02$). Proliferation using IL-6+10 was still significantly greater than IL-10 alone ($p<0.05$) suggesting that IL-6 was not able to overcome the effect of IL-10 completely. While IL-6 appeared to be less potent than IL-2 in reversing the effect of IL-10 (Figure 4.1.1), the differences between proliferation in IL-2+10 and IL-6+10 cultures was not significant.

The findings of this study support previous studies showing that IL-6 induces proliferation and enhances survival of B-CLL cells through inhibition of spontaneous apoptosis [58]. However, this mechanism is controversial [58]. Some authors suggested that IL-6, acting in an autocrine manner, may inhibit DNA synthesis but prolong survival of B-CLL cells [113]. IL-6 spontaneously increases DNA synthesis but in the presence of high concentration of TNF-alpha, it inhibits TNF induced DNA synthesis in a dose dependent manner [113].

Tangye et al [114] suggested that the enhancement of cell viability and suppression of apoptosis were associated with a delay in down-regulation of bcl-2 [114]. In the studies reported here, where IL-10 was added to IL-6 cultures, IL10 appears to demonstrate its ability to suppress proliferation. Fayad et al [115] suggested that IL-6 inhibit proliferation but prolongs survival of B-CLL cells by suppressing apoptosis [115]. This is in contrast with the findings of this study on proliferation but agrees with the survival of B-CLL report.

5.1.1.3 Interleukin 7

In this study B-CLL cells cultured with IL-7 demonstrated a significant increase in proliferative effect when compared to B-CLL cells without any interleukins (controls) and was as effective as the mitogens used as positive controls

(p>0.05) (Figure 4.1.1). Although IL-7 appeared to be less potent than IL-2, the difference was not significant (p>0.1).

Although the addition of IL-10 to IL-7 cultures reduced proliferation to levels seen in controls, the reduction was not significant and proliferation in cultures with IL-10 alone was still significantly less than with the combination IL-7+10 (p<0.05). In this study IL-7 inhibited apoptosis and this support the previous data relating to IL-7 which suggested that IL-7 played a role in protecting lymphoid progenitors and mature tumour cells [62, 63 & 116]. This protection was suggested to be partly mediated by IL-7 induction of Bcl-2 [62].

The addition of IL-2 to IL-7 did not have any effect on proliferation of B-CLL cultured cells. The addition of IL-10 did not inhibit the effect of IL-7 and also the addition of PWM did not enhance the proliferation. When IL-2 was added to IL-7 culture, it also did not have any additive effect on proliferation and data previously shown that combination of IL-2, IL-7 and TNF had an additive proliferative effect [63].

5.1.1.4 Interferon alpha 2b

In this study Interferon with or without PWM, significantly enhanced proliferation and promoted the survival of B-CLL cells. These results supported previous reports [37, 40, 60, 117 & 118]. In this study it may be

suggested that IFN enhanced proliferation through up regulation of Bcl-2 proto-oncogene. This assumption is based on the previous proven data [37, 40, 94, 118 & 119]. Interferon demonstrated similar proliferation effect as IL-2+P in B-CLL culture. Addition of PWM did not make any significant changes in the proliferation. IFN significantly enhanced proliferation of B-CLL cells when compared to IL-10, IL-2+10, GM-CSF, IL-10+P, IL-6+10 and IL-7+10.

Addition of PWM in IFN culture with B-CLL cells did not enhance proliferation but did slightly slow down the proliferation effect, but this was not significant. The addition of PWM was expected to enhance proliferation. Data on this experiment shows that the effect of IFN and IFN+P is similar. It also had the same effect as IL-2P and IL-2. This further proves that addition of PWM does not enhance proliferation of B-CLL when cultured with the interleukins tested in this experiment. IFN demonstrated to have a non-significant proliferation effect when compared to IL-2+6, GM-CSF, IL-6, and IL-7+P and with IL-6+7, IL-6+P and IL-7 was just not significant.

5.1.2 Enhancers of apoptosis

5.1.2.1 Interleukin 10

Although proliferation in IL-10 cultures was less than that seen in controls (Figure 4.1.1), the difference did not reach statistical significance ($p>0.1$). However, when compared to cultures with other interleukins and with the positive controls, proliferation in IL-10 cultures was considerably reduced

($p<0.01$). This difference was greatest when compared to IL-2 and INF cultures ($p< 0.0001$).

IL10 had a significant negative proliferative effect when combined with other interleukins and reduced proliferation to levels no different from controls ($p>0.05$) (See section 4.1). The most significant difference was seen when IL-10 was combined with IL-2. Proliferation was markedly lower than with IL-2 alone ($p< 0.01$) and with INF alone ($p<0.001$). These results show that IL-10 has a significant negative effect on proliferation when combined with an interleukin, which would generally be expected to enhance proliferation. On the other hand, INF and IL-2 inhibited apoptosis induced by IL-10. Addition of PWM to IL-10 culture decreased the apoptotic effect of IL-10 but the results were not significant ($p<0.5$). IL-10 significantly inhibited proliferation in B-CLL cell culture. The effect was significantly reversed by the addition of IL-2 or IFN in the culture and these finding were in agreement with previous findings [40]. The IL-10 findings in this study were in accordance with those found in the literature [52 & 69].

It has previously been demonstrated that IL-10 mediated apoptosis showed decreased Bcl-2 levels [40]. Bcl-2 expression was found to be down regulated by IL-10 and up-regulate by IL-2 [94 and 112].

In this study IL-10 promoted apoptosis (see section 4.1). A mechanism to explain the phenomenon may be through up regulation of proto-oncogene Bax levels and by down regulating expression of proto-oncogene Bcl-2. Levels of Bax protein were not measured in this experiment, but up regulation of Bax proto-oncogene and down regulation of Bcl-2 by IL-10 have been previously proven [94, 40 & 120]. The effect of IL-10 in combination with GM-CSF and IFN were not done.

5.1.3 Combined Mitogens

Combination of different combinations of interleukins was done (see table: 3.4.1.). Combination of these ILs did not enhance proliferation as expected. It was thought if you add two cytokines or mitogens that lead to B-CLL cells proliferation the proliferation will be enhance. This phenomenon was observed in most combinations tested in this study. For example: IL-6 had the similar proliferation effect as IL-7+P, IL2+7, IL2+6, and IFN+P. In another example where IL-2 and IL-6 where combined, a change in proliferation effect was noticed but again this was not statistically significant ($p<0.3$ compared to IL-2 and $p<0.5$ compared to IL-6). These results support the finding of Marinou-Fowler et al [6] even though there were different cytokines used. IL-2 was combined with IL-4, IL-5 and IL-1 did not enhance proliferation of B-CLL cells compared to IL-2 alone [6].

When IL-10 was added to IL-6 B-CLL culture, proliferation was reduced but not to statistically significant levels. IFN demonstrated an increased proliferation of B-CLL cells greater than that of IL-6, but the effect was also not significant. The above findings were not expected and unfortunately there was no enough published literature to compare the results.

5.2 EFFECT OF CYTOKINES ON CELL SURVIVAL

It has been suggested that several cytokines play role in growth of leukaemic cells or in prolonging cell survival of B-CLL cells *in vitro*. These cytokines may be produced by the leukaemic cells themselves or cells of the immuno-competent system [6, 37, 58 & 72]. In this study we tested for B-CLL survival, noting prominent morphological changes including cell shrinkage, condensation of the nuclear chromatin, fragmentation of the nucleus and clearing of the chromosomal DNA in B-CLL cells tested. B-CLL survival is expressed in percentage in this study, calculated from number of viable cells versus total number of cell counted. B-cells cultured at 37°C without cytokines were considered as negative controls and B-cells cultures at 4°C without cytokines and those cultured with GM-CSF were considered as positive controls.

Morphological changes were observed when IFN treated B-CLL cells were examined under a fluorescent microscope. They appear to survive better than with any other growth factor / mitogen used in this study. At day five of cells

treated with IFN survived better than the positive controls. IFN was also compared with IL-2 and showed to survive better than IL-2, which was highly significant compared to all mitogens used in this study. These results concur with previous reports presented in the literature [3, 70, 117 & 118]. A significant difference was noted mainly at Day 5. These results show that IFN is the one of the best growth factors promoting B-CLL survival.

B-CLL cells treated with IL-10 appeared to have the most apoptotic changes compared to all B-CLL cells tested with different mitogens in this study. These changes were more prominent at Day 5. These results prove that IL-10 lead to a poor survival in B-CLL cells [40, 113 & 120]. In this study some of these cells responded to IL-10 effect faster than others. This may be due to the age of the B-CLL cells at the time blood was taken or whether the patients had been treated before collection of blood samples [40].

Negative controls (B-CLL cells cultured at 37°C without cytokine and growth factors) were examined and prominent morphological changes were observed. These morphological changes were less prominent compared to that of IL-10 treated cells but were more compared to other mitogens testes.

B-CLL cells cultured with IL-2 had better survival than negative controls and better than positive controls. In general, IL-2 has proved to enhance survival of B-CLL cells in culture and supported the previous findings [3, 52, 40, 53 & 119].

It is suspected that IL-7 also promotes B-CLL cells survival since it promotes B-CLL proliferation by preventing loss of expression of BCL-2 *in vitro*. According to the results of these studies, IL-7 had less survival effect compared to positive controls GM-CSF, IL-6, IL-2 and IFN (Bonferroni t, $p< 0.05$. Compared to negative controls, IL-7 had similar. (Bonferroni t, $p> 0.05$). These results showed that IL-7 does not have any effect on the B-CLL cells survival.

