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CYTOKINE EXPRESSION IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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CYTOKINE EXPRESSION IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

by

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ABSTRACT

B-cell chronic lymphocytic leukaemia (B-CLL) results from a clonal accumulation of CD5+ve B-lymphocytes. This expansion of B-lymphocytes is due to increased proliferation and extended survival secondary to decreased apoptosis. Both proliferation and apoptosis are regulated by a group of soluble proteins, termed cytokines, which are secreted by cells and play a key role in the regulation of immunological responses. In view of this, the role of cytokines in the pathogenesis of B-CLL was studied.

Interleukin 4 (IL-4) is an anti-apoptotic cytokine which up-regulates bcl-2 in malignant B-lymphocytes. Peripheral blood samples, from untreated B-CLL patients were investigated, together with a group of ten healthy controls. Flow cytometric analysis was used to quantify (i) the expression of intracellular IL-4 by CD19+ and CD3+ lymphocytes, and (ii) IL-4R expression of CD19+ and CD3+ lymphocytes. The proportion of CD19+ve lymphocytes expressing intracellular IL-4 was significantly higher in patients with B-CLL than in controls ($p=0.03$). Similarly, the proportion of CD3+ve lymphocytes expressing intracellular IL-4 was significantly higher in B-CLL patients than controls ($p=0.01$). No significant difference was found in the overall proportion of CD19+ve lymphocytes expressing IL-4R between patients and controls. However, the proportion of CD3+ve lymphocytes expressing IL-4R was significantly greater in patients than in controls ($p=0.001$).

A number of anti-apoptotic cytokines, including IL-2, IL-4, IL-7, IL-9 and IL-15 share a sub-unit known as the common gamma chain (γc). A nested RT-PCR technique was used to analyse full-length γc receptor RT-PCR products in B and T-lymphocytes from patients with B-CLL and controls. The presence and concentration of full size IL-2, IL-4, IL-7, IL-9, IL-15, γc, IL-4R α chain, as well as IL-2 delta 2 (IL-2δ2) and IL-4 delta 2 (IL-2δ2) spliced RT-PCR products were measured. IL-2 full-length RT-PCR products (using exon 1-4 amplifiers) were present ta a significantly more present at higher concentration in patient when compared to control B-lymphocyte samples ($p=0.04$). No significant difference was found between IL-4 full-length RT-PCR products in patients than in controls. IL-7 RT-PCR products were present at higher concentration in patient B- and T-lymphocytes than in controls ($p=0.034$ and $p=0.041$ respectively). IL-15 RT-PCR products were present at a lower concentration in patient than in control T-lymphocytes ($p=0.001$). IL-2 and IL-4 wild RT-PCR products (using exon 1-3 amplifiers) were present at significantly higher concentration in patient B-lymphocytes ($p=0.0001$ and $p=0.026$ respectively). The concentration of IL-4δ2 and the IL-4Rα RT-PCR products were significantly lower in patient than in control T-lymphocytes

(p=0.0001 for both). There was a significant difference in the concentration of the IL-2 δ2 RT-PCR products between patient and control B-lymphocytes (p=0.029).

In view of the above results, the apoptotic effect of the addition of IL-2 and IL-4 antisense oligonucleotides (ONs) to B-CLL and control cells was investigated. ONs were designed to block wild type IL-2 and IL-4 mRNA transcript expression as well as their spliced variants IL-2δ2, and IL-4δ2. The percentages of CD19+/Annexin V+, CD3+/Annexin V+ or Propidium iodide (PI)+/Annexin V+ cells, as well as the percentage of CD19+ve and CD3+ve cells expressing intracellular IL-2, was measured by flow cytometry. The results demonstrated that, when B-CLL cells were incubated with IL-2 ONs, the percentage of CD19+/Annexin V+, as well as PI+/Annexin V+, lymphocytes were significantly increased (p=0.002). The addition of IL-4 and control antisense ONs had a similar, but not statistically significant effect. This pro-apoptotic effect was not seen in B-CLL CD3+ve lymphocytes. ELISA analysis showed that addition of IL-2 ONs decreased the level of IL-2 protein secreted by PHA-stimulated B-CLL cells but the addition of IL-4 antisense ONs increased the level of IL-4 protein. Finally, addition of ONs decreased the percentage of CD19+ve and CD3+ve B-CLL lymphocytes expressing intracellular IL-2 and IL-4.

In summary, the results obtained in this study imply that IL-2, IL-4, IL-7 and IL-15 may play a role in the survival of malignant B-lymphocytes in B-CLL.

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Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

All the work presented in this study was performed by the author.

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PUBLICATIONS & CONFERENCE PRESENTATIONS

Peer reviewed publications

1. Kaminski A, Demaine A, Prentice A.
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Published abstracts

2. Prentice AG, Kaminski A, Hurlock N, Copplestone JA & Demaine A.
   Increased IL-4 accumulation in B-CLL; evidence for autocrine or paracrine involvement in leukaemogenesis.
   Blood 1997, 90: 354a

3. Kaminski A, Demaine A, Prentice AG.
   In chronic lymphocytic leukaemia, both leukaemic B and normal T cells express IL4-m-RNA, but decreased amounts of the IL-4 spliced variant, IL-4 delta 2.
   Blood 1998, 92 (suppl 1): 434a.

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   Blood 1999, 94 (suppl 1): 300

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   British Journal of Haematology 2000, 108 (suppl 1): 74

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   The Haematology Journal 2001 (suppl 1): 38

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1. International Society for Experimental Haematology (ISEH), 26th meeting, 1997, Cannes (poster)
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3. American Society for Haematology (ASH), 39th Annual Meeting, 1997, San Diego (poster)
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Abbreviations

A	Adenine
APC	Antigen presenting cell
Apaf-1	Apoptotic protease-activating factor-1
B-CLL	B cell chronic lymphocytic leukaemia
bp	Base pairs
BSA	Bovine serum albumin
BLV	Bovine leukaemia virus
Caspases	Cysteine aspartate specific proteases
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CDI	Cyclin dependent inhibitors
cFLIP	FLICE-like inhibitory protein
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CyD1	Cyclin D1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	2' deoxyribonucleotide 5'-triphosphates
DMSO	Dimethyl sulphoxide
EDTA	Ethylene diamine tetra-acetic acid
EB	Ethidium bromide
EBV	Epstein-Barr virus
ELISA	Enzyme Linked Immunoabsorbent Assay
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FASL	Fas-ligand

FCL	Follicular Lymphoma
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G	Guanosine
HGPRT	Hypoxanthine guanine phosphoribosol trasferase
HMDS	Haematological Malignancies Diagnostic Services
HLA	Human leukocyte antigen
HCL	Hairy cell leukaemia
HCL-v	HCL-variant
HS	Human serum
IAP	Inhibitor of apoptosis
ICAM	Intracellular adhesion molecule
ICE	Interleukin-1β converting enzyme
INF	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin 1 receptor
IU	International units
Kb	Kilo-base
kDa	Kilodalton
LPD	Chronic lymphoproliferative disease
MCL	Mantle cell lymphoma
MHC	Major histocompatibility antigen
MTT	3-(4,5.dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide)
mRNA	Messenger ribonucleic acid

NK	Natural killer cell
ON	Oligonucleotide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
PMA	Phorbol myristic acetate
PI	Propidium iodine
PL	Persistent lymphocytosis
PLL	Prolymphocytic leukaemia
PS	Phosphatidylserine
PTO	Phosphorothioate
rRNase	Ribonuclease
RT	Room temperature
RT-PCR	Riverse transcriptase PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrilamide gel electrophoresis
Smac/DIABLO	Mitochondrial protein
SSC	Side scatter
T	Thymine
TBS	Tris-buffered saline
TCR	T cell receptor
Tc	T cytotoxic cell
Th	T helper cell
TGF	Transforming growth factor
TdT	Terminal deoxynucleotydil transferase
Tm	Melting temperature

tRNA	Transfer RNA
TRAIL	Tumour necrosis factor-related apoptosis-inducing factor
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
ug	Microgram
UV	Ultraviolet
U	Uracyl
XSCID	X-linked combined immunodeficiency

CHAPTER 1: INTRODUCTION

1.1. NATURE OF HAEMATOLOGICAL MALIGNANCIES

Blood is the very essence of life, the "circulatory computer tape'' that carries various coded cellular and humoral messages in order to protect the organs and tissues of the body against foreign antigens (Page, 1972). However for a very long time, nothing was known about its real purpose and function, until the construction of the first microscope by Antonj van Leeuwenhoek (1632-1723), when red blood cells were described as "ruddy globules''. Subsequently, William Hewson (1739-1774) discovered lacteal and lymphatic vessels in birds and reptiles, and he was the first to discover red blood cells and leukocytes. In addition, he noticed that the cellular content of the lymph was nucleated and believed that these cells were formed in the lymph nodes. He also showed that the thymus discharges huge numbers of cells into a thoracic duct and into the blood (Hewson, 1774).

Using the newly discovered microscope, various types of white cells in the blood were further described. This is attributed to Paul Ehrlich (Ehrlich, 1880). He drew the distinction between lymphocytes and other leukocytes by emphasising their different origins and proposed that lymphocytes originate in lymph nodes and spleen while other classes of leukocyte are produced in the bone marrow. He also developed a theory of antibody production (Ehrlich, 1900). However, the rather uniform appearence of lymphocytes did not reveal their astonishing diversity until the mid-1960s, when they were described as thymus derived T-lymphocytes and "bursa equivalent" or thymus independent B-lymphocytes (Roitt et al. 1964). Equally long and laborious is the story of haematological malignancies. Initially, described as a pathological curiosity, leukaemia was almost simultaneously discovered by Virchow in Berlin (Virchow, 1845) and Bennett in Edinburgh (Bennett, 1845), although others reported seeing patients with large spleens and characteristic blood changes. Both saw the changes in the blood of leukaemic patients but described them differently. Bennett called the condition, "pyemia'', meaning blood containing pus. Virchow however, could find no inflammation that might lead to the formation of the pus. He described the cell as "white cells'' which,

translated to Greek later became known as *leukaemia*. It was soon apparent that leukaemia is not one disease. Virchow himself proposed to subdivide leukaemia into two forms. One was "splenic" or "lienal" leukaemia, characterised by great swelling of the spleen and the other form characterised by the enlargement of the lymph nodes was called "lymphatic" leukaemia. Ebstein in 1889 (Ebstein, 1889) proposed to further subdivide leukaemia into acute and chronic forms. The term acute and chronic lymphatic is still in use for the classification of leukaemias. Leukaemia is a malignant neoplasm, the term used to describe abnormal cellular growth. The term 'malignant' is used because of the ability of the abnormally dividing cells to invade normal tissues and spread to distant sites in the host. Malignant neoplasms have abnormal nuclear divisions and chromosomes.

Cancer can be divided into three broad subgroups: carcinomas, sarcomas and leukaemias and lymphomas. The leukaemias and lymphomas arise in the blood-forming cells in the lymph nodes and bone marrow and then invade the mononuclear phagocytic system and remaining body structures. The development of many cancers is a co-ordinated and logical process with a multi-factorial aetiology.

1.2. CLASSIFICATION OF HAEMATOLOGICAL MALIGNANCIES

Chronic lymphoproliferative diseases include different entities characterised by distinct clinico-pathological features (Bennet et al. 1989). Over the past 30 years, the classification of haematological malignancies has undergone continuous change. Described below is a Revised European-American Classification of Lymphoid Neoplasms (R.E.A.L) of the lymphoproliferative disorders (Harris et al. 1994).

1.2.1. Lymphoproliferative disorders

- Lymphoblastic leukaemias
- Mature (peripheral) B-cell tumours
- Mature (peripheral) T-cell disorders
- Hodgkin's lymphoma

1.2.1.1. Sub-division of lymphoproliferative disorders

Lymphoproliferative disorders can be further sub-divided. With the revolution of molecular biological techniques as well as the application of multiparametric and quantitative flow cytometry, which has improved the diagnostic utility of immunophenotypic analysis, more thorough analyses of T- and B-lymphocyte monoclonality, chromosomal abnormalities and phenotypes are possible (Rothe et al. 1996).

Mature (peripheral) B-cell tumours:

- Chronic lymphocytic leukaemia
- Atypical CLL
- Mantle Cell Lymphoma
- B-prolymphocytic Leukaemia
- Marginal Zone B-cell Lymphoma
- Large cell transformation of CLL
- Follicular Lymphoma
- Non-endemic Burkitt's Lymphoma
- Diffuse Large B-cell Lymphoma
- Plasma Cell Myeloma, Plasmacytoma
- Hairy Cell Leukaemia

1.3. B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

1.3.1. General description

B cell chronic lymphocytic leukaemia (B-CLL) was first described in 1903 by Turk (Turk, 1903). Minot and Isaacs wrote about its clinical features in 1924 and Dameshek further described it in 1967 (Minot et al. 1924 and Dameshek, 1967). Since then knowledge of this disease has expanded considerably. B-CLL is the most common single haematological malignancy, accounting for more than 25% of all leukaemias in the Western world. It is far less common in the Far East. Its incidence among all adult leukaemias ranges from 2.5% in Japan to 38% in Denmark (Brinker, 1982). In West Africa, two peaks among the low rates of B-CLL are observed in women aged 40-44 and in males aged 70 and over. In general, B-CLL is rare before the age of 40, peaks around 60 and affects twice as many males than females. Elderly patients tend to have a more advanced form of the disease.

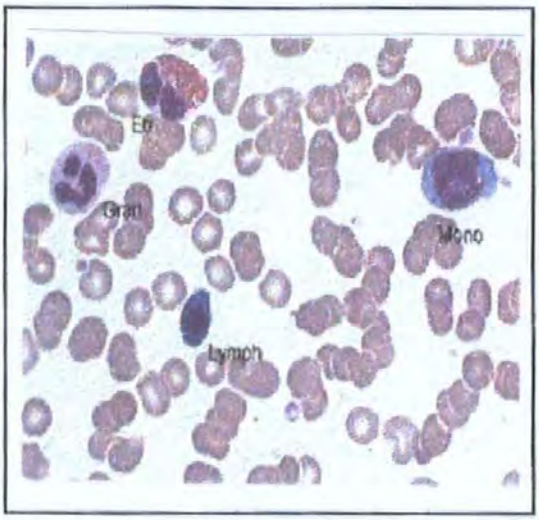
The cause of the disease is unknown, but viruses and environmental factors are suspected. B-CLL has been linked to several occupations and exposures. Increase in B-CLL incidence has been reported among farmers engaged in soybean production, cattle raising and herbicide use (Burmeister et al. 1982 and Blair et al. 1985). Rubber manufacturers as well as asbestos workers also have an increased incidence of B-CLL (Kagan et al. 1983). Many cases are diagnosed following a routine blood test. Of all the leukaemias, B-CLL has the highest familial incidence, with first-degree relatives having a 2 to 7-fold higher chance of developing the disease (Capalbo et al. 2000). Since Japanese individuals, including those who migrated to Hawaii, have a much lower incidence of the disease there is speculation that genetic influences are stronger than environmental influences (Capalbo et al. 2000).

Although classified as a proliferative disorder, in the majority of patients, B-CLL is a disease with little evidence of an aggressive proliferation of the abnormal lymphocytes at least in early stage (Binet et al. 1981, Baccarini et al. 1982, and Catovsky et al. 1989). The malignant lymphocytes are the result of a clonal expansion which has been arrested at an early stage of

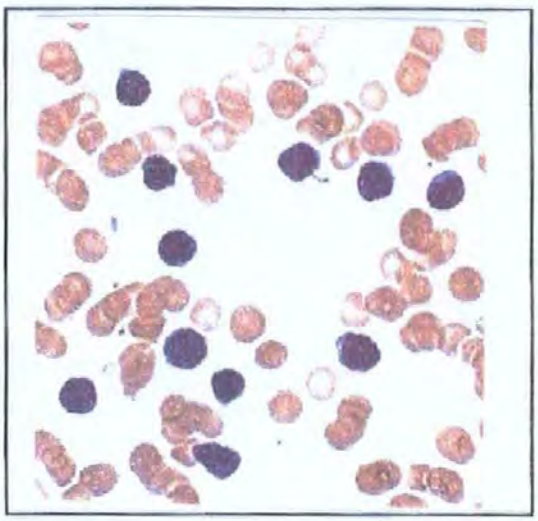
differentiation and as a result appears cytologically mature but biologically immature (Dameshek et al. 1967, Dighiero et al. 1991). The cells are an expansion of a rare CD5+ve B-lymphocyte population, normally seen in the mantle zone of normal peripheral lymph nodes (Bannerji, 2000). This leads to an accumulation of lymphocytes, a progressive enlargement of tumour burden and eventually the death of the host. The progressive accumulation of lymphocytes starts in lymph nodes and/or bone marrow gradually expands to most of the haemopoietic organs (Dameshek et al. 1967). Involvement of other organs such as central nervous system or peripheral nerves, skin (Bonvalet et al. 1984), lungs (Palosarri et al. 1986) kidneys (Dabbs et al. 1986), and bones and lachrymal glands (Hansen, 1973) is also possible but rare at diagnosis.

Figure 1.1: Morphology of normal and B-CLL lymphocytes

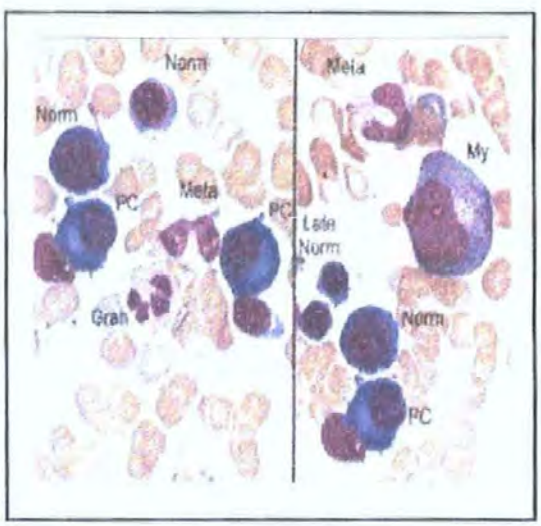
A) Normal blood film



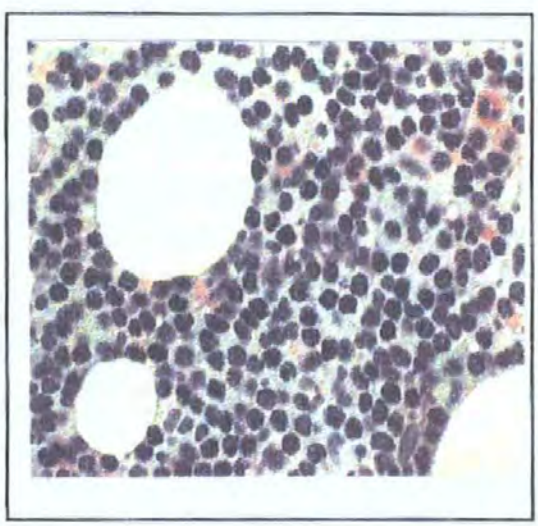
B) B-CLL blood film



C) Normal bone marrow stain



D) B-CLL bone marrow stain



Normal and B-CLL peripheral blood and trephine biopsy stain. A) Normal peripheral blood May-Grunwald-Giemse (MGG) stain: Gran=granulocytes, Eo=eosinophil, Mono=monocytes, Lymph, lymphocytes B) B-CLL stain: peripheral blood film shows lymphocytes with rims of cytoplasm, coarse condensed nuclear chromatin and rare nucleoli C) Normal bone marrow stain: Nor=normoblast, Gran=granulocyte, My=myelocyte, PC=plasma cell, Meta=metamyelocyte D) Bone marrow trephine biopsy: infiltration by small leukaemic B-CLL cells. Adapted from Hoffbrand et al. 1993 and Haematological malignancy Diagnostic Service (HMDS) classification, Website.

1.3.2. Clinical features of B-CLL

In many patients, symmetrical enlargement of superficial cervical and axilliary lymph nodes is found, but the nodes are usually discrete, smooth, mobile and non-tender and vary from 1 to 2 cm. It is possible to document lymph enlargement in the hilar regions on routine radiography, or in the retroperitoneal regions by ultrasound or CT scanning. Increasing tiredness and excessive sweating or night sweats are present with malaise and weight loss. Splenomegaly and hepatomegaly are found in 50% of the patients and an enlarging abdomen could be the primary complaint. Due to reduced humoral and cellular responses, patients experience a greater tendency to bacterial and viral (mainly herpes zoster and simplex) but also mycobacterial and fungal infections than normal healthy individuals (Bannerji, 2000). Hypogammaglobulinaemia, specific antibody deficiency, depleted cell-mediated immunity, neutropenia and poor opsonisation are the main causes of immunodeficiency (Chapel et al. 1987). A severe chest infection or pneumonia could be the first manifestation of B-CLL (Bannerji, 2000).

Associated autoimmune phenomena are related to the presence of autoantibodies directed mainly against blood components (Hamblin et al. 1986). This can lead to the development of autoimmune haemolytic anaemia or thrombocytopenia. Autoimmune haemolytic anaemia occurs in 10-25% of patients at some time during the course of the disease (Sthoeger et al. 1993). With time, anaemia, thrombocytopenia and neutropenia develop as a result of bone marrow failure.

1.3.3. Laboratory findings

The diagnosis should be considered in any patient in whom the lymphocyte count consistently exceeds 4.5 x 10^9/l. The peripheral blood film reveals a lymphocytosis, predominantly due to a population of small lymphocytes with a high nuclear to cytoplasmic ratio similar in appearance to normal, mature peripheral blood lymphocytes. The autoantibodies described

are warm reactive polyclonal IgG and display activity against monomorphic antigens of the rhesus system (Dighiero et al. 1988). Reduced concentrations of immunoglobulins are found and this becomes more marked with advancement of the disease. This decline starts with IgA levels. Monoclonal bands, often IgM (M band) are seen in less than 10% of cases. The appearance of an M band and discovery of free light chains in the urine (Bence-Jones proteinuria) may be an indication of transformation of the disease. Bone marrow examination is an essential investigation in B-CLL. The aspirate is useful to confirm morphological features particularly in-patients with low white blood cells (WBC) counts and to assess haematopoiesis. B-CLL does not transform into acute leukaemia, although immunoblastic transformation may occur as a terminal lymphoma (Richter syndrome) (Bannerji, 2000).

1.3.4. Diagnosis of B-CLL

B-CLL is diagnosed by the presence of an absolute and persistent lymphocytosis greater than $5x10^9$ lymphocytes per litre with bone marrow infiltration by these cells equal to or greater than 30% of all nucleated cells (Bennet et al. 1987, Cheson et al. 1988, Bannerji, 2000). Morphologically, the lymphocytes in the blood films are small, showing scanty cytoplasm and a characteristic pattern of nuclear chromatin clumping (Bannerji, 2000). Some variation in size and shape may be seen and occasionally cells with immature or even blast cell characteristics may be noted. A feature of the blood film is also the appearance of 'smear cells'. Smear cells are artefacts due to rupture of the nuclear and cell membranes during preparation of the blood film (Figure 1.1). However, the proportion of smear cells in the blood film correlates directly with the lymphocyte count. Lymphoid infiltration described as nodular, interstitial mixed (nodular and interstitial) and diffuse coincides with disease progression (Rozman et al. 1984, Pangalis et al. 1988).