On the other hand, IL-6 had similar survival compared to positive controls (GM-CSF) and better survival compared to IL-7, IL-10 and negative control (Bonferroni t, $p <0.05$). These results showed that IL-6 was not different from the positive controls.

5.3 AGAROSE GEL ELECTROPHORESIS AND DNA DEGRADATION

Previous studies have demonstrated that B-CLL cells spontaneously die from apoptosis during *in vitro* culture, although the rate remains low compared to the important cell death observed with normal B cells (10% *versus* 35%, respectively) [40, 117 & 120]. In this study the spontaneous apoptosis of B-CLL cells was found to be enhanced by IL-10 rather than any other cytokines or growth factors used. This is indicated by agarose gel electrophoresis showing internucleosomal DNA fragmentation, characteristic of apoptosis.

Furthermore, DNA electrophoresis revealed that IL-10 enhances apoptosis of cultured B-CLL cells with supported Wickremasinghe et al and Fluckinger et al findings [37 & 40]. The degradation of the DNA extracted from B-CLL cells cultured without cytokine (positive control) was significantly less than that found in the presence IL-10. This observation is in agreement with the previous literature [40].

There were either no ladder patterns observed with B-CLL cells treated with IL-2, IFN, IL-6, GM-CSF, PWM, and IL-7 [4, 6, 40, 62, 117]. There was less evidence of DNA degradation observed. This evidence of no degradation was more prominent with the negative controls and IFN and IL-2. These results confirm that INF, IL-2, IL-6 are B-CLL cells apoptosis inhibitors, and that they all prevented spontaneous apoptosis in B-CLL cells treated with these cytokines. GM-CSF and L-7 has also demonstrated to be inhibitors of spontaneous apoptosis in B-CLL cells.

The above information supports data presented in the literature reviewed [6, 40, 64, 113, 116 & 117]. However some of the gels were not clear and some of the ladder pattern might have been missed due to necrotic masking and/or undergraded DNA which was also confirmed by Basil and et al [21].

It was previously proven that conventional ethidium bromide staining of DNA may not be sufficiently sensitive to reveal a classic DNA ladder. The method

involving the gentle isolation of genomic DNA with a silica-based resin and radiolabeled with taq DNA polymerase, the sensitivity was found to be better than that of ethidium bromide staining technique [21].

CHAPTER 6

6 CONCLUSION

These results indicate that IFN and IL-2 promote the survival and proliferation of B-CLL cells in culture. IFN and IL-2, are potent inhibitors of apoptosis and, in their absence, B-CLL cells *in-vitro* will die by means of apoptosis. These features are consistent with up-regulation of the Bcl-2 proto-oncogene. In contrast, IL-10 was demonstrated to induce apoptotic cell death, inhibit proliferation, even in the presence of promoting interleukins, and to reduce survival of B-CLL cells. It is believed that IL-10 down regulates Bcl-2 and up regulates Bax proto-oncogenes. These findings demonstrate the array of IL-10 activities and constitute an example of the pro-apoptotic effect of cytokines.

The effect of the other stimulatory factors tested is less easy to interpret. PWM and GM-CSF, which were included as positive controls, enhanced proliferation but not to the same extent as IL-2 and INF. In fact, the addition of PWM demonstrated no additional effects. IL-6 and IL-7, used alone in culture, did promote proliferation but appeared to be less potent inhibitors of apoptosis. On the other hand, IL-7 did not promote survival of B-CLL cells while IL-6 did. The combination of these cytokines and when added individually to IL-2 cultures did not result in any difference in proliferation or apoptosis. The mechanism of action of these cytokines is uncertain.

CHAPTER 7

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CHAPTER 8

8 APPENDICES

Appendix I: Proliferation assay results of B-CLL cells in response to cytokines and growth factors. Absorbance was measured to express the effect of these cytokines and these results are with the background and Graph 8.1: Absorbance results for proliferation Assay of B-CLL cells in response to cytokines and growth factors with background.

Appendix II: B-CLL cells survival results based on the morphological changes. Percentage survival results on addition of IL-2, IL-6, IL-7, IL-10, IFN, GM-CSF, PWM and Controls cultured at 37 degrees Celsius and cells cultured at 4 degrees Celsius.

Appendix III: The ANOVA procedure and Bonferroni (Dunn) t Test results comparing the difference in survival between day 1 and day 5 on treatment with different mitogen comparing day1 versus day 5. Furthermore, comparing difference in survival between day1, day3 and day5 between the mitogen tested in B-CLL cells culture.

Appendix IV: The University of the Witwatersrand, Johannesburg, Committee for Research on Human Subjects (Medical) Clearance Certificate for Protocol Number 00/05/24

Appendix I : Table 8.1. Proliferation assay results of B-CLL cells in response to cytokines and growth factors. Absorbance was measured to express the effect of these cytokines and these results are with the background.

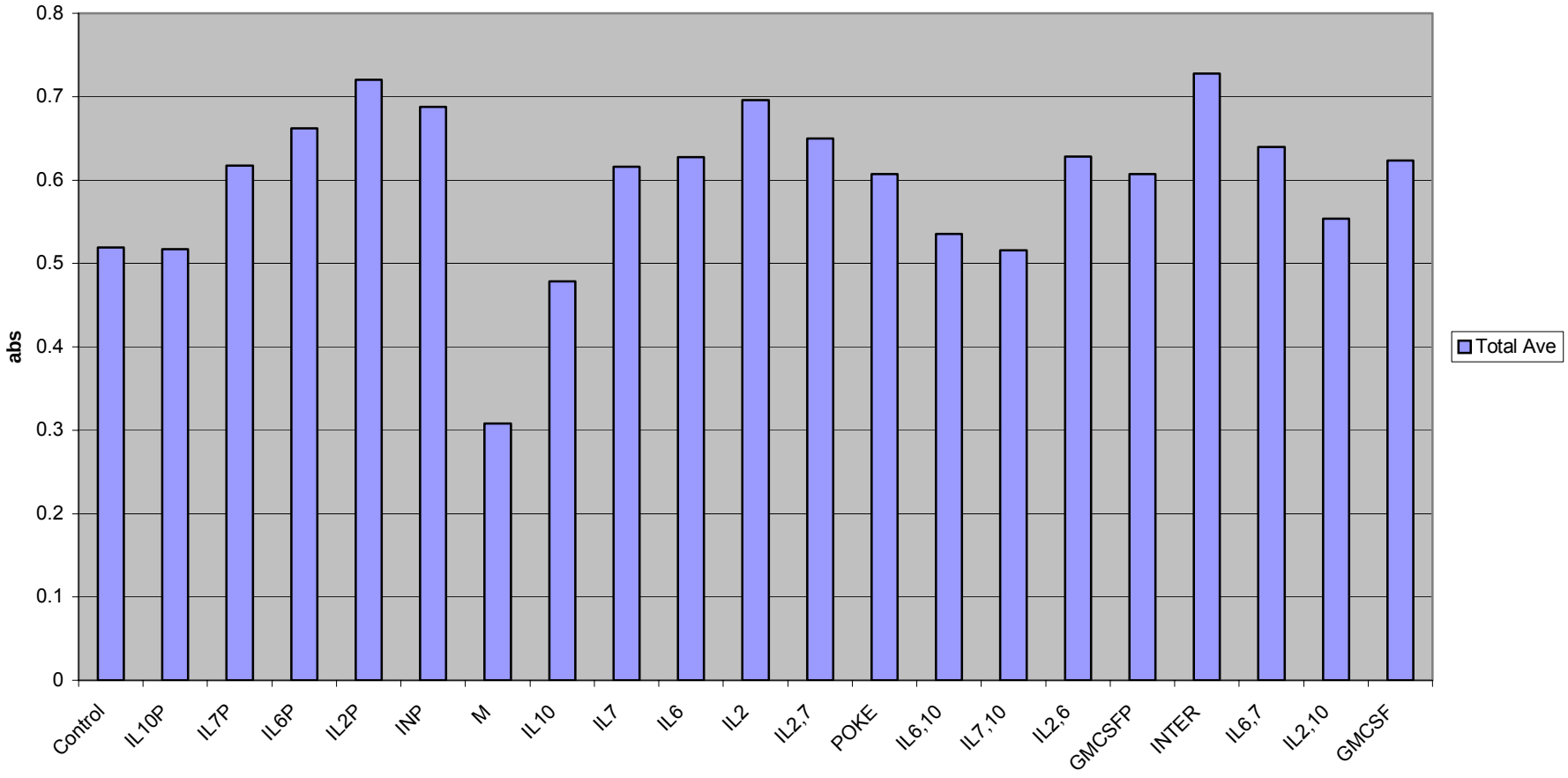
PT 001	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.051	0.903	1.32	1.399	1.425	1.401	0.663	1.001	1.357	1.409	1.388	1.348	1.368	1.157	1.112	1.4	1.33	1.667	1.299	1.157	1.286
B	1	1.305	1.346	1.472	1.371	1.364	0.649	1.299	1.4	1.489	1.376	1.277	1.362	1.191	1.269	1.62	1.387	1.782	1.368	1.17	1.4
Average	1.0255	1.104	1.333	1.4355	1.398	1.3825	0.656	1.15	1.3785	1.449	1.382	1.3125	1.365	1.174	1.1905	1.51	1.3585	1.7245	1.3335	1.1635	1.343
PT 002	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.189	0.163	0.148	0.222	0.267	0.163	0.158	0.174	0.229	0.25	0.233	0.16	0.253	0.268	0.123	0.309	0.205	0.299	0.276	0.199	0.205
B	0.144	0.124	0.253	0.233	0.225	0.23	0.1	0.122	0.192	0.269	0.287	0.197	0.266	0.181	0.2	0.207	0.208	0.321	0.28	0.115	0.225
Average	0.1665	0.1435	0.2005	0.2275	0.246	0.1965	0.129	0.148	0.2105	0.2595	0.26	0.1785	0.2595	0.2245	0.1615	0.258	0.2065	0.31	0.278	0.157	0.215
PT 003	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.344	0.274	0.302	0.349	0.389	0.4	0.155	0.211	0.412	0.41	0.363	0.46	0.409	0.348	0.287	0.351	0.322	0.628	0.423	0.349	0.449
B	0.35	0.317	0.314	0.37	0.361	0.363	0.149	0.233	0.358	0.499	0.432	0.353	0.3	0.398	0.291	0.405	0.434	0.555	0.439	0.421	0.46
Average	0.347	0.2955	0.308	0.3595	0.375	0.3815	0.152	0.222	0.385	0.4545	0.3975	0.4065	0.3545	0.373	0.289	0.378	0.378	0.5915	0.431	0.385	0.4545
PT 004	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.341	0.366	0.452	0.401	0.513	0.461	0.145	0.251	0.393	0.359	0.393	0.441	0.432	0.349	0.197	0.322	0.312	0.433	0.338	0.219	0.345
B	0.322	0.341	0.548	0.43	0.405	0.437	0.133	0.279	0.407	0.344	0.464	0.354	0.428	0.346	0.21	0.325	0.328	0.359	0.334	0.365	0.366
Average	0.3315	0.3535	0.5	0.4155	0.459	0.449	0.139	0.265	0.4	0.3515	0.4285	0.3975	0.43	0.3475	0.2035	0.3235	0.32	0.396	0.336	0.292	0.3555
PT 005	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.011	0.937	1.333	1.506	1.351	1.416	0.792	0.911	1.3	1.053	1.322	1.212	1.112	0.864	1.105	1.288	1.151	1.225	1.105	1.307	1.265
B	1.162	1.163	1.182	1.429	1.49	1.392	0.6	1.001	1.204	1.289	1.306	1.188	1.126	0.998	0.964	1.256	1.247	1.489	1.211	1.389	1.114
Average	1.0865	1.05	1.2575	1.4675	1.4205	1.404	0.696	0.956	1.252	1.171	1.314	1.2	1.119	0.931	1.0345	1.272	1.199	1.357	1.158	1.348	1.1895
PT 006	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.311	1.402	1.512	1.522	1.578	1.529	0.86	1.448	1.377	1.5	1.396	1.396	1.317	1.238	1.323	1.339	1.401	1.458	1.487	1.39	1.149
B	1.375	1.445	1.437	1.659	1.455	1.498	0.969	1.432	1.395	1.519	1.376	1.376	1.41	1.385	1.314	1.348	1.399	1.568	1.384	1.227	1.276
Average	1.343	1.4235	1.4745	1.5905	1.5165	1.5135	0.9145	1.44	1.386	1.5095	1.386	1.386	1.3635	1.3115	1.3185	1.3435	1.4	1.513	1.4355	1.3085	1.2125
PT 007	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.461	1.373	1.387	1.495	1.441	1.446	0.912	1.288	1.412	1.519	1.554	1.515	1.404	1.374	1.381	1.471	1.367	1.479	1.414	1.403	1.46
B	1.478	1.42	1.476	1.472	1.466	1.379	0.914	1.252	1.424	1.532	1.485	1.471	1.371	1.368	1.368	1.401	1.33	1.747	1.641	1.609	1.637
Average	1.4695	1.3965	1.4315	1.4835	1.4535	1.4125	0.913	1.27	1.418	1.5255	1.5195	1.493	1.3875	1.371	1.3745	1.436	1.3485	1.613	1.5275	1.506	1.5485

PT 008	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.306	1.255	1.375	1.32	1.381	1.414	0.801	1.369	1.427	1.523	1.482	1.324	1.279	1.336	1.361	1.363	1.328	1.447	1.422	1.353	1.443
B	1.364	1.381	1.393	1.48	1.383	1.403	0.785	1.376	1.434	1.479	1.431	1.35	1.275	1.339	1.353	1.381	1.326	1.622	1.457	1.345	1.515
Average	1.335	1.318	1.384	1.4	1.382	1.4085	0.793	1.3725	1.4305	1.501	1.4565	1.337	1.277	1.3375	1.357	1.372	1.327	1.5345	1.4395	1.349	1.479
PT 009	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.251	1.239	1.309	1.324	1.393	1.389	0.818	1.109	1.342	1.321	1.411	1.338	1.446	1.253	1.205	1.336	1.265	1.448	1.371	1.35	1.455
B	1.253	1.285	1.295	1.329	1.377	1.451	0.799	1.141	1.332	1.318	1.478	1.326	1.37	1.235	1.217	1.366	1.323	1.519	1.45	1.388	1.436
Average	1.252	1.262	1.302	1.3265	1.385	1.42	0.8085	1.125	1.337	1.3195	1.4445	1.332	1.408	1.244	1.211	1.351	1.294	1.4835	1.4105	1.369	1.4455
PT 010	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	1.39	1.315	1.43	1.433	1.479	1.447	0.859	1.244	1.371	1.393	1.505	1.479	1.314	1.379	1.227	1.443	1.394	1.457	1.486	1.4	1.385
B	1.374	1.347	1.438	1.514	1.561	1.488	0.966	1.326	1.391	1.365	1.521	1.447	1.446	1.284	1.312	1.448	1.417	1.575	1.39	1.328	1.429
Average	1.382	1.331	1.434	1.4735	1.52	1.4675	0.9125	1.285	1.381	1.379	1.513	1.463	1.38	1.3315	1.2695	1.4455	1.4055	1.516	1.438	1.364	1.407
PT 011	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.367	0.303	0.319	0.399	0.383	0.44	0.179	0.227	0.369	0.401	0.37	0.345	0.355	0.304	0.288	0.397	0.275	0.42	0.441	0.404	0.275
B	0.399	0.391	0.498	0.361	0.421	0.373	0.105	0.312	0.321	0.358	0.383	0.359	0.374	0.357	0.291	0.44	0.312	0.399	0.395	0.346	0.255
Average	0.383	0.347	0.4085	0.38	0.402	0.4065	0.142	0.2695	0.345	0.3795	0.3765	0.352	0.3645	0.3305	0.2895	0.4185	0.2935	0.4095	0.418	0.375	0.265
PT 012	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.293	0.331	0.326	0.375	0.494	0.378	0.103	0.219	0.415	0.469	0.539	0.447	0.393	0.338	0.273	0.443	0.473	0.505	0.525	0.308	0.525
B	0.264	0.281	0.379	0.393	0.445	0.434	0.164	0.205	0.419	0.479	0.542	0.499	0.387	0.312	0.303	0.42	0.484	0.532	0.505	0.428	0.45
Average	0.2785	0.306	0.3525	0.384	0.4695	0.406	0.1335	0.212	0.417	0.474	0.5405	0.473	0.39	0.325	0.288	0.4315	0.4785	0.5185	0.515	0.368	0.4875
PT 013	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.253	0.206	0.267	0.25	0.348	0.342	0.145	0.215	0.272	0.318	0.361	0.29	0.296	0.199	0.159	0.322	0.281	0.382	0.345	0.25	0.394
B	0.281	0.213	0.2801	0.216	0.294	0.423	0.112	0.224	0.367	0.312	0.445	0.301	0.231	0.198	0.21	0.315	0.339	0.369	0.321	0.235	0.332
Average	0.267	0.2095	0.2736	0.233	0.321	0.3825	0.1285	0.2195	0.3195	0.315	0.403	0.2955	0.2635	0.1985	0.1845	0.3185	0.31	0.3755	0.333	0.2425	0.363
PT 014	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.206	0.192	0.322	0.315	0.348	0.395	0.162	0.192	0.289	0.313	0.388	0.366	0.312	0.215	0.211	0.299	0.313	0.368	0.369	0.206	0.3
B	0.199	0.194	0.301	0.255	0.351	0.328	0.159	0.179	0.257	0.281	0.343	0.371	0.381	0.191	0.195	0.376	0.278	0.364	0.291	0.209	0.345
Average	0.2025	0.193	0.3115	0.285	0.3495	0.3615	0.1605	0.1855	0.273	0.297	0.3655	0.3685	0.3465	0.203	0.203	0.3375	0.2955	0.366	0.33	0.2075	0.3225
PT 015	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.203	0.178	0.333	0.385	0.417	0.395	0.165	0.182	0.259	0.293	0.448	0.271	0.293	0.199	0.144	0.311	0.351	0.423	0.301	0.27	0.32
B	0.199	0.171	0.356	0.305	0.466	0.453	0.151	0.203	0.291	0.317	0.355	0.312	0.287	0.177	0.199	0.358	0.361	0.402	0.359	0.247	0.391
Average	0.201	0.1745	0.3445	0.345	0.4415	0.424	0.158	0.1925	0.275	0.305	0.4015	0.2915	0.29	0.188	0.1715	0.3345	0.356	0.4125	0.33	0.2585	0.3555