At least 70% of cases presenting with lymphocytosis are identified as B-CLL (Catovsky, 1997). Their immunophenotype typically shows a low expression of surface IgM and IgD

with clonal restricted light chains (Bannerji, 2000). B-lymphocyte antigens like CD19, CD20 and CD23 (the hallmark of the disease with expression correlating with the disease prognosis) are co-expressed with CD5 in almost all cases (Kneitz et al. 2000). CD10 and CD22 are negative, whereas CD11c and CD25 may be expressed but in most cases are only weakly positive (Dameshek et al. 1967, Knapp et al. 1989, Ginaldi et al. 1998). In general, antigen density is weaker than on normal lymphocytes. The surface immunoglobulin (sIg) is often IgM with frequent co-expression of IgD. Infrequently IgD predominates. This is shown to be monoclonal because of the expression of one form of light chain, either kappa (κ) or lambda (λ) (Dighiero et al. 1993).

CD38 is a transmembrane glycoprotein expressed on the surface of leukaemic cells in a significant percentage of patients with B-CLL (40% of the patients). Patients with high expression (20% or more) have significantly shorter survival times (Ibrahim et al. 2001). Malignant B-lymphocytes were considered antigen-inexperienced 'virgin' lymphocytes but there is some evidence that at least half of these cases represent expansion of previously triggered, post germinal centre (GC) 'memory' B-lymphocytes. This is based on the presence of somatic mutations in the heavy (H) chain variable region (V) genes (Damle et al. 1999).

1.3.5. Malignant B-lymphocyte markers and adhesion molecules

1.3.5.1. CD5

This is a marker expressed on the majority of peripheral blood T-lymphocytes and a sub-population of B-lymphocytes. In 1991, a new nomenclature for CD5+ve B-lymphocytes was suggested. B-lymphocytes expressing the CD5 antigen were defined as B1 lymphocytes and conventional B-lymphocytes as B2 (Kantor, 1991).

The CD5 molecule is tyrosine phosphorylated and co-precipitates following cross linking of the B-cell receptor (Lankester et al. 1994). In adult humans, 5% to 30% of the circulating B-lymphocytes are B1, with fewer than 10% in the spleen and less than 30% in lymph nodes

(Ladyard et al. 1987). The population of CD5+ve B-lymphocytes use a restricted set of Ig genes and produce natural auto antibodies (mostly IgM) against single stranded (ss) DNA or IgG (Hardy et al. 1994). CD27 has been shown to be the native ligand for CD5 and is expressed on normal and leukaemic cells (Garllard et al. 1994). It was hypothesised that CD5+ve B-lymphocyte antigen presenting cells could interact with CD27 on the B2 lymphocytes and this would augment the signal delivered through the specific antigen receptor by thymus-independent antigens (Gordon, 1994). Mice deficient in CD5+ve B-lymphocytes are not able to mount responses to thymus independent antigens (Gordon, 1994).

1.3.5.2. CD19

CD19 is expressed during all stages of B-cell differentiation and maturation, except on plasma cells (Tedder et al. 1989 and Zhou et al. 1995). It is also present on follicular dendritic cells, but is not found on T-lymphocytes or normal granulocytes. CD19 plays a role in regulation of B-lymphocyte proliferation. It associates with the complement receptor 2 (CD21), CD81, Leu-13 and / or MHC class II to form a signal transduction complex on the surface of B-lymphocytes (Bradbury et al. 1992 and Tedder et al. 1994). CD19 closely resembles the T-cell receptor and belongs to the Ig superfamily.

1.3.5.3. CD20

CD20 is expressed on pre-B-lymphocytes, resting and activated B-lymphocytes but not on plasma cells. Low level of expression is observed on a small subset of normal circulating T-lymphocytes (Genot et al. 1991). The CD20 molecule is involved in the regulation of B lymphocyte activation. In malignant B-CLL cells, CD20 is hyperphosphorylated when compared to CD20 on normal resting B-lymphocytes. However, its expression on malignant B-lymphocytes is relatively less intense (Genot et al. 1991).

1.3.5.4. CD21

CD21 is a receptor for the C3d complement fragment and for Ebstein-Barr virus. It is expressed on mature B-lymphocytes, follicular dendritic cells and some epithelial cells. It is also weakly expressed on a subset of T lymphocytes and thymocytes. CD21 plays a role in B-lymphocyte activation and proliferation (Timems et al. 1995). Normal and malignant B lymphocyte when activated via CD21 increase their DNA synthesis and the level of soluble CD19 is increased in B-CLL (Lowe et al. 1989).

1.3.5.5. CD23

CD23 is identical to the low affinity receptor for IgE (FcεRII). It is mainly expressed on mature B-lymphocytes, mantle zone B-lymphocytes and follicular dendritic cells, but not on proliferating cells in the germinal centre. In fact its expression is rapidly lost upon isotype switching (Sarfati et al. 1989). CD23 plays a role in the regulation of IgE responses. Two spliced variants of CD23 (CD23 A and CD23 B) exist (Yokota et al. 1988). CD23 A is expressed on B-lymphocytes and CD23 B on a variety of cell types and its expression is IL-4 dependent. The level of soluble CD23 antigen is increased in B-CLL (Lowe et al. 1989) and its expression is most probably dependent on other cell types (Fournier et al. 1995 and Fournier et al. 1992).

1.3.5.6. CD24

CD24 is expressed on the surface of B-lymphocytes, granulocytes and most B-cell lines. CD24 may play a role in the regulation of B-cell proliferation and maturation.

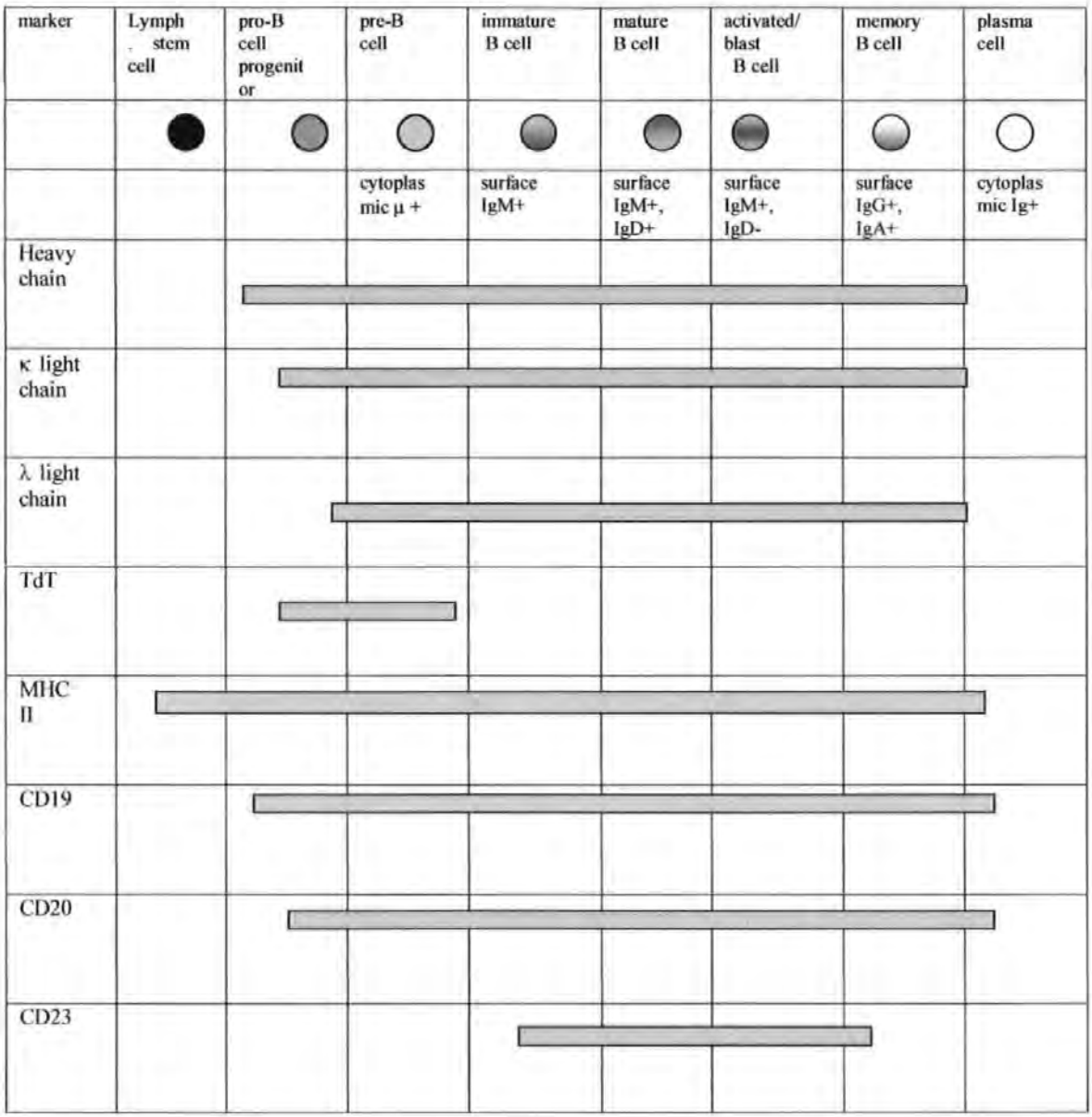
1.3.5.7. CD25

CD25 is expressed on activated T and B-lymphocytes. CD25 associates with the p75 βchain (CD122) and the common γ chain (CD123) to form the high affinity IL-2 receptor complex.

1.3.5.8. Adhesion molecules

It was found that the expression of L-selectin (CD62) is significantly increased in B-CLL but not in other cases. Also, the beta-1 integrin VLA-4 (CD49d-CD29) is expressed only in a minority of B-CLL cases and the beta-2 integrin LFA-1 (CD11a/CD18) was shown to be a marker of bad prognosis (Baldini et al. 1992). The presence of high CD44 density predicts for longer survival compared to cases with low CD44 density (Csanaki et al. 1998). An increased serum level of intracellular adhesion molecule 1 (ICAM-1) identifies stage B patients with poorer survival and stage A patients with higher risk of progression (Molica et al. 1997).

Figure 1.2: B-lymphocyte differentiation



The sequence of immunoglobulin gene rearrangement, antigen and immunoglobulin expression during B-lymphocyte development. The immunoglobulin heavy-chain and κ and λ light-chain genes occur on chromosomes 14, 2 and 22 respectively. CD19 is expressed during all stages of B-lymphocyte differentiation and maturation but not on plasma cells. CD20 is expressed on pre-B-lymphocytes, resting and activated B-lymphocytes but no on plasma cells. CD23 is mainly expressed on mature B-lymphocytes and mantle zone B-lymphocytes. CD22 is also a feature of very early B-lymphocytes. TdT=terminal deoxynucleotidyl transferase, κ= kappa, λ=lambda
Adapted from Hoffbrand et al. 1993.

1.3.6. Animal models of B-CLL

An animal model for B-cell chronic lymphocytic leukaemia is the aged H-2 congenic NZB (New Zealand Black), NZW (New Zealand White) and (NZB x NZW) F1 mouse strain (Marti et al. 1995). The spleens of all the congenic strains, homozygous for H-2z locus, contain extremely high frequencies of CD5+ve B-lymphocytes. These cells proliferate in a monoclonal and polyclonal fashion and B-CLL develops in some cases. The high CD5+ve B-lymphocytes frequencies are apparently controlled by the homozygosity of a locus or cluster of loci closely linked to the H-2z complex (Okada et al. 1991). B-CLL in these mice has a surface phenotype typical of CD5+ve B lineage cells, which express high levels of proto-oncogene bcl-2. This was also observed in pre-malignant B-lymphocytes in the aged mice (Okamoto et al. 1993).