PT 016	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.197	0.171	0.345	0.384	0.466	0.453	0.16	0.203	0.287	0.263	0.399	0.381	0.276	0.219	0.262	0.311	0.351	0.423	0.279	0.27	0.33
B	0.205	0.186	0.333	0.383	0.417	0.395	0.151	0.21	0.269	0.256	0.355	0.366	0.268	0.187	0.219	0.358	0.288	0.452	0.273	0.3	0.311
average	0.201	0.1785	0.339	0.3835	0.4415	0.424	0.1555	0.2065	0.278	0.2595	0.377	0.3735	0.272	0.203	0.2405	0.3345	0.3195	0.4375	0.276	0.285	0.3205
PT 017	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.187	0.186	0.239	0.287	0.381	0.309	0.101	0.182	0.217	0.259	0.302	0.357	0.234	0.199	0.214	0.2307	0.282	0.295	0.289	0.199	0.29
B	0.212	0.206	0.3	0.36	0.317	0.359	0.161	0.179	0.209	0.292	0.329	0.291	0.226	0.189	0.201	0.276	0.35	0.267	0.311	0.197	0.345
average	0.1995	0.196	0.2695	0.3235	0.349	0.334	0.131	0.1805	0.213	0.2755	0.3155	0.324	0.23	0.194	0.2075	0.2534	0.316	0.281	0.3	0.198	0.3175
PT 018	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A	0.262	0.266	0.366	0.256	0.396	0.371	0.14	0.239	0.311	0.25	0.332	0.351	0.317	0.278	0.234	0.294	0.289	0.349	0.311	0.274	0.301
B	0.266	0.271	0.329	0.268	0.409	0.289	0.146	0.237	0.239	0.262	0.334	0.343	0.32	0.247	0.242	0.296	0.332	0.358	0.321	0.278	0.289
average	0.264	0.2685	0.3475	0.262	0.4025	0.33	0.143	0.238	0.275	0.256	0.333	0.347	0.3185	0.2625	0.238	0.295	0.3105	0.3535	0.316	0.276	0.295
PT 019	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.401	0.421	0.543	0.591	0.741	0.654	0.145	0.318	0.511	0.637	0.661	0.5	0.51	0.358	0.399	0.469	0.435	0.536	0.489	0.332	0.544
B	0.423	0.408	0.481	0.63	0.623	0.742	0.13	0.321	0.609	0.65	0.72	0.488	0.518	0.371	0.345	0.48	0.456	0.533	0.448	0.362	0.487
average	0.412	0.4145	0.512	0.6105	0.682	0.698	0.1375	0.3195	0.56	0.6435	0.6905	0.494	0.514	0.3645	0.372	0.4745	0.4455	0.5345	0.4685	0.347	0.5155
PT 020	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.409	0.494	0.499	0.7	0.773	0.766	0.118	0.393	0.498	0.442	0.535	0.5	0.57	0.402	0.476	0.424	0.445	0.667	0.444	0.275	0.455
B	0.55	0.453	0.545	0.648	0.776	0.722	0.111	0.344	0.566	0.479	0.487	0.581	0.51	0.467	0.356	0.566	0.522	0.567	0.459	0.189	0.437
average	0.4795	0.4735	0.522	0.674	0.7745	0.744	0.1145	0.3685	0.532	0.4605	0.511	0.5405	0.54	0.4345	0.416	0.495	0.4835	0.617	0.4515	0.232	0.446
PT 021	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.224	0.246	0.268	0.325	0.391	0.314	0.127	0.203	0.299	0.386	0.496	0.456	0.266	0.306	0.301	0.315	0.254	0.353	0.39	0.224	0.254
B	0.252	0.197	0.189	0.328	0.382	0.321	0.113	0.206	0.323	0.395	0.455	0.412	0.356	0.326	0.323	0.311	0.246	0.328	0.322	0.236	0.246
average	0.238	0.2215	0.2285	0.3265	0.3865	0.3175	0.12	0.2045	0.311	0.3905	0.4755	0.434	0.311	0.316	0.312	0.313	0.25	0.3405	0.356	0.23	0.25
PT 022	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.305	0.339	0.429	0.47	0.551	0.453	0.145	0.308	0.479	0.458	0.426	0.492	0.553	0.429	0.434	0.453	0.497	0.597	0.609	0.368	0.486
B	0.354	0.398	0.456	0.437	0.489	0.429	0.152	0.281	0.458	0.406	0.511	0.511	0.489	0.387	0.401	0.489	0.223	0.697	0.662	0.285	0.505
average	0.3295	0.3685	0.4425	0.4535	0.52	0.441	0.1485	0.2945	0.4685	0.432	0.4685	0.5015	0.521	0.408	0.4175	0.471	0.36	0.647	0.6355	0.3265	0.4955
PT 023	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.396	0.349	0.567	0.612	0.703	0.641	0.27	0.336	0.689	0.784	0.884	0.789	0.621	0.673	0.501	0.621	0.599	0.749	0.682	0.521	0.65
B	0.384	0.331	0.499	0.749	0.812	0.688	0.266	0.322	0.738	0.772	0.813	0.851	0.655	0.553	0.49	0.602	0.681	0.694	0.588	0.506	0.624
average	0.39	0.34	0.533	0.6805	0.7575	0.6645	0.268	0.329	0.7135	0.778	0.8485	0.82	0.638	0.613	0.4955	0.6115	0.64	0.7215	0.635	0.5135	0.637

PT 024	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.589	0.654	0.809	1.08	1.262	0.999	0.306	0.661	0.844	0.88	0.936	0.954	0.875	0.475	0.689	0.861	0.796	0.972	0.892	0.73	0.747
B	0.607	0.584	0.79	0.947	1.088	0.944	0.262	0.616	0.819	0.728	0.871	0.904	0.799	0.52	0.538	0.819	1.02	1.106	0.827	0.745	0.822
average	0.598	0.619	0.7995	1.0135	1.175	0.9715	0.284	0.6385	0.8315	0.804	0.9035	0.929	0.837	0.4975	0.6135	0.84	0.908	1.039	0.8595	0.7375	0.7845
PT 025	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.278	0.251	0.3778	0.399	0.57	0.474	0.192	0.211	0.301	0.297	0.684	0.552	0.377	0.494	0.299	0.438	0.488	0.666	0.459	0.487	0.502
B	0.296	0.226	0.345	0.467	0.577	0.476	0.156	0.2	0.372	0.349	0.599	0.548	0.396	0.452	0.32	0.436	0.5	0.615	0.389	0.333	0.504
average	0.287	0.2385	0.3614	0.433	0.5735	0.475	0.174	0.2055	0.3365	0.323	0.6415	0.55	0.3865	0.473	0.3095	0.437	0.494	0.6405	0.424	0.41	0.503
PT 026	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.255	0.397	0.389	0.273	0.414	0.419	0.167	0.217	0.282	0.273	0.325	0.367	0.358	0.189	0.189	0.36	0.371	0.399	0.388	0.336	0.367
B	0.26	0.293	0.278	0.283	0.432	0.352	0.112	0.173	0.279	0.273	0.359	0.371	0.32	0.256	0.297	0.381	0.339	0.381	0.41	0.388	0.346
average	0.2575	0.345	0.3335	0.278	0.423	0.3855	0.1395	0.195	0.2805	0.273	0.342	0.369	0.339	0.2225	0.243	0.3705	0.355	0.39	0.399	0.362	0.3565
PT 027	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
A.	0.219	0.273	0.379	0.349	0.545	0.469	0.108	0.248	0.408	0.297	0.373	0.301	0.298	0.292	0.231	0.389	0.307	0.426	0.318	0.273	0.302
B	0.257	0.263	0.347	0.421	0.399	0.478	0.118	0.293	0.435	0.336	0.463	0.328	0.252	0.312	0.176	0.328	0.297	0.435	0.263	0.202	0.31
average	0.238	0.268	0.363	0.385	0.472	0.4735	0.113	0.2705	0.4215	0.3165	0.418	0.3145	0.275	0.302	0.2035	0.3585	0.302	0.4305	0.2905	0.2375	0.306
PT 028	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
	0.167	0.199	0.48	0.459	0.68	0.579	0.158	0.172	0.359	0.368	0.513	0.538	0.369	0.262	0.272	0.455	0.384	0.471	0.366	0.259	0.305
	0.241	0.184	0.468	0.344	0.648	0.584	0.167	0.186	0.392	0.367	0.527	0.468	0.398	0.269	0.279	0.414	0.415	0.544	0.362	0.274	0.361
average	0.204	0.1915	0.474	0.4015	0.664	0.5815	0.1625	0.179	0.3755	0.3675	0.52	0.503	0.3835	0.2655	0.2755	0.4345	0.3995	0.5075	0.364	0.2665	0.333
PT 029	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
	0.18	0.192	0.347	0.354	0.339	0.29	0.178	0.185	0.275	0.228	0.255	0.284	0.249	0.277	0.307	0.252	0.271	0.289	0.234	0.196	0.231
	0.201	0.183	0.3	0.379	0.462	0.27	0.135	0.173	0.254	0.188	0.226	0.282	0.201	0.226	0.211	0.244	0.32	0.322	0.254	0.2	0.264
average	0.1905	0.1875	0.3235	0.3665	0.4005	0.28	0.1565	0.179	0.2645	0.208	0.2405	0.283	0.225	0.2515	0.259	0.248	0.2955	0.3055	0.244	0.198	0.2475
PT 030	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
	0.238	0.302	0.342	0.488	0.428	0.519	0.176	0.218	0.401	0.358	0.578	0.468	0.44	0.351	0.288	0.395	0.396	0.482	0.433	0.288	0.441
	0.208	0.283	0.348	0.417	0.479	0.471	0.145	0.238	0.42	0.344	0.61	0.363	0.404	0.38	0.372	0.343	0.315	0.448	0.476	0.311	0.472
average	0.223	0.2925	0.345	0.4525	0.4535	0.495	0.1605	0.228	0.4105	0.351	0.594	0.4155	0.422	0.3655	0.33	0.369	0.3555	0.465	0.4545	0.2995	0.4565
overall	Control	IL10P	IL7P	IL6P	IL2P	INP	M	IL10	IL7	IL6	IL2	IL2,7	POKE	IL6,10	IL7,10	IL2,6	GMCSFP	INTER	IL6,7	IL2,10	GMCSF
Total Ave	0.5194	0.517	0.617	0.6617	0.7205	0.6877	0.3081	0.4783	0.616	0.6276	0.6956	0.6495	0.607	0.5355	0.516	0.6278	0.6068	0.7277	0.6396	0.5538	0.6236

Graph 8.1 : Absorbance results for proliferation Assay of B-CLL cells in response to cytokines and growth factors with background



Appendix II: B-CLL Cell Survival results based on the morphological changes.

Table 8.2 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of IL-2

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	288	252	217	112	148	183	400	400	400	72	63	54.25
2	269	253	255	131	147	145	400	400	400	67.25	63.25	63.75
3	317	257	216	83	143	184	400	400	400	79.25	64.25	54
4	332	291	297	68	109	103	400	400	400	83	72.75	74.25
5	279	277	258	121	123	142	400	400	400	69.75	69.25	64.5
6	271	249	248	129	151	152	400	400	400	67.75	62.25	62
7	280	238	246	120	162	154	400	400	400	70	59.5	61.5
8	333	289	258	67	111	142	400	400	400	83.25	72.25	64.5
9	327	273	257	73	127	143	400	400	400	81.75	68.25	64.25
10	282	277	239	118	123	161	400	400	400	70.5	69.25	59.75
11	245	223	216	155	177	184	400	400	400	61.25	55.75	54
13	282	244	217	118	156	183	400	400	400	70.5	61	54.25
14	292	252	215	108	148	185	400	400	400	73	63	53.75
15	297	256	213	103	144	187	400	400	400	74.25	64	53.25
16	256	239	215	144	161	185	400	400	400	64	59.75	53.75
17	302	261	210	98	139	190	400	400	400	75.5	65.25	52.5
18	289	244	213	111	156	187	400	400	400	72.25	61	53.25
19	291	237	211	109	163	189	400	400	400	72.75	59.25	52.75
20	255	236	212	145	164	188	400	400	400	63.75	59	53
21	269	217	213	131	183	187	400	400	400	67.25	54.25	53.25
22	284	254	177	116	146	223	400	400	400	71	63.5	44.25
23	266	217	289	134	183	111	400	400	400	66.5	54.25	72.25
24	288	232	214	112	168	186	400	400	400	72	58	53.5
25	274	216	253	126	184	147	400	400	400	68.5	54	63.25
26	294	255	254	106	145	146	400	400	400	73.5	63.75	63.5
Mean	286.48	249.56	232.52	113.52	150.44	167.48	400	400	400	71.62	62.39	58.13
Median	284	252	217	116	148	183	400	400	400	71	63	54.25
SD	22.8129	20.9545	27.9108	22.8129	20.95447	27.91075	0	0	0	5.703215	5.238619	6.9777

Table 8.3 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of IL-6

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	238	208	216	162	192	184	400	400	400	59.5	52	54
2	205	241	217	195	159	183	400	400	400	51.25	60.25	54.25
3	222	204	176	178	196	224	400	400	400	55.5	51	44
4	201	170	173	199	230	227	400	400	400	50.25	42.5	43.25
5	209	174	172	191	226	228	400	400	400	52.25	43.5	43
6	242	176	210	158	224	190	400	400	400	60.5	44	52.5
7	288	252	279	112	148	121	400	400	400	72	63	69.75
8	255	239	216	145	161	184	400	400	400	63.75	59.75	54
9	214	189	171	186	211	229	400	400	400	53.5	47.25	42.75
10	250	211	173	150	189	227	400	400	400	62.5	52.75	43.25
11	295	255	242	105	145	158	400	400	400	73.75	63.75	60.5
13	204	174	176	196	226	224	400	400	400	51	43.5	44
14	252	215	172	148	185	228	400	400	400	63	53.75	43
15	216	177	210	184	223	190	400	400	400	54	44.25	52.5
16	214	169	172	186	231	228	400	400	400	53.5	42.25	43
17	251	244	209	149	156	191	400	400	400	62.75	61	52.25
18	243	211	168	157	189	232	400	400	400	60.75	52.75	42
19	213	200	177	187	200	223	400	400	400	53.25	50	44.25
20	215	198	211	185	202	189	400	400	400	53.75	49.5	52.75
21	246	189	210	154	211	190	400	400	400	61.5	47.25	52.5
22	213	175	162	187	225	238	400	400	400	53.25	43.75	40.5
23	286	215	211	114	185	189	400	400	400	71.5	53.75	52.75
24	214	210	212	186	190	188	400	400	400	53.5	52.5	53
25	261	249	244	139	151	156	400	400	400	65.25	62.25	61
26	236	210	171	164	190	229	400	400	400	59	52.5	42.75
Mean	235.32	206.2	198	164.68	193.8	202	400	400	400	58.83	51.55	49.5
Median	236	208	209	164	192	191	400	400	400	59	52	52.25
SD	27.3948	27.7399	29.6156	27.3948	27.73986	29.61559	0	0	0	6.848707	6.934966	7.4039

Table 8.4 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of IL-7

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	202	134	129	198	266	271	400	400	400	50.5	33.5	32.25
2	240	203	175	160	197	225	400	400	400	60	50.75	43.75
3	215	135	133	185	265	267	400	400	400	53.75	33.75	33.25
4	176	129	119	224	271	281	400	400	400	44	32.25	29.75
5	217	203	171	183	197	229	400	400	400	54.25	50.75	42.75
6	210	171	162	190	229	238	400	400	400	52.5	42.75	40.5
7	173	163	155	227	237	245	400	400	400	43.25	40.75	38.75
8	212	168	156	188	232	244	400	400	400	53	42	39
9	174	159	134	226	241	266	400	400	400	43.5	39.75	33.5
10	210	164	150	190	236	250	400	400	400	52.5	41	37.5
11	189	129	128	211	271	272	400	400	400	47.25	32.25	32
13	179	132	124	221	268	276	400	400	400	44.75	33	31
14	210	199	173	190	201	227	400	400	400	52.5	49.75	43.25
15	198	170	172	202	230	228	400	400	400	49.5	42.5	43
16	174	166	136	226	234	264	400	400	400	43.5	41.5	34
17	200	174	169	200	226	231	400	400	400	50	43.5	42.25
18	209	173	170	191	227	230	400	400	400	52.25	43.25	42.5
19	159	133	130	241	267	270	400	400	400	39.75	33.25	32.5
20	163	131	125	237	269	275	400	400	400	40.75	32.75	31.25
21	189	134	133	211	266	267	400	400	400	47.25	33.5	33.25
22	173	161	135	227	239	265	400	400	400	43.25	40.25	33.75
23	193	176	155	207	224	245	400	400	400	48.25	44	38.75
24	175	168	155	225	232	245	400	400	400	43.75	42	38.75
25	161	134	120	239	266	280	400	400	400	40.25	33.5	30
26	207	173	163	193	227	237	400	400	400	51.75	43.25	40.75
Mean	192.32	159.28	146.88	207.68	240.72	253.12	400	400	400	48.08	39.82	36.72
Median	193	164	150	207	236	250	400	400	400	48.25	41	37.5
SD	20.9518	23.5735	19.1079	20.9518	23.5735	19.10785	0	0	0	5.237943	5.893376	4.777

Table 8.5 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of IL-10

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	185	96	99	215	304	301	400	400	400	46.25	24	24.75
2	156	94	67	244	306	333	400	400	400	39	23.5	16.75
3	159	90	69	241	310	331	400	400	400	39.75	22.5	17.25
4	150	105	35	250	295	365	400	400	400	37.5	26.25	8.75
5	133	92	31	267	308	369	400	400	400	33.25	23	7.75
6	188	132	66	212	268	334	400	400	400	47	33	16.5
7	151	94	58	249	306	342	400	400	400	37.75	23.5	14.5
8	174	91	66	226	309	334	400	400	400	43.5	22.75	16.5
9	174	159	102	226	241	298	400	400	400	43.5	39.75	25.5
10	129	65	26	271	335	374	400	400	400	32.25	16.25	6.5
11	188	129	70	212	271	330	400	400	400	47	32.25	17.5
13	165	90	101	235	310	299	400	400	400	41.25	22.5	25.25
14	135	96	30	265	304	370	400	400	400	33.75	24	7.5
15	126	85	29	274	315	371	400	400	400	31.5	21.25	7.25
16	180	122	62	220	278	338	400	400	400	45	30.5	15.5
17	150	83	29	250	317	371	400	400	400	37.5	20.75	7.25
18	126	81	26	274	319	374	400	400	400	31.5	20.25	6.5
19	130	77	24	270	323	376	400	400	400	32.5	19.25	6
20	125	45	21	275	355	379	400	400	400	31.25	11.25	5.25
21	151	89	100	249	311	300	400	400	400	37.75	22.25	25
22	122	78	22	278	322	378	400	400	400	30.5	19.5	5.5
23	131	83	28	269	317	372	400	400	400	32.75	20.75	7
24	175	133	62	225	267	338	400	400	400	43.75	33.25	15.5
25	161	80	62	239	320	338	400	400	400	40.25	20	15.5
26	160	77	65	240	323	335	400	400	400	40	19.25	16.25
Mean	152.96	94.64	54	247.04	305.36	346	400	400	400	38.24	23.66	13.5
Median	151	90	62	249	310	338	400	400	400	37.75	22.5	15.5
SD	21.8069	24.2828	27.3054	21.8069	24.28285	27.30537	0	0	0	5.45172	6.070712	6.8263