A similar condition to human B-CLL was described in cattle. Bovine leukaemia virus (BLV) infection causes persistent lymphocytosis in cows (Trueblood et al. 1998). BLV-induced persistent lymphocytosis (PL) is characterised by a polyclonal expansion of CD5+ve lymphocytes (Trueblood et al. 1998). In this condition, increased T-lymphocyte expression of IL-2 contributes to the development and possibly maintenance of a persistent lymphocytosis. There is also some evidence suggesting that abnormal expression of GM-CSF receptor could play an important role in PL (Murakami et al. 1999).

Permanent, EBV-negative, B-CLL lines were also established from the peripheral blood of patients with B-CLL. These cells can grow as suspensions in liquid culture, express IgG lambda and other B lymphocyte markers and show Ig heavy chain and light chain rearrangements. The lymphocytes have been injected subcutaneously into severe combined immune deficient (SCID) mice and the growing tumour used as a xenograft model to test new chemotherapeutic agents (Mohammad et al. 1996).

1.3.7. Normal counterpart of the malignant B-CLL B-lymphocytes

B-lymphocytes found at the edge of germinal centres and within the mantle zone of secondary follicles are considered to be normal counterparts of malignant B-lymphocytes (Caligaris-Capio et al. 1985). However further work has shown that many specific differences exist between normal CD5+ve lymphocytes and malignant B-lymphocytes. Furthermore, a substantial number of B-lymphocytes in 20-week-old foetal lymph nodes and spleen express the CD5 marker (Bofil et al. 1985, Antin et al. 1986). They share with malignant B-lymphocytes lectin non-responsiveness and the inability to cap sIg.

1.3.8. Chromosomal abnormalities

Different neoplasms are characterised by karyotypic changes in tumour cells that are unevenly distributed throughout different chromosomes, regions and bands. After the discovery of the Philadelphia chromosome in CML in 1960 by Novell and Hungeford it became apparent that chromosomal changes in human cancers are well organised events.

Conventional cytogenic techniques demonstrate clonal chromosome abnormalities in about 50% of patients and molecular cytogenetic techniques in 80% of the patients (Monserrat et al. 1997, Dohner et al. 1998). Before 1993, the main recurrent cytogenetic alteration described in B-CLL was trisomy of chromosome 12 (Bienz et al. 1993). Karyotypic abnormalities have been described most of them involving chromosomes 6, 11, 12, 13 and 14. These are now found in 50% of cases studied. Trisomy of the chromosome 12 (10-15% of cases) is associated with a poor prognosis and atypical B-CLL (Han et al. 1984a, Juliusson et al. 1990a, Juliusson et al. 1990b). Several oncogenes are found on chromosome 12 (ATFI, ETV6, K-ras, and MGF) but none seem to be important in the pathogenesis of B-CLL. Trisomy 12 is found only in malignant B-lymphocytes suggesting the change occurred after commitment to B-lymphocyte lineage (Han et al. 1984 b, Knuutila et al. 1986, Escudier et al. 1993). Structural modifications of the long arm of chromosome 13 at or near band 13q14 (15-

20% of cases) are the most common. In many patients, an abnormality involving band 13q14 was the only chromosomal alteration (Peterson et al. 1992). Chromosome 14 t(11;14) abnormalities are associated with a poor prognosis (Que et al. 1993). Other chromosomal abnormalities include chromosome 6 deletions, where breakpoints are very heterogeneous, although q15, q23 and q25-27 are the most frequently reported. Monosomies of chromosome 17 and 18 are seen rarely.

1.3.9. Staging of B-CLL

Based on the assumption of slow progression, the disease has been divided into five stages (Rai et al 1975) (Table 1.1). Rai staging system takes the view that malignant lymphocytes first accumulate in the blood and bone marrow then in lymph nodes and spleen, and finally result in bone marrow failure.

Table 1.1: Rai system of staging B-CLL

Stage 0	Lymphocytosis (greater than $5x10^6$/l) in blood and bone marrow, no anaemia or thrombocytopenia or physical signs
Stage I	Lymphocytosis with palpable adenopathy. No hepatosplenomegaly, anaemia or thrombocytopenia.
Stage II	Lymphocytosis with hepatic and/or splenic enlargement, with or without lymph node enlargement and without anaemia and thrombocytopenia
Stage III	Lymphocytosis with anaemia (haemoglobin < than 11g/dl), irrespective of physical signs
Stage IV	Lymphocytosis with thrombocytopenia (platelets < $100x10^9$/l) with or without lymphadenopathy, hepatomegaly, splenomegaly or anaemia

Binet staging modified the Rai system (Table 1.2). The International Workshop on CLL accepted this latter proposal in 1981. Patients are staged according to the number of the organs,

involved, considering each of the following nodal areas as one: neck, axillae, inguinal regions spleen and liver.

Table 1.2: Binet system of staging B-CLL

Stage A	No organ enlargement or up to two nodal areas, no anaemia or thrombocytopenia
Stage B	Patients have three involved areas, no anaemia or thrombocytopenia
Stage C	Anaemia (haemoglobin (Hb), 10g/dl) and thrombocytopenia (platelets $<100x10^9/l$) regardless of the number of areas of lymphoid enlargement

The International Workshop proposed to retain the Rai stages as subgroups for A, as A(0), A(I) and A(II). This is to identify the most benign group of Rai system, stage 0. There is a more favourable prognosis in stage A(0). These stages correlate with different prognosis. However about 20% of low risk patients will progress to more advanced disease and those staging systems are presently unable to predict these evolutions.

1.3.10. B-CLL prognostic factors

The most significant prognostic feature of the disease is staging by either system. The important individual features like Hb, spleen size, lymph nodes or platelet count are already incorporated in the staging system. Other factors such as sex (males), age (>70 years), poor response to initial therapy or some key laboratory findings, (initial lymphocyte count, doubling time (< 12 months, trisomy 12) also indicate poor prognosis.

1.3.11. Treatment of B-CLL

The National Institute of Health sponsored a Working Group, which has published guidelines for the diagnosis and treatment of B-CLL (Cheson et al. 1996). Treatment depends on the stage of the disease, with no treatment needed at the stage A(0) (Kalil et al. 2000). For stages A(0), A(I) and for A(II) a period of observation may be necessary to establish the pattern of

the disease (stability or progression) (Keating, 1999). However, evidence of bone marrow failure, symptomatic involvement of lymph nodes or skin, autoimmune anaemia or splenomegaly may be indications to start treatment (Zwiebel et al. 1998). In the context of B-CLL, complete remission (CR) is defined by normalisation of blood and bone marrow, partial remission (PR) by the regression of at least 50% of the physical signs, lymphocyte counts less than $15x10^9$ /l and normal Hb and platelet count.

Autoimmune haemolytic anaemia and / or thrombocytopenia are very often controlled by corticosteroids, before administering chemotherapy. Alternatively, patients may receive high dose immunoglobulins, cyclosporin, splenectomy or low-dose radiation to the spleen (Rozman et al. 1995).

Due to lack of humoral defence against bacterial and viral agents, infectious complications are very common in advanced stages of B-CLL. Intravenous immunoglobulins (400 mg/kg every 3 weeks for 1 year) can significantly reduce bacterial infections.

Many drugs and combinations have been used in the treatment of B-CLL:

A) Corticosteroids are used as an initial treatment for stage C patients. However, prednisolone as a single agent has only a limited effect on B-CLL. Because prednisolone can produce a reduction in lymphocytic infiltration of bone marrow that can result in significant improvement in cytopenia, it is useful for treatment with advanced stage of disease (Catovsky et al. 1991).

B) Chlorambucil is an alkylating agent, which remains one of the most commonly used drugs in B-CLL. It may be helpful for elderly patients with poor performance status and for patients who do not tolerate fludarabine (Kalil et al. 2000). It is well absorbed after oral administration and produces less side effects such as gastrointestinal upset or alopecia. However, it does have a cumulative toxicity. Chlorambucil delays the rate of disease progression but does not improve survival (Catovsky et al. 1991).

C) Combination chemotherapy has been attempted in advanced forms of the disease (Kalil et al. 2000). The first used was CHOP (cyclophosphamide, vincristine and prednisolone) which gave a response rate about 70%. French Co-operative Group studies found that modified CHOP therapy produces a higher response rate and better median survival (Hansen et al. 1991).

D) Purine analogues such as fludarabine, cladribine and deoxycoformycin have been used in B-CLL. Fludarabine inhibits DNA polymerase and ribonucleotide reductase and promotes apoptosis of malignant B as well as normal T-lymphocytes and is an acceptable option for first-line therapy in B-CLL (Kalil et al. 2000).

E) Splenic irradiation has been used succesfully in B-CLL (Rubin et al. 1981).

F) Splenectomy may be indicated in autoimmune haemolytic anaemia refractory to corticosteroids or thrombocytopenia and massive painful splenomegaly (Seymour et al. 1997).

G) Allogeneic bone marrow transplantation gives promising results, but is only available to patients with matched, related donors. Autologous transplantation of bone marrow purged by a mixture of anti-B-cell monoclonal antibodies, after conditioning with cyclophosphamide and total body irradiation, produced a high proportion of complete responses (Rabinowe et al. 1993)

H) Immune response modifiers are potential therapies of the future. Aberrant expression by malignant cells of genes that regulate growth and survival of cells can give rise to ''altered-self'' antigens that could potentially be targeted by the host immune system. In addition, tumour cells generally maintain expression of tissue-specific ''differentiation antigens''. Such antigens can be used to target tumour cells.

-Using this passive immunotherapy, monoclonal antibodies specific for tumour associated ''differentiation antigens'' can be administered to patients. CD20 and CD52 are targets for such monoclonal antibody therapy (MoAb) (Rawstron et al. 1997 and Nguyen et al 1999).

The use of antibody may include unconjugated antibody (anti-CD20), chimeric anti-CD20 antibody (Rituximab), radiolabelled antibody, and immunotoxins. CD20 expression is limited to B-lymphocytes but CD52 occurs on T and B-lymphocytes as well as monocytes. Chimeric/humanised antibodies have been developed against these antigens using the human IgG1 backbone for optimum complement fixation (Multani et al. 1998). Campath 1-H is a humanised antibody directed at CD52. It binds to CD52 and induces antibody-dependent lysis of the target cells. Campath therapy has now been approved for patients with B-CLL who received alkylating agents, and who failed fludarabine therapy (Osterborg et al. 1997).

-Interferon-α has no effect on patients with advanced disease and has only a minor effect on patients with early disease (O'Brien et al. 1995)

-Therapeutic vaccination involves introducing modified cancer cells, cancer particles or antigen-modified cytotoxic T-lymphocytes to the patient, which in turn provoke an anti-cancer response. Dendritic cell-based vaccines are gaining popularity and these cells have the particular ability to effectively present tumour-associated antigens (TAA) to the immune system. Dendritic cells can be loaded with TAA by their natural endocytic capability or by genetic modification (Morse et al. 2000).

-Cancer is a genetic disease and thus gene transfer may correct genetic defects. It can be used to selectively kill tumour cells or it may be used to induce host anti-tumour immunity. This can be achieved by using vectors as genetic adjuvants. Vectors can encode stimulatory cytokines such us IL-2, IL-12 or granulocyte macrophage colony-stimulatory factor (GM-CSF) or vectors encoding immune stimulatory surface molecules such as B7-1 (CD80) or CD40 ligand (CD154) (Buhmann et al. 1999). Naked DNA vaccines represent an attractive approach for generating antigen-specific immunity because of their stability and simplicity of delivery. However, there are some concerns about DNA vaccines such as integration into host genome, cell transformation and limited potency (Hsu et al. 2001). The use of RNA as a tumour vaccine has also been proposed and has several potential advantages. Total cellular

RNA or mRNA can be utilised and it is not necessary to know the molecular nature of the putative tumour antigen(s) (Mitchell et al. 2000, Saens-Badillos et al. 2001).

-Antisense oligonucleotides are short synthetic, single stranded stretches of DNA molecules that can interfere with complementary sequences within target mRNAs. Antisense mediated reduction in bcl-2 protein level has been shown to accelerate the rate of death in cultures of pre-B cell leukaemia, T-ALL or non-Hodgkin B-cell lymphoma. Bcl-2 antisense therapeutics may find a role in the practice of clinical oncology (Reed et al. 1997).

1.3.12. Differential diagnosis

The differential diagnosis of lymphoproliferative disorders is based on many important factors. Physical signs of the disease as well as many laboratory finding have to be taken into consideration. Flow cytometry is commonly used to detect the percentage of cells positive or negative for a particular antigen as well as the intensity of the antigen on a particular cell type. For example, low expression of surface immunoglobulins (sIg) in B-CLL is considered an important diagnostic criterion (Tefferi et al. 1996). In addition identification of markers such as TdT in the nucleus or CD25 provide further information about cellular maturation and the activation state of the malignant cells (Jenings et al. 1997). Based on the most frequent B-CLL antigenic profile (CD5+, CD23+, FMC7$^-$, low sIg expression and weak or negative expression of CD22), a scoring system was proposed (Matutes et al. 1994). Each of the above markers has 1 or 0 value (typical or atypical for CLL). According to the criteria used by the National Cancer Institute (NCI) typical cases of B-CLL are characterised by the following immunophenotypes: CD5+, CD23+, CD11a-, FMC7-, IgM+ and /or IgD+, and weak expression of CD20. All cases with this phenotype and lymphocyte count greater than $5x10^9$/l should be diagnosed as B-CLL. Table 1.3 describes the differential diagnosis of B-CLL.

Table 1.3: Differential diagnosis of B-CLL

Disease	Characterisation
Atypical CLL	Atypical CLL is characterised by the presence of CD5+ve and CD23-ve cells. However, FISH results for t(11;14) are needed to distinguish it from mantle cell lymphoma. Trisomy 12 and any 2 of the following: CD11a, FMC7, strong CD20, CD28 and Ig/CD79b are also seen in atypical CLL.
Mantle cell lymphoma	Mantle cell lymphoma is characterised by the presence of CD5+ve and CD23-ve lymphocytes with moderate to strong sIg/CD79b expression (Jennings et al. 1997). Evidence of t(11;14) must be present, demonstrated by nuclear expression of cyclin D1 gene (CCND1).
B-cell prolymphocytic leukaemia	In B-cell prolymphocytic leukaemia (B-PPL) the cells are large, with a prominent nucleolus and lower nuclear/cytoplasmic ratio. They are also strongly sIg/ CD79b+ve but also CD5-ve, and FMC7 positive (Sole et al. 1998, Ginaldi et al. 1998).
Large transformation of CLL	This term is used when there is evidence of pre-existing B-CLL and partial or complete replacement of lymph nodes and/or bone marrow by cohesive sheets of large B-lymphocytes.
Marginal zone lymphoma	Marginal zone lymphoma is characterised by bone marrow infiltration with small to medium sized lymphocytes with some evidence of plasma cell differentiation. The B-cell immunophenotype should be CD5-ve, CD10-ve, CD19+ve, CD20+ve and CD23+ve. In extranodal marginal zone lymphoma cells are: sIgM+ve, sIgD-ve, CD5-ve, CD10-ve, CD20+ve, CD23-ve.
Follicular lymphoma	Follicular lymphoma is characterised by follicular growth pattern, as well as the presence of centrocytes and centroblasts. Cells are CD10+ve, CD19+ve, CD20+ve, CD23-ve, CD45RA+ve and CD75+ve. T(14;18) and bcl-2 rearrangements are present in the majority of follicular lymphomas (Bordeleau et al. 2000).
Myeloma	Myeloma plasma cells can be distinguished by CD56 expression and/or low or absent CD19 expression (Mulligan 2000).
Hairy cell	Hairy cell leukaemia (HCL) requires cells with typical morphology in

leukaemia	blood and in bone marrow). The immunophenotype of Hairy cell leukaemia includes clonal Ig with CD11, CD25, CD103 and strong CD22 expression (Bennett et al. 1989, Mulligan et al. 1990, Matutes et al. 1994).

1.4. LYMPHOCYTES

1.4.1. General introduction

A variety of cells are important in mediating immune responses by directly interacting with each other and secreting soluble molecules. These cells include lymphocytes, phagocytes, auxiliary cells (basophils, mast cells, platelets) and tissue cells. It is only in the last 40 years that it has become apparent that the lymphocyte is a crucial player in the immune system. Lymphocytes are immunologically competent cells that assist phagocytes in defence of the body against infection and other foreign antigens. To understand B-CLL, it is important to view the characteristics of the human lymphoid system. In primary lymphopoietic organs such as bone marrow and thymus, lymphocytes derived from stem cells undergo division in a manner which is not dependent on antigenic stimulation. These organs provide a unique microenvironment controlled in part by soluble factors (cytokines, growth hormones and hormones). After being released from their primary organs, lymphocytes migrate to secondary (or peripheral) lymphoid organs, such as the spleen, lymph nodes and tonsils. Here, from the resting state (G_0 phase of the cycle) after encounter with antigen, they undergo activation, leading to cell division and differentiation. T lymphopoiesis in the thymus continues until about the time of puberty but B-lymphocytes continue to be produced throughout life. The normal human body contains about 10^{12} lymphocytes. Under light microscopy they appear to have uniform appearance. Lymphocytes are small, round (5-12 um in diameter) with a roughly spherical nucleus, and densely compacted nuclear chromatin.

However on the basis of their functional properties and the expression of specific proteins, they can be divided into several important groups. Two major types are B- and T-lymphocytes. B- and T-lymphocytes lineage cells arise from a common subset of haematopoietic stem cells that become committed to the lymphoid pathway. They are responsible for the specific recognition of antigen.

1.4.2. Normal B-lymphocytes

All lymphocytes are derived from pluripotential haematopoietic bone marrow stem cells. B-lymphocytes develop in the bone marrow, which provides a specialised microenvironment allowing B-lymphocytes to develop. The microenvironment achieves this by interacting with B-lymphocytes by means of adhesion molecules, and also by producing factors such as IL-7 (Figure 1.3, Table 1.4). B-lymphocyte can be divided into two groups, which are distinguished by the presence of the CD5 antigen on the surface of the cell. B-lymphocytes expressing CD5 molecules are called B1 cells and conventional CD5- ve B-lymphocytes are named B-2 cells. B-1 cells, which are present in B-CLL, are characterised by self-renewal, with spontaneous production of immunoglobulins, polyreactive specificity and low or no somatic hypermutation. With time, they are no longer produced by bone marrow but are maintained by division of existing B-1 cells (Kearney, 1993, Parham, 2000).

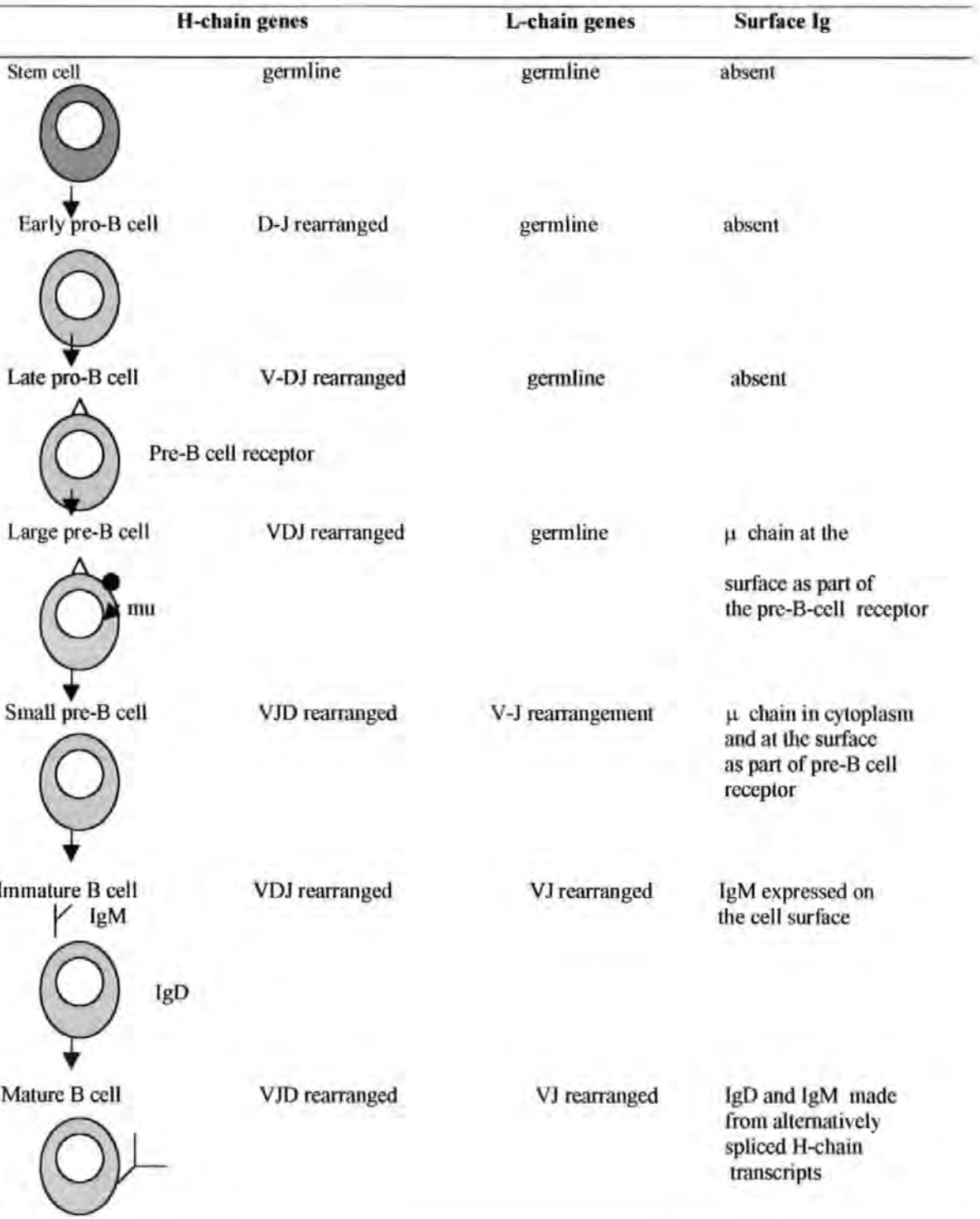
Each B-lymphocyte is genetically programmed to encode a surface receptor for a specific antigen. After initial recognition of an antigen, B-lymphocytes multiply and differentiate into plasma cells and are able to produce and secrete antibody. Antibodies are divided into five classes or isotypes IgA, IgD, IgE, IgG and IgM consisting of two heavy chains (α, δ, ε, γ or μ) and two light chains (kappa (κ) and lambda (λ). In humans, two-thirds of the antibody molecules contain κ chain and one-third λ chains. Each antibody molecule contains either κ or λ chains but not both. Cell surface IgM and IgD are the antigen receptors on circulating B-lymphocytes that have yet to encounter antigen. The first antibody to be secreted in the

immune response is IgM (also IgD but in very small amounts). IgM, which is displayed on B-CLL B-lymphocytes, is secreted as a circular pentamer of immunoglobulin monomers. IgM binds strongly to surface antigen but its effector mechanisms are limited.

The response of B-lymphocytes to pathogens and foreign substances is mediated through the cell-surface B-cell receptor (BCR). Signalling through this receptor can also lead to activation, tolerance and/or differentiation. Two ligand receptor systems have been identified that exert profound influence on the outcome of B-T lymphocyte interactions. The first system ligates CD28/CTLA-4 on T-lymphocytes to B7.1 and B7.2 on B cell signals the TCR-activated cell to produce cytokines and avoid anergy (Gimmi et al. 1991, Razi-Wolf et al. 1992, Freeman et al. 1993). In the second system the expression of CD40L by activated T helper cells triggers B-lymphocyte activation through binding to CD40 (Armitage et al. 1992) (Figure 1.4).