Table 8.6 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of IFN

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	352	322	281	48	78	119	400	400	400	88	80.5	70.25
2	334	291	277	66	109	123	400	400	400	83.5	72.75	69.25
3	312	254	241	88	146	159	400	400	400	78	63.5	60.25
4	256	275	251	144	125	149	400	400	400	64	68.75	62.75
5	333	292	273	67	108	127	400	400	400	83.25	73	68.25
6	336	294	289	64	106	111	400	400	400	84	73.5	72.25
7	335	324	308	65	76	92	400	400	400	83.75	81	77
8	330	281	271	70	119	129	400	400	400	82.5	70.25	67.75
9	311	255	224	89	145	176	400	400	400	77.75	63.75	56
10	325	295	240	75	105	160	400	400	400	81.25	73.75	60
11	293	286	252	107	114	148	400	400	400	73.25	71.5	63
13	278	245	229	122	155	171	400	400	400	69.5	61.25	57.25
14	329	294	246	71	106	154	400	400	400	82.25	73.5	61.5
15	368	322	295	32	78	105	400	400	400	92	80.5	73.75
16	323	254	222	77	146	178	400	400	400	80.75	63.5	55.5
17	331	291	248	69	109	152	400	400	400	82.75	72.75	62
18	292	253	250	108	147	150	400	400	400	73	63.25	62.5
19	368	336	331	32	64	69	400	400	400	92	84	82.75
20	375	364	330	25	36	70	400	400	400	93.75	91	82.5
21	332	255	242	68	145	158	400	400	400	83	63.75	60.5
22	335	323	293	65	77	107	400	400	400	83.75	80.75	73.25
23	293	276	288	107	124	112	400	400	400	73.25	69	72
24	330	286	283	70	114	117	400	400	400	82.5	71.5	70.75
25	289	249	236	111	151	164	400	400	400	72.25	62.25	59
26	365	344	294	35	56	106	400	400	400	91.25	86	73.5
Mean	325	290.44	267.76	75	109.56	132.24	400	400	400	81.25	72.61	66.94
Median	330	291	271	70	109	129	400	400	400	82.5	72.75	67.75
SD	29.5212	32.6025	31.1786	29.5212	32.60251	31.17863	0	0	0	7.380295	8.150626	7.7947

Table 8.7 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of GM-CSF

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	249	233	175	151	167	225	400	400	400	62.25	58.25	43.75
2	204	172	136	196	228	264	400	400	400	51	43	34
3	240	209	173	160	191	227	400	400	400	60	52.25	43.25
4	238	189	137	162	211	263	400	400	400	59.5	47.25	34.25
5	285	215	176	115	185	224	400	400	400	71.25	53.75	44
6	252	224	169	148	176	231	400	400	400	63	56	42.25
7	277	210	172	123	190	228	400	400	400	69.25	52.5	43
8	291	273	250	109	127	150	400	400	400	72.75	68.25	62.5
9	213	188	157	187	212	243	400	400	400	53.25	47	39.25
10	212	193	169	188	207	231	400	400	400	53	48.25	42.25
11	250	241	172	150	159	228	400	400	400	62.5	60.25	43
13	245	229	211	155	171	189	400	400	400	61.25	57.25	52.75
14	284	271	216	116	129	184	400	400	400	71	67.75	54
15	244	215	210	156	185	190	400	400	400	61	53.75	52.5
16	212	189	173	188	211	227	400	400	400	53	47.25	43.25
17	210	188	166	190	212	234	400	400	400	52.5	47	41.5
18	208	175	165	192	225	235	400	400	400	52	43.75	41.25
19	277	250	242	123	150	158	400	400	400	69.25	62.5	60.5
20	253	244	216	147	156	184	400	400	400	63.25	61	54
21	245	229	137	155	171	263	400	400	400	61.25	57.25	34.25
22	253	241	211	147	159	189	400	400	400	63.25	60.25	52.75
23	250	277	249	150	123	151	400	400	400	62.5	69.25	62.25
24	291	270	247	109	130	153	400	400	400	72.75	67.5	61.75
25	279	231	215	121	169	185	400	400	400	69.75	57.75	53.75
26	255	214	205	145	186	195	400	400	400	63.75	53.5	51.25
Mean	248.68	222.8	189.96	151.32	177.2	210.04	400	400	400	62.17	55.7	47.49
Median	250	224	175	150	176	225	400	400	400	62.5	56	43.75
SD	27.4723	31.0403	35.2792	27.4723	31.0403	35.27922	0	0	0	6.868072	7.760074	8.8198

Table 8.8 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of PWM

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	189	135	117	211	265	283	400	400	400	47.25	33.75	29.25
2	210	175	156	190	225	244	400	400	400	52.5	43.75	39
3	172	133	121	228	267	279	400	400	400	43	33.25	30.25
4	209	170	192	191	230	208	400	400	400	52.25	42.5	48
5	172	160	155	228	240	245	400	400	400	43	40	38.75
6	179	161	152	221	239	248	400	400	400	44.75	40.25	38
7	186	161	123	214	239	277	400	400	400	46.5	40.25	30.75
8	249	215	210	151	185	190	400	400	400	62.25	53.75	52.5
9	211	202	188	189	198	212	400	400	400	52.75	50.5	47
10	171	166	134	229	234	266	400	400	400	42.75	41.5	33.5
11	209	201	190	191	199	210	400	400	400	52.25	50.25	47.5
13	212	199	123	188	201	277	400	400	400	53	49.75	30.75
14	201	190	134	199	210	266	400	400	400	50.25	47.5	33.5
15	212	166	123	188	234	277	400	400	400	53	41.5	30.75
16	177	168	152	223	232	248	400	400	400	44.25	42	38
17	170	175	190	230	225	210	400	400	400	42.5	43.75	47.5
18	252	211	189	148	189	211	400	400	400	63	52.75	47.25
19	200	172	155	200	228	245	400	400	400	50	43	38.75
20	176	215	186	224	185	214	400	400	400	44	53.75	46.5
21	209	173	149	191	227	251	400	400	400	52.25	43.25	37.25
22	199	165	127	201	235	273	400	400	400	49.75	41.25	31.75
23	174	166	163	226	234	237	400	400	400	43.5	41.5	40.75
24	211	198	188	189	202	212	400	400	400	52.75	49.5	47
25	215	170	161	185	230	239	400	400	400	53.75	42.5	40.25
26	216	150	215	184	250	185	400	400	400	54	37.5	53.75
Mean	199.24	175.88	159.72	200.76	224.12	240.28	400	400	400	49.81	43.97	39.93
Median	201	170	155	199	230	245	400	400	400	50.25	42.5	38.75
SD	22.6335	22.6133	30.0521	22.6335	22.61327	30.05207	0	0	0	5.658364	5.653318	7.513

Table 8.9 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on addition of Control

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**	**D1**	**D3**	**D5**
1	210	199	119	190	201	281	400	400	400	52.5	49.75	29.75
2	173	149	129	227	251	271	400	400	400	43.25	37.25	32.25
3	209	159	124	191	241	276	400	400	400	52.25	39.75	31
4	209	170	156	191	230	244	400	400	400	52.25	42.5	39
5	214	192	130	186	208	270	400	400	400	53.5	48	32.5
6	211	197	126	189	203	274	400	400	400	52.75	49.25	31.5
7	171	163	150	229	237	250	400	400	400	42.75	40.75	37.5
8	169	188	133	231	212	267	400	400	400	42.25	47	33.25
9	213	200	167	187	200	233	400	400	400	53.25	50	41.75
10	216	230	211	184	170	189	400	400	400	54	57.5	52.75
11	210	159	127	190	241	273	400	400	400	52.5	39.75	31.75
13	205	196	132	195	204	268	400	400	400	51.25	49	33
14	204	192	134	196	208	266	400	400	400	51	48	33.5
15	210	125	95	190	275	305	400	400	400	52.5	31.25	23.75
16	206	231	200	194	169	200	400	400	400	51.5	57.75	50
17	202	170	129	198	230	271	400	400	400	50.5	42.5	32.25
18	231	158	97	169	242	303	400	400	400	57.75	39.5	24.25
19	200	150	155	200	250	245	400	400	400	50	37.5	38.75
20	209	166	127	191	234	273	400	400	400	52.25	41.5	31.75
21	213	206	189	187	194	211	400	400	400	53.25	51.5	47.25
22	244	201	128	156	199	272	400	400	400	61	50.25	32
23	177	120	112	223	280	288	400	400	400	44.25	30	28
24	214	241	170	186	159	230	400	400	400	53.5	60.25	42.5
25	208	152	133	192	248	267	400	400	400	52	38	33.25
26	214	121	126	186	279	274	400	400	400	53.5	30.25	31.5
Mean	205.68	177.4	139.96	194.32	222.6	260.04	400	400	400	51.42	44.35	34.99
Median	209	170	130	191	230	270	400	400	400	52.25	42.5	32.5
SD	17.2499	33.0593	28.8826	17.2499	33.05929	28.88264	0	0	0	4.312482	8.264823	7.2207