The process of isotype switching or class switching produces antibodies of the IgG, IgA or IgE isotypes. These antibodies have more specialised effector functions than IgM. Antibodies secreted by plasma cells in secondary lymphoid tissues and bone marrow finds their way into the extracellular fluid where recognition of antigen takes place. Their binding sites interact with a component of the pathogen surface such as glycoproteins and proteoglycans. Antibodies can also coat bacteria (opsonisation) facilitating their ingestion and destruction by phagocytes or activation of complement prior to destruction by phagocytes.

Figure 1.3: Stages of B-lymphocyte development defined by the rearrangement and expression of immunoglobulin genes.



In the stem cell, the immunoglobulin (Ig) genes are in the germline configuration. The first rearrangements are heavy-chain (H-chain) genes. Joining D_H to J_H defines the early pro- cell, which becomes a late pro-B cell on joining V_H to DJ_H. Expression of a functional μ chain and its expression at the cell surface as part of the pre-B receptor define the large pre-B lymphocyte. Successful light-chain gene rearrangement and expression of IgM on the cell surface define the immature B-lymphocyte. The mature B-lymphocyte is defined by the use of alternative splicing if heavy chain mRNA to place IgD on the cell surface as well as IgM. Adapted from: ' The Immune system' by P Parham, 2000.

1.4.3. Malignant B-lymphocytes

B-CLL malignant B-lymphocytes express many cell surface antigens but their expression differs from normal B-lymphocytes. 95% of all B-CLL B-lymphocytes express CD5, a 6kDa antigenic determinant initially found on the surface of T-lymphocytes (Boumsell et al. 1978). Almost all appear to react with CD19, CD20 (with low density), CD24, CD37 and CD21 monoclonal antibodies (Ranheim, 1995, Nadler, 1986). In addition most cases express CD18, CD23, CD27, CD32, CD37, CD39, CD40, CD44, CD45RA and Ddw75. A minority of cases express other antigens such as CD1c, CD11a, b, and c, CD14, CD22, CD25, CD29, CD35, CD49c, CD54, CD61, CD62L, CD70, CD71 and CD72 (Morabito et al. 1987, Keller et al. 1987, Delia et al. 1988, Merle-Beral et al. 1989, Wormsley et al. 1990, Kipps, 1995).

Their immunophenotype typically shows a low expression of surface IgM and IgD with clonal restricted light chains (Bannerji, 2000). The surface immunoglobulin (sIg) is often IgM with frequent co-expression of IgD. Infrequently IgD predominates. This is shown to be monoclonal because of the expression of one form of light chain, either kappa (κ) or lambda (λ) (Dighiero et al. 1993).

Malignant B-lymphocytes are inefficient antigen presenting cells because they lack expression of the T cell co-stimulatory molecule B7-1 (CD80) and B7-2 (CD86) (Van der Hove et al. 1997) although they may also down-regulate the expression of CD154, the CD40 ligand in T-lymphocytes (Cantwell et al. 1997). Leukaemic B-lymphocytes have been show to interfere with the interaction of normal T-lymphocytes with B-lymphocyte in B-CLL.

1.4.4. Normal T-lymphocytes

T-lymphocytes are also derived from the bone marrow but then mature in the thymus. They have a variety of functions. One role is to interact with B-lymphocytes and to help them to mature into antibody secreting cells. The other is to interact with mononuclear phagocytes and help them destroy intracellular pathogens. T-lymphocytes recognize degraded proteins bound by major histocompatibity complex (MHC). There are two types of T lymphocytes, T-helper lymphocytes (Th) and T cytotoxic lymphocytes (Tc). Th cells have been further subdivided into Th1 and Th2 subtypes, depending on the profile of cytokines they secrete. Tc lymphocytes are responsible for the destruction of host cells, which have been infected with viruses or other intracellular pathogens (Mossman et al. 1996).

Table 1.4: Distribution of B- and T-lymphocytes in normal human tissue

Tissue	B lymphocytes (%)	T lymphocytes (%)
Peripheral blood	10-15	70-80
Bone marrow	80-90	5-10
Thymus	<1	99
Lymph node	20-30	70-80
Spleen	50-60	30-40

1.4.4.1. The Th1/Th2 paradigm

The Th1/Th2 pattern of cytokine production was first described in mice (Mossman et al. 1986) and later in man (del Prete et al. 1991). Th1 lymphocytes are characterised by the secretion of cytokines such as interferon-gamma (IFN-γ), IL-2 and tumour necrosis factor alpha (TNF-α), and Th2 lymphocytes by the secretion of IL-4, IL-5, IL-10 and IL-13 (Romagnamini et al. 1999). Th1 and Th2 subsets secrete IL-3, TNFα, granulocyte-

macrophage colony stimulating factor (GM-CSF) and members of the chemokine family. The functions of Th1 and Th2 lymphocytes correlate with the pattern of their cytokines production.

Th1 lymphocytes are involved in cell-mediated immunity. Th1 clones induce delayed type hypersensitivity (DTH) reaction, with IFN-γ commonly expressed at the sites of DTH reactions (Yamamura et al. 1991 and Tsicopoulos et al. 1992). Th2 cytokines are involved in antibody production, particularly IgE responses by promoting class switching from IgM to IgG1 and IgE, as well as enhancement of eosinophil production and function (Mossman et al. 1989). Th1 and Th2 lymphocytes are not pre-programmed as separate lineages in the thymus, instead they develop from a common peripheral precursor T-lymphocyte (Kamagava et al. 1993). Th1 or Th2 commitment appears to be dependent on the cytokine milieu at the time of activation, antigen concentration (Hosken et al. 1995), strength of TCR binding by antigen-MHC complex (Constant et al. 1997) and the type of APC interacting with the T-lymphocyte (Langhorne et al. 1998).

IL-4 stimulates differentiation of uncommitted T-lymphocyte precursors into Th2 lymphocytes, whereas INF-γ, IL-12 and TGF-β enhance Th1 development. Once established, each of these patterns of response is able to suppress the other: INF-γ secreted by Th1 cells inhibits proliferation of Th2 lymphocytes, IL-10 secreted by Th2 lymphocytes inhibits cytokine secretion by Th1 lymphocytes (Florentino et al. 1989). While Th1 and Th2 lymphocytes are the major sources of their respective cytokines, many other cells within and outside the immune system contribute to the production of these cytokines, e.g. NK cells can produce IFN-γ while IL-4 can be produced by mast cells and basophils.

1.4.5. T-lymphocytes in B-CLL

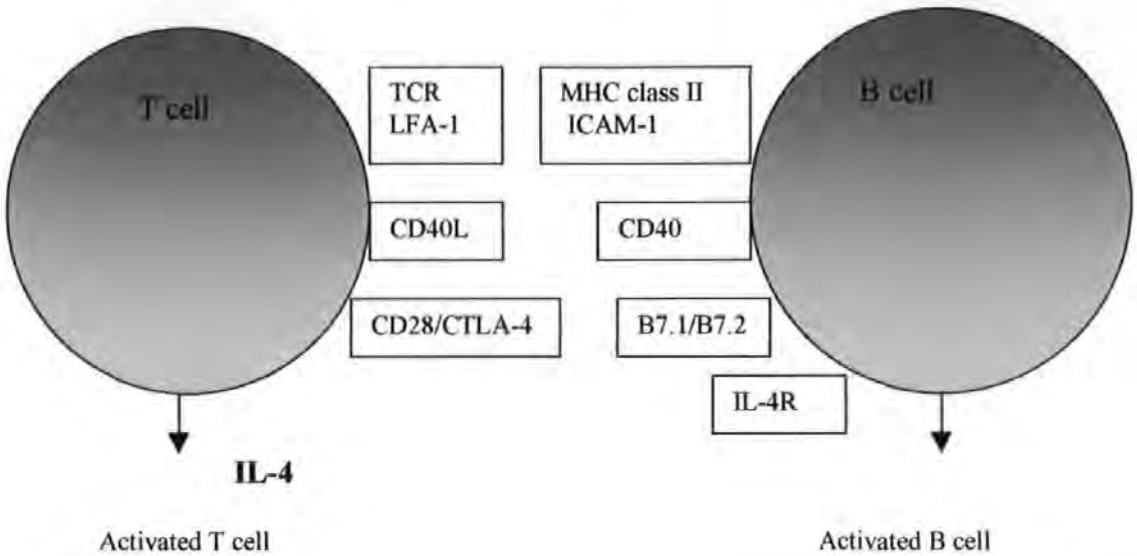
Although the neoplastic process in B-CLL involves a clonal proliferation of B-lymphocytes, there are many functional defects within the T-lymphocyte population. These defects may be

primary or secondary and may play a role in the pathogenesis of the disease. The first of these changes involves an alteration in T-lymphocyte sub-populations. They include an increase in total T-lymphocyte numbers as well as an inverted CD4:CD8 ratio (Hautekeete et al. 1987). B-CLL T-lymphocytes also demonstrate a different pattern of activation and proliferation potential compared to normal controls.

CD8 T-lymphocytes have been found in B-CLL to have a lower proliferation rate than their normal counterparts (Antica et al. 1993). Decreased T helper function and increased T suppressor function have also been reported (Chiorazzi et al. 1979, Hersey et al. 1980, Kay et al. 1981). The T-cell receptor repertoire in B-CLL was more frequently abnormal in CD4 T-lymphocytes (Rezvany et al. 1999).

Cytokine production by T-lymphocytes from B-CLL patients differs from normal individuals. Reduced IL -2 production as well as impaired IL-2 receptor production has been reported. However, some differences in IL-2 production kinetics by CD2 lymphocytes have been reported but no differences were found when the levels of both cytokines reach their peak (Ayanlar-Batuman et al. 1986). CD2-stimulated B-CLL T-lymphocytes produced higher levels of INF-γ and TNF-α (TNFSF2) but not TNF-β (TNFSF1) when compared to normal T-lymphocytes (Reyes et al. 1998). Altered T-lymphocyte function in B-CLL patients is also suggested by reduced levels of the activation markers CD28 and CTLA-4 (CD152) and the IL-2 receptor, when compared with T lymphocytes of healthy donors (Scrivener, et al. 2001).

Figure 1.4: Interactions between normal B- and T-lymphocytes



Ligation of the T cell receptor complex with antigen-specific major histocompatibility complex (MHC) leads to sequential expression of CD40L, B7.1 and B7.2. This leads to up-regulation of CD40L expression on T-lymphocytes and its sequential interaction with CD40 on B-lymphocytes. Signalling via CD40 up-regulates the expression of B7.1/B7.2. At this stage B-lymphocyte becomes an antigen-presenting cell (APC) and fully activates T-lymphocyte.

1.5. CYTOKINES

1.5.1. Introduction

Over 30 years ago it was shown that antigen inhibited migration of neutrophils and macrophages in culture. Subsequently a variety of biological activities in lymphoid culture supernatants were described and a cytotoxic lymphocyte-derived mediator called lymphotoxin was isolated in 1968 (Kolb, 1968). These biochemically undefined, lymphocyte-derived factors were thus called 'lymphokines' (Dumonde et al. 1969) and later becomed known as **interleukins**. To qualify as an interleukin, a cytokine must be shown to have a unique amino acid sequence and functional activities involving leukocytes. Whereas many cytokines are now termed interleukins, others continue to be known by their older names [e.g. interferon -alfa/beta (IFN α/β), IFN- gamma (IFN-γ), tumour necrosis factor (TNF)].

Cytokines are peptide molecules, some with sugar attached (glycoproteins), that act as soluble cell-to-cell messengers. Many form high molecular weight oligomers and one cytokine (IL-12) is a heterodimer. They have regulatory effects on haematopoietic and many other cell types that participate in host defence and repair systems. Cytokines include lymphocyte-derived factors known as 'lymphokines', monocyte-derived factors called 'monokines', haematopoietic factors called 'colony stimulating factors', connective tissue 'growth factors' and chemotactic chemokines. They have relatively low molecular weights (8-25kDa), are distinguished by their overlapping activity and ability to act on many cell types and are produced by many different cells, so they can act in both an autocrine and a paracrine manner. Depending on their target cell type they can activate or supress gene in the target cell (Arai et al. 1990). Cytokines are involved in a variety of processes such as viral infections, inflammation, haemopoiesis, immunity, tissue repair and fibrosis. Some of them are now termed chemokines because of their chemotactic role for specific cell types. Rapid secretion in response to appropriate signals makes them very powerful cell-to-cell communicators. Despite been structurally distinct they can be organised into groups that exhibit functional similarities based on shared receptor utilisation. For instance, the IL-2 common gamma (γc) receptor chain is shared by interleukins 2, 4, 7, 9 and 15 (Kondo et al. 1993, Russell et al. 1993, Noguchi et al. 1993, and Giri et al. 1994). IL-6, IL-11, LIF and CNTF (Taga et al. 1997) share the gp 130 chain of the IL-6 receptor. IL-3, IL-5 and colony stimulating factor share a common beta (β) chain receptor (Scott et al. 1999).

1.5.2. Classification of cytokines

Cytokines can be divided into different categories such as interferons, interleukins and colony stimulating factors.

-Interferons (IFNs) are particularly important in defence against viral infections, being the first line of resistance. These include IFN-α and IFN-β that are produced by virally infected cells and IFN-γ, which is responsible for the resistance to infection of uninfected cells.

-Interleukins (ILs) are mainly produced by T-lymphocytes but they can also be produced by B-lymphocytes, mononuclear phagocytes, mast cells and other tissue cells. They are involved in cell-to-cell interactions.

-Colony stimulating factors play a role in directing the division and differentiation of bone marrow stem cells (Jakubowski et al. 1996).

-Other cytokines include tumour necrosis factors {TNFα (TNFSF2), TNFβ (TNFSF1)} and transforming growth factor (TGF-β) with a variety of functions (Fortunel et al. 2000, R&D System mini review, 2001).

1.5.3. Role of cytokines

The interactions between cytokines can be described as synergistic or antagonistic and stimulatory or inhibitory. Cytokine action is also contextual, in other words, dependent on the microenvironment (other cytokines, hormones, growth factors, and prostaglandins) (Sporn et al. 1988). The induction of IgM production by IL-2 and IL-5 (Matsui et al. 1989) and potentiation of the cytotoxic action of TNFSF2 on tumour cells by INF-γ are examples of cytokine synergy (Lee et al. 1984) while IL-4 is antagonistic to INF-γ influence on Ig subclasses synthesis by B cells (Snapper et al. 1988).

Among the first examples of the ability of cytokines to stimulate the production of other cytokines was the discovery of IL-1 that stimulates the production of IL-2 by murine thymocytes and effects their proliferation (Smith et al. 1980). Similarly, many examples of inhibitory actions of cytokines on the production of other cytokines are known. For example IL-4 inhibits the production of TNFSF by monocytic cells (Hart et al. 1989).

However, the regulation of cytokine interactions is still not fully understood. Evidence suggests that transcriptional and post-transcriptional mechanisms are involved (Aman et al. 1993 and Oliveira et al. 1994). Cytokine receptor expression may also play an important role with reduced levels of cytokine receptor or the expression of higher affinity receptors (Holtman et al. 1987).

The great variety of cytokines, their abundance in the organism and the complicated way they interact with each other help define their characteristics. These include pleiotropy (they tend to have multiple targets and multiple actions), redundancy (different cytokines may have similar actions), synergism/antagonism (different responses with more than one cytokines) and formation of cytokine cascades.

1.5.4. The cytokine receptor family

The common structural features of cytokine receptors allow them to be grouped into families and subfamilies. The class I cytokine receptor family includes receptors for many important cytokines including IL-2, IL-4, IL-12. Most of these receptors form heterodimers but some are monodimers or even heterotrimers (Heim et al., 1996). Some form subfamilies with the common receptor to all its members (Taga et al. 1995) (Table 1.5).

Table 1.5: Human cytokines and their receptors

Cytokine	Molecular weight	Exons	Introns	Gene location	Receptor MW	Gene colation
IL-1α	17.5 kDa	5	4	2q(q12-21)	80 and 60 kDa shared with IL-1β	2q12
IL-1β	17.3 kDa	6	5	2q(q13-q21)	80 to 60 kDa	
IL-2	15.5 kDa	4	3	4q(q26-q27)	55kDa, 70 kDa and 64-kDa	α -Xp22.3 γ-Xq13.1
IL-3	14-30 kDa			5q(q23-31)	120 kDa	
IL-4	15-19 kDa	4	3	5q(q23-31)	50-60kDa and 64kDa	α-16p11.2-16p12 γ-Xq13.1
IL-5	2x22.5kDa (homodimer)	4	3	5q(q23-q31)	46.5 kDa and 114 kDa	3p24-26
IL-6	26 kDa			7p(p21-24)	80 kDa	1q21
IL-7	14.9 kDa	6	5	8q(q12-q13)	70kDa	α-5p13 γ-Xq13.1
IL-8	8.5 kDa	4	3	4	58kDa and 67kDa	2q35
IL-9	32-39 kDa	5	4	5q31-q35	64kDa	Yq28 γ-Xq13.1
IL-10	18kDa			1	90-120kDa	11q23.3
IL-11	23kDa	5	4	19		
IL-12	35kDa and 40kDa (heterodimer)			5q31-q33 3p12-p13.2	100kDa	1p31.2
IL-13	12kDa	4	3	5q31	140kDa	Xq13.1-q28
IL-14						
IL-15	34kDa	8	7	4q31		α-10p14-p15 γ-Xq13.1
IL-16	14-17kDa			15q26.1		
IL-17					97.8kDa	
IL-18	22.3kDa	3	4			

1.5.5. Role of cytokines in the development and function of lymphoid cells

Each cytokine has a unique function in homeostasis. The following is a short outline of their main characteristics, emphasising the role they play in B and T lymphocyte activities.

Macrophages, endothelial cells, B-lymphocytes and fibroblasts, produce IL-1 (Kasahara et al. 1992). Its role is to induce inflammatory responses such as the production of prostaglandins and degenerative enzymes (collagenase) that take part in the destruction of cartilage and bone. During injury, IL-1 induces production of acute phase proteins by the liver and is also responsible for inducing fever and augmenting release of corticosteroids. IL-1 exerts its actions through IL-1 receptors, which are present on most cells (Dover et al. 1990).

IL-2 is known as T-cell growth factor because it activates and induces proliferation of T-lymphocytes. It is also produced in response to activation of T cells. IL-2 augments B cell growth and immunoglobulin production as well as IFN-γ production.

IL-3 is produced primarily by activated T lymphocytes, activated NK cells, activated mast cells and possibly epidermal cells (Luger et al. 1985). The activities of IL-3 include stimulating the proliferation of mast cell lines, differentiation of neutrophils, macrophages, basophils, eosinophils and mast cells, and potentiation of the activities of eosinophils, basophils and monocytes (Schrader et al. 1983).

IL-4 is produced by mast cells, basophils, a subpopulation of T-lymphocytes bearing NK1.1 markers and naive CD4, as well as CD8 T-lymphocytes and some IL-3 dependent bone marrow cell lines (Howard et al. 1982, Brown et al. 1987, Rodriques-Traduce et al. 1992 and Seder et al. 1992).

IL-5 is a product of activated T-lymphocytes and acts on eosinophils, basophils, B-lymphocytes and thymocytes. It has been shown that IL-5 enhances IgE synthesis and CD23 expression in IL-4 stimulated cells (Pene et al. 1988). IL-5 exerts its activity by interacting with a specific multi-subunit receptor present on normal B-lymphocytes and eosinophils (Mita et al. 1989).

IL-6 is produced by activated monocytes, endothelial cells, a variety of tumour cells, activated T and B-lymphocytes and fibroblasts and acts on a variety of cell types including fibroblasts and hepatocytes (Wong et al. 1988 and Roodman et al. 1992). Among other roles, IL-6 increases B lymphocyte differentiation and stimulation of IgG secretion and induces cytotoxic T lymphocyte differentiation (Taga et al. 1987).

IL-7 is a product of bone marrow stromal cells and thymic stromal cells (Goodwin et al. 1989). It has some T cell growth factor activity with augmentation of T cell cytotoxicity (Hickman et al. 1990).

IL-8 is a neutrophil chemotactic factor produced by LPS-stimulated mononuclear cells (Yoshimura et al. 1987). It is released at the site of injury where there is recruitment of monocytes and neutrophils. It can be produced by stimulated normal eosinophils and induced by histamine (Jeannin et al. 1994). It is also able to inhibit IgE production by B-lymphocytes (Kimata et al. 1992).

IL-9 is a T-cell-derived cytokine that interacts with a specific receptor associated with the IL-2 receptor γ chain. Interleukin-9 potentiates IL-4 induced immunoglobulin (IgG, IgM and IgE) production by normal human B-lymphocytes and shares common signal transduction pathways with IL-4 (Renauld et al. 1995a).

IL-10 was initially named cytokine synthesis inhibitory factor (Fiorentino et al. 1989). It is produced by T helper clones, but also, by EBV-positive B cells lines and LPS transformed macrophages (Benjamin et al. 1992 and Vaal Melafyt et al. 1993). It has a range of activities, the most important being a growth and differentiation factor for activated B-lymphocytes, but also the induction of MHC II antigen expression and increased viability of B cells (Rousset et al. 1992). IL-10 also co-operates with TGF-β to induce IgA production by naive B-lymphocytes co-cultured with anti-CD40 antibody. *In vitro* it can inhibit the production of IFN-γ and IL-2, cytokines produced mainly by Th1 cells.

IL-12 is a key cytokine in immune regulation. IL-12 is produced by dendritic cells, macrophages, neutrophils and activated T-lymphocytes (Ma et al. 1998). It promotes both natural killer cell and cytotoxic T-lymphocyte activity and therefore is an important factor in the development of immune responses against tumours and infectious agents. Priming with IFN-γ increases production of IL-12 by PBMC (Harrison et al. 1997, Lee et al. 1998).

IL-13 is produced by Th2 lymphocytes and together with IL-4 and IL-10 share the capacity to inhibit cytokine synthesis by monocytes. It is essential for B lymphocyte activation (Defrance et al. 1998), and induces CD23 expression and MHC class II expression, as well as playing a role in allergic reactions (Punnomen et al. 1993). IL-4 and IL-13 receptors share a common subunit involved in signal transduction (Vita et al. 1995).

The major sources of IL-15 are monocytes, epithelial cells and fibroblast cell lines. It shares many biological activities with IL-2 but shows no homology to IL-2. These two cytokines, at least in part, use components of the IL-2R system, β and γ chain, for binding and signalling.

IL-16 was originally identified as a product of CD8+ve cells. CD4+ve cells, after mitogenic stimulation, can also produce IL-16 as well as eosinophils, bronchial epithelial cells, mast cells and fibroblasts. IL-16 is a potent chemotactic factor for CD4+ve cells and eosinophils but also induces cell surface expression of high affinity IL-2 receptors (Rand et al. 1991 and Center et al. 1995).

Human IL-17 is secreted primarily by activated CD4+ve lymphocytes (predominantly by the memory cell subset) (Fossiez et al. 1996). Activities of IL-17 include the ability to induce IL-6, IL-8, G-CSF and PGE_2 secretion, thus having pro-inflammatory as well as indirect haematopoietic activities (Fine et al. 1997).