Table 8.10 Results of the B-CLL survival counted at day 1 to day 5 and percentage survival on B-CLL cells cultured $ 4 degrees without cytokines

	Number of S cells			Number of A cells			Total			% of Survival		
PT No	D1	D3	D5	D1	D3	D5	D1	D3	D5	D1	D3	D5
1	252	231	205	148	169	195	400	400	400	63	57.75	51.25
2	249	203	157	151	197	243	400	400	400	62.25	50.75	39.25
3	289	280	253	111	120	147	400	400	400	72.25	70	63.25
4	245	211	191	155	189	209	400	400	400	61.25	52.75	47.75
5	253	251	244	147	149	156	400	400	400	63.25	62.75	61
6	250	249	241	150	151	159	400	400	400	62.5	62.25	60.25
7	253	244	246	147	156	154	400	400	400	63.25	61	61.5
8	213	208	199	187	192	201	400	400	400	53.25	52	49.75
9	251	243	230	149	157	170	400	400	400	62.75	60.75	57.5
10	253	245	198	147	155	202	400	400	400	63.25	61.25	49.5
11	239	211	216	161	189	184	400	400	400	59.75	52.75	54
13	257	245	203	143	155	197	400	400	400	64.25	61.25	50.75
14	215	211	197	185	189	203	400	400	400	53.75	52.75	49.25
15	255	243	213	145	157	187	400	400	400	63.75	60.75	53.25
16	215	211	168	185	189	232	400	400	400	53.75	52.75	42
17	217	248	210	183	152	190	400	400	400	54.25	62	52.5
18	255	212	169	145	188	231	400	400	400	63.75	53	42.25
19	209	209	167	191	191	233	400	400	400	52.25	52.25	41.75
20	212	251	199	188	149	201	400	400	400	53	62.75	49.75
21	213	200	176	187	200	224	400	400	400	53.25	50	44
22	255	241	236	145	159	164	400	400	400	63.75	60.25	59
23	216	211	166	184	189	234	400	400	400	54	52.75	41.5
24	256	239	214	144	161	186	400	400	400	64	59.75	53.5
25	215	216	173	185	184	227	400	400	400	53.75	54	43.25
26	214	210	199	186	190	201	400	400	400	53.5	52.5	49.75
Mean	238.04	228.92	202.8	161.96	171.08	197.2	400	400	400	59.51	57.23	50.7
Median	249	231	199	151	169	201	400	400	400	62.25	57.75	49.75
SD	21.7591	20.9203	27.9717	21.7591	20.92033	27.97171	0	0	0	5.439765	5.230081	6.9929

Appendix III: The ANOVA procedure and Bonferroni (Dunn) t Test results comparing the difference in survival at day 5 on B-CLL cells treated with different mitogen.

Difference in Survival at Day 5 on treatment of B-CLL cells cultured with different mitogens

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
GF	8	CONTROL GM-CSF IFN IL-10 IL-2 IL-6 IL-7 PWM

Number of Observations Read	200
Number of Observations Used	200

Dependent Variable: Day 1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	33563.72875	4794.81839	132.80	<.0001
Error	192	6932.22000	36.10531		
Corrected Total	199	40495.94875			

R-Square	Coeff Var	Root MSE	Day 1 Mean
0.828817	10.41787	6.008770	57.67750

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GF	7	33563.72875	4794.81839	132.80	<.0001

The ANOVA Procedure

Dependent Variable: Day 3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	39022.56969	5574.65281	119.23	<.0001
Error	192	8976.98500	46.75513		
Corrected Total	199	47999.55469			

R-Square	Coeff Var	Root MSE	Day 3 Mean
0.812978	13.88204	6.837772	49.25625

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GF	7	39022.56969	5574.65281	119.23	<.0001

Dependent Variable: Day 5

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	46161.10000	6594.44286	125.59	<.0001
Error	192	10081.27500	52.50664		
Corrected Total	199	56242.37500			

R-Square	Coeff Var	Root MSE	Day 5 Mean
0.820753	16.69619	7.246147	43.40000

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GF	7	46161.10000	6594.44286	125.59	<.0001

The ANOVA Procedure: Bonferroni (Dunn) t Tests at Day 1

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	192
Error Mean Square	36.10531
Critical Value of t	3.16806
Minimum Significant Difference	5.3842

Means with the same letter are not significantly different.			
Bon Grouping	**Mean**	**N**	**GF**
A	81.250	25	IFN
B	71.620	25	IL-2
C	62.170	25	GM-CSF
C			
C	58.830	25	IL-6
D	51.420	25	CONTRO
D			
D	49.810	25	PWM
D			
D	48.080	25	IL-7
E	38.240	25	IL-10

The ANOVA Procedure : Bonferroni (Dunn) t Tests at Day 3

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	192
Error Mean Square	46.75513
Critical Value of t	3.16806
Minimum Significant Difference	6.1271

Means with the same letter are not significantly different.			
Bon Grouping	**Mean**	**N**	**GF**
A	72.610	25	IFN
B	62.390	25	IL-2
C	55.700	25	GM-CSF
C			
C	51.550	25	IL-6
D	44.350	25	CONTRO
D			
D	43.970	25	PWM
D			
D	39.820	25	IL-7
E	23.660	25	IL-10

The ANOVA Procedure : Bonferroni (Dunn) t Tests at Day 5

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	192
Error Mean Square	52.50664
Critical Value of t	3.16806
Minimum Significant Difference	6.493

Means with the same letter are not significantly different.			
Bon Grouping	**Mean**	**N**	**GF**
A	66.940	25	IFN
B	58.130	25	IL-2
C	49.500	25	IL-6
C			
C	47.490	25	GM-CSF
D	39.930	25	PWM
D			
D	36.720	25	IL-7
D			
D	34.990	25	CONTRO
E	13.500	25	IL-10

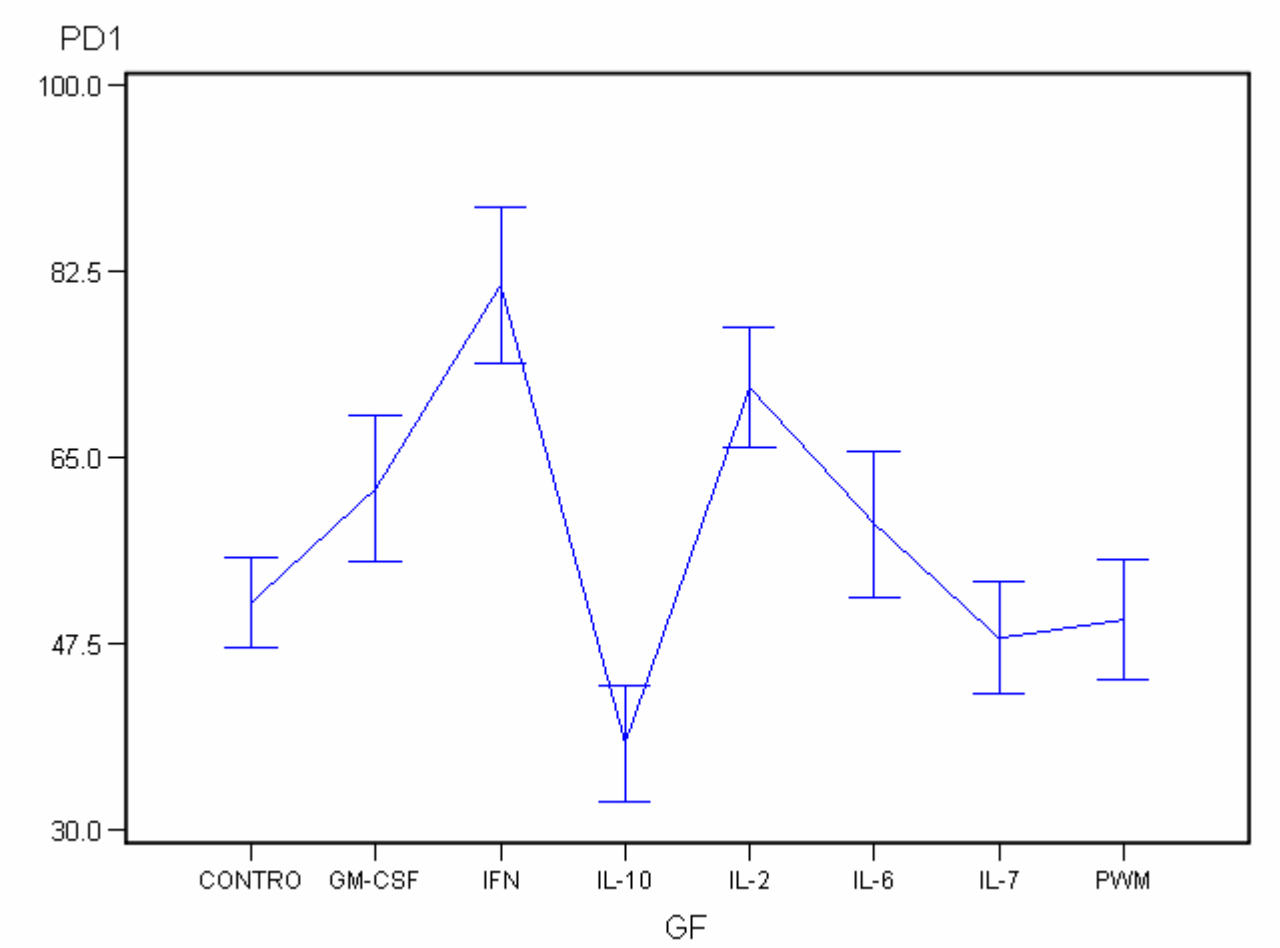
Difference in Survival at Day 5 on B-CLL cells treated with different mitogens

Means and Descriptive Statistics

GF	Mean of Day 1	Mean of Day 3	Mean of Day 5	Std. Dev. of Day 1	Std. Dev. of Day 3	Std. Dev. of Day 5
	57.6775	49.2563	43.40	14.2652	15.5307	16.8115
CONTRO	51.4200	44.3500	34.99	4.3125	8.2648	7.2207
GM-CSF	62.1700	55.7000	47.49	6.8681	7.7601	8.8198
IFN	81.2500	72.6100	66.94	7.3803	8.1506	7.7947
IL-10	38.2400	23.6600	13.50	5.4517	6.0707	6.8263
IL-2	71.6200	62.3900	58.13	5.7032	5.2386	6.9777
IL-6	58.8300	51.5500	49.50	6.8487	6.9350	7.4039
IL-7	48.0800	39.8200	36.72	5.2379	5.8934	4.7770
PWM	49.8100	43.9700	39.93	5.6584	5.6533	7.5130

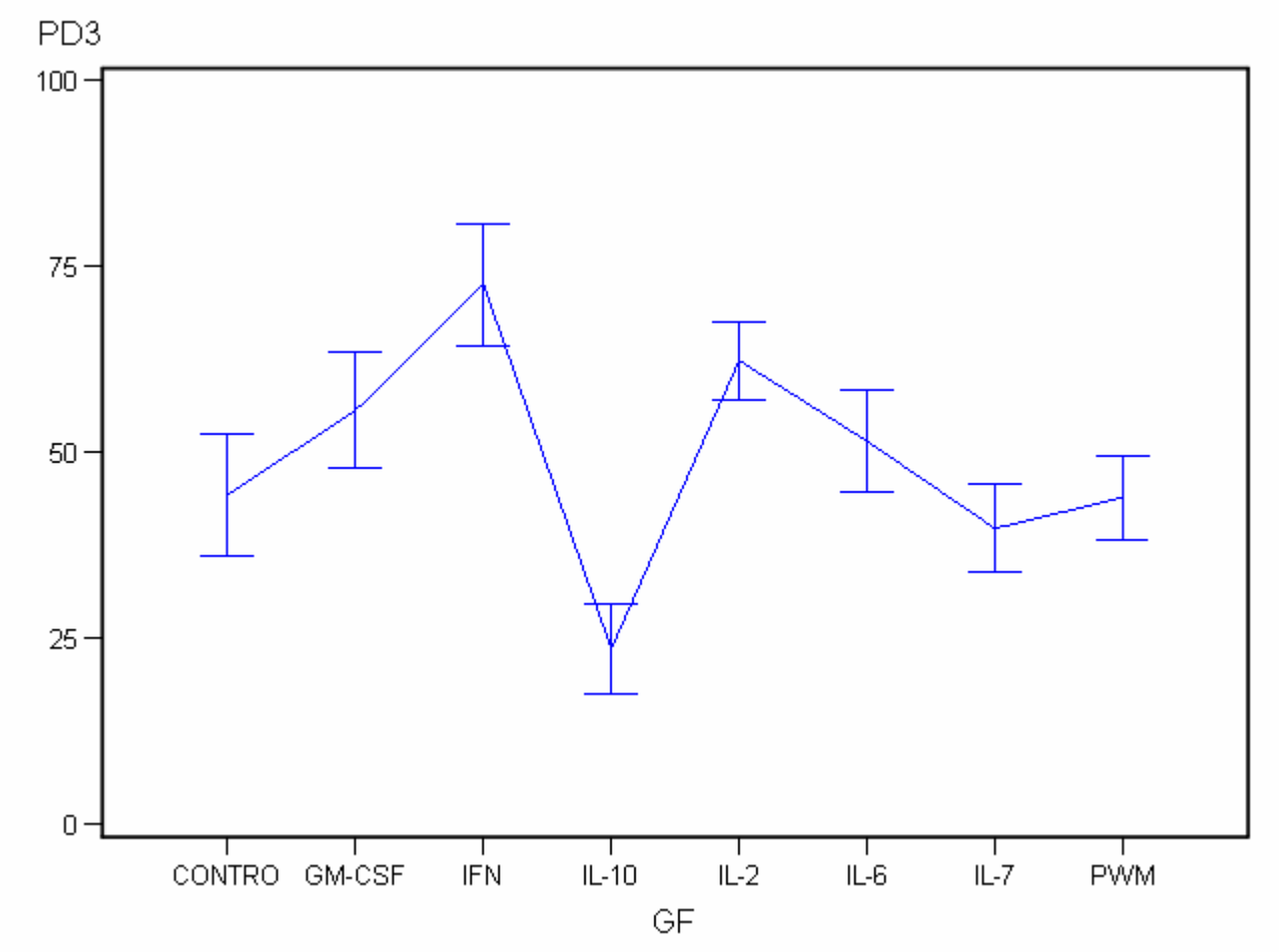
Difference in Survival at Day 1 on B_CLL cells treated with different mitogens

Means Plot of Day 1 by GF



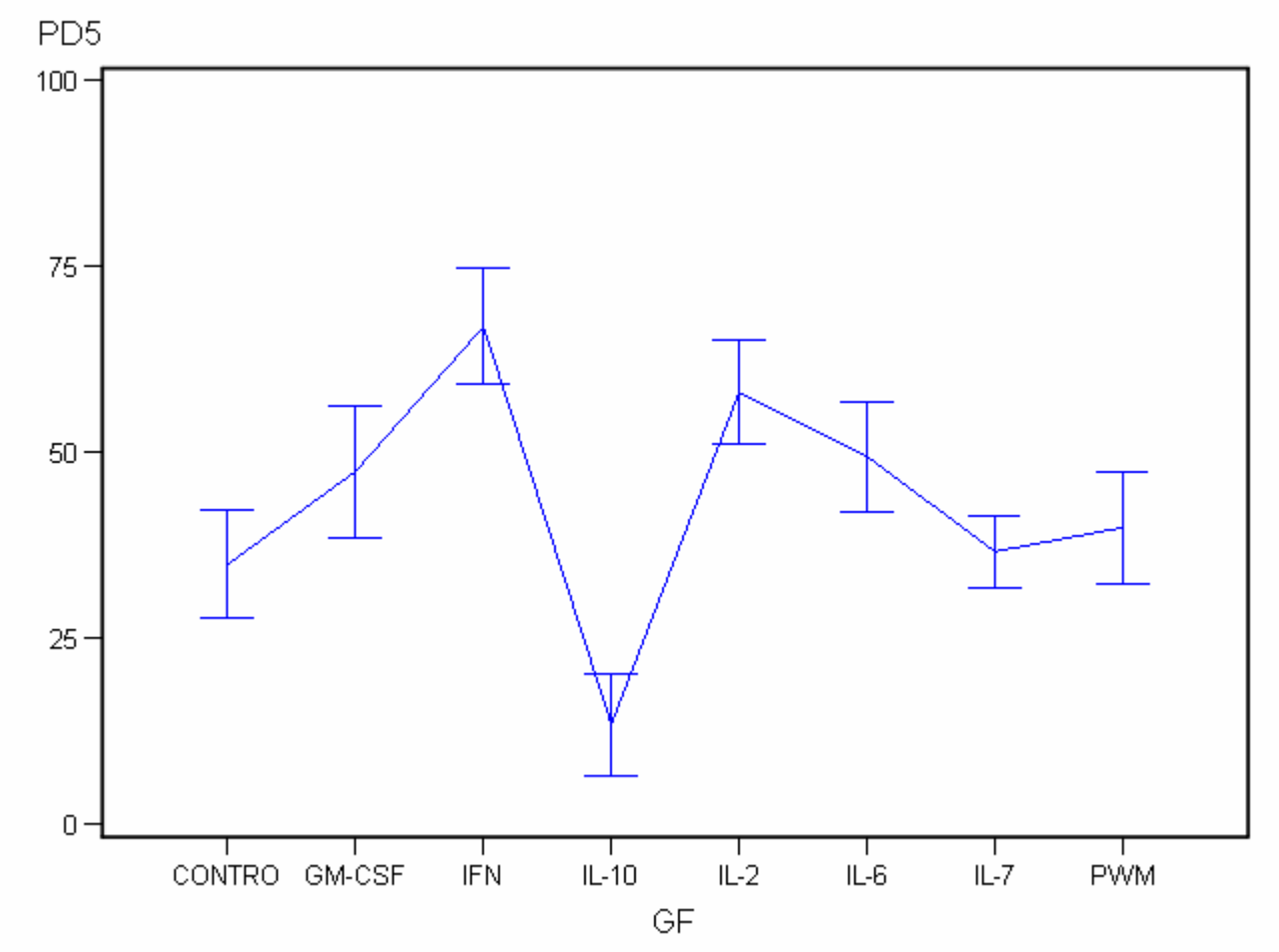
Difference in Survival at Day 3 on B-CLL cells treated with different mitogens

Means Plot of Day 3 by GF



Difference in Survival at Day 5 on B-CLL cells treated with different mitogens

Means Plot of Day 5 by GF



APPENDIX IV:

The University of the Witwatersrand, Johannesburg, Committee for Research on Human Subjects (Medical) Clearance Certificate for Protocol Number 00/05/24