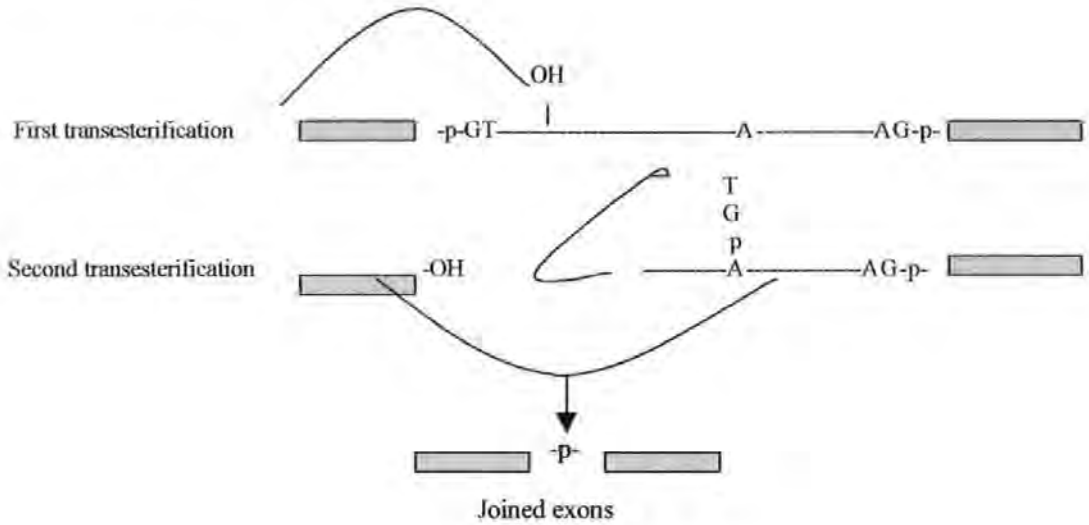
Finally, IL-18 has been shown to be a widely produced cytokine. IL-18 mRNA is detected in macrophages, adrenal epithelial cells, liver, lungs and unstimulated PBMC. It was originally designated as IFN-gamma inducing factor (IGIF), because it was cloned as a cytokine that strongly induces IFN-gamma production (Okamura et al 1995).

1.5.6. Splice variants of cytokine genes

The function of individual cytokines and of the overall network is further complicated by alternative splicing. Spliced variants have been described for many cytokines (Atamas et al 1997). These can be expressed as soluble or membrane bound proteins and can be tissue specific. Alternative splicing is a tightly controlled process by specific factors whose expression is highly restricted.

Pre-mRNA splicing take place on the spliceosomes, a dynamic complex of small nuclear ribonucleoprotein particles (snRNPs) and extrinsic (nonsnRNP) protein factors assembled on the juxtaposed 5' and 3' splice sides (Figure 1.5). Intron excision proceeds in two successive transesterification reactions whereby the upstream exon is cleaved from the intron and ligated to the downstream exon (Lopez, 1998). First the 2' hydroxyl group of the adenosine residue of the branch site attacks the phosphate group linking the last nucleotide of the 5' exon with the guanosine residue of the GU intronic dinucleotide, thus forming the branched intermediate (lariat formation). Then the 3' hydroxyl group that has been formed at the last nucleotide of the 5' exon attacks the phosphate group that links the guanosine residue of the AG intronic dinucleotide with the first nucleotide of the 3' exon, resulting in release of the lariat and joining the two exons (Madhani et al. 1992 and Nielson, 1994). Terminal exons, that are the first and last exon, require special mechanisms for their recognition that appear to involve cap and poly A+ binding proteins. In addition, the terminal exons are generally longer with the average size being 600 nucleotides. Under certain conditions exclusion of exons can occur, suggesting that the juxtaposition events do not necessarily have to occur between neighbouring exons. Tissue specific exons, may be excluded from mRNA by exon skipping, whereby the splice sites for the optional exon are ignored and the upstream and downstream exons are ligated to each other directly (Sorg et al. 1993, Korte et al. 1999). Splicing is regulated by sequence elements that are distant from a splice site.

Figure 1.5: Design of a splice variant



Two-transesterification steps involved in the splicing process. Pre-mRNA splicing takes place on the spliceosomes. Intron excision proceeds in two successive transesterification reactions. Exons are indicated as boxes and introns as solid lines. The conserved GT, A and AG nucleotides are shown. These variants can act as a natural inhibitor of the wild type protein (Atamas, 1997).

Splice variants have been described for TGF β1, TGFβ2, IL-1αM-SSF, G-CSF, IL-2, IL-4, IL-5, IL-6, IL-7 and IL-15 (Webb et al. 1988, Chiu et al. 1990, Sorg et al. 1991, Tanihara et al. 1993, Frishman et al. 1993, Kestler et al. 1995 and Onu et al. 1997). However in only a few cases has the actual translation of the spliced variant mRNA into protein been demonstrated.

Such variants can be created for IL-2 and IL-4 by alternative splicing of certain exons that may in turn give rise to the generation of proteins with differential expression in tissue. IL-4 and IL-2 splice variants, IL-4δ2 and IL-2δ2 and IL-2δ3 respectively, are reported to function as natural inhibitors of the wild type protein (Alms et al. 1996). The IL-4 variant inhibits co-stimulation of T cell proliferation through competitive binding to its receptors. Similarly, both IL-2 spliced variants inhibit IL-2 co-stimulation of T-lymphocytes proliferation and cellular

binding of recombinant IL-2 to high affinity IL-2 receptors (Atamas et al. 1996). They may do so by engaging only β and γ c chains but not the α chain. Thus the altered expression of a wild type and alternatively spliced variant protein may lead to disease (Glare et al. 1999 and Sakkas et al. 1999).

Alternative splicing has also been described for IL-7 (Korte et al. 1999). Also spliced variants lacking exons 3 and 5 or both in combination with exon 4 have been described (Korte et al. 1999). Similarly secretion of IL-15 is controlled via alternative splicing in the region of the signal peptide of the precursor protein, although both mature variants have the same amino acid composition (Onu et al. 1997).

1.5.7. Structure and function of the γc dependent cytokines and their receptors

1.5.7.1. Introduction

As well as other receptor families, the γc chain family is an important example of the common features of cytokine receptors such us IL-2R, IL-4R, IL-7R, IL-9R and IL-15R (Kondo et al. 1993, Russell et al. 1993, Noguchi et al. 1993 and Giri et al. 1994) (Table 1.8). Each receptor has additional individual subunits, which add specificity to their signalling The γc chain was identified in 1990 and a complementary cDNA clone encoding this member of the cytokine receptor family was isolated (Takeshita et al. 1990). Co-immunoprecipitation studies performed in the presence of IL-2 led to the identification of a 64-kDa protein that was associated with IL-2R β. The γc chain is composed of a 255-amino-acid extracellular domain, a 29-amino-acid hydrophobic transmembrane region, and 86-amino-acid C-terminal cytoplasmic tail.

The extracellular domain contains many features, which are common to receptors such as the IL-2 β, IL-4R α, IL-7R α and IL-9R α chains (Bazan et al. 1990) (Table 1.6). These include a region having four Cys residues located in the N-terminal half of the extracellular domain and a second WS motif (WSXWS) (Noguchi et al. 1993). This region also contains two

fibronectin type III-like domains of unknown significance. The γ chain is necessary for the formation of high (equilibrium dissociation constant: $K_D=10^{11}$M) and intermediate ($K_D=10^{10}$ – 10^9 M) affinity receptors and increases receptor affinity by 3 to 10 fold (Smith, 1980). It is required for the receptor-mediated internalisation of IL-2.

The γc chain receptor is constitutively expressed on essentially all cells of haematopoietic origin. However they display two types of γc transcripts, differing in their carboxyl terminal coding regions. The newly identified sequence display a deletion of 72 nucleotides close to the 3'-end of the open reading frame. The result is the loss of 24 amino-acids, which include a conserved tyrosine residue shared by several members of the cytokine receptor family (Shi et al. 1997). The IL-2 receptor complex signals by rapidly increasing tyrosine phosphorylation of β and γc chains of the complex (Mills et al. 1990). Signalling is mediated through a group of receptor-associated tyrosine-kinases termed Janus or JAK kinases (Schindler et al. 1995).

The importance of the γ chain was identified in the study of X-linked combined immunodeficiency (XSCID) where a mutation of the γc chain leads to impaired T and NK lymphocyte function and consequently to impaired immune responses (Noguchi et al. 1993).

Table 1.6: Characteristics of cytokines belonging to γc chain receptor family

Cytokine	Mature proteins (kDa)	Secreted peptide (aa)	Exons	Size of an exon (kb)	Introns	Chromosomal location
IL-2	15	133	4	4	3	4q26-28
IL-4	20	129	4	10	3	5q26-28
IL-7	17	152	6	33	5	8q12-13
IL-9	14	126	5	4	4	5q31-q35
IL-15	15	114	8	5	7	4q25-35

aa=Amino-acid, kDa=kilodalton, kb=kilo-bases

1.5.7.2. Interleukin-2 (IL-2)

IL-2 was first identified as an essential growth-promoter for bone marrow-derived T lymphocytes (Morgan et al., 1976). IL-2 is a polypeptide of approximately 15.5 kDa. Cloning of IL-2 revealed that 153 amino-acid human IL-2 translation products undergo several post-translational processing events, including cleavage of a 20-residue signal peptide, addition of carbohydrate to the threonine residue at position 3 (Thr-3), and formation of a disulphide bond between cysteines 58 and 105 (De Vos et al. 1983, Taniguchi et al. 1983). The three-dimensional structure of IL-2 predicts the existence of four core α helices and two crossover loops containing β strands (Bazan et al. 1992). The IL-2 gene is located on chromosome 4 and contains four exons and three introns (Fujita et al. 1983). Exon 1 encodes Ala^{1}-Asn^{29}, which forms a short strand plus helix A. Exon 2 encodes Asn^{30}-Lys^{49}, which forms a connecting strand with short α helix and β pleated sheet. Exon 3 encodes Ala^{50}-Lys^{97}, which forms helix B+B', a short connecting strand and helix C. And finally exon 4 encodes Gly^{98}-Thy^{133}, to make a short connecting strand, a β-pleated sheet

and helix D (Brandhuber et al, 1987). To exert its biological effects, IL-2 must interact with specific high-affinity receptors. Helix A contacts the β-chain receptor. The minor α helix and connecting strand encoded by exon 2 contact the α chain and then a loop around the γc chain. Helix B+B' lie on top of the contact residue without connecting the IL-2R. Finally Helix D plays an important role in the engagement of the gamma c receptor and lies in a groove between the beta and γc chain (Voss et al. 1993).

Th1 cells, in response to activation by mitogen, alloantigens or antigens presented in context with appropriate MHC molecules, produce IL-2. Its expression is controlled predominantly at the levels of mRNA transcription and message stabilisation (Lindstain et al. 1989 and Fraser et al. 1991). It can also be produced after stimulation by B-lymphocytes but in very small amounts (Mouzaki et al. 1993). The effect of IL-2 on different cell type of the immune system is shown in Table 1.7.

Table 1.7: IL-2 effect on the immune system

On B-lymphocytes

- Augments B lymphocyte growth and increases immunoglobulin production (Ceuppens et al. 1985)

On T-lymphocytes

- Autocrine growth factor for T-lymphocytes and supports the development of cytotoxic T-lymphocytes (Ruscetti et al. 1977)
- Augments gamma interferon production (Ortaldo et al. 1984)
- Modulates the expression of IL-2R (Smith et al. 1985)

On other cells

- Participates in the activation, tumoricidal activity and growth of NK / LAK cells (Grimm et al. 1983)
- Induces IL-6 production by human monocytes (Musso et al. 1992)
- Modulates histamine production by stimulated basophils (White et al. 1992)
- Affects effector function of fibroblasts (Plaisance et al. 1992) and normal and malignant epithelial cells (Ciacci et al. 1993), myeloid cells including polymorphonuclear and monomorphonucler phagocytes as well as normal and myelopoietic progenitor cells (Pizzolo et al. 1993).

1.5.7.2.1. IL-2 receptor (IL-2R)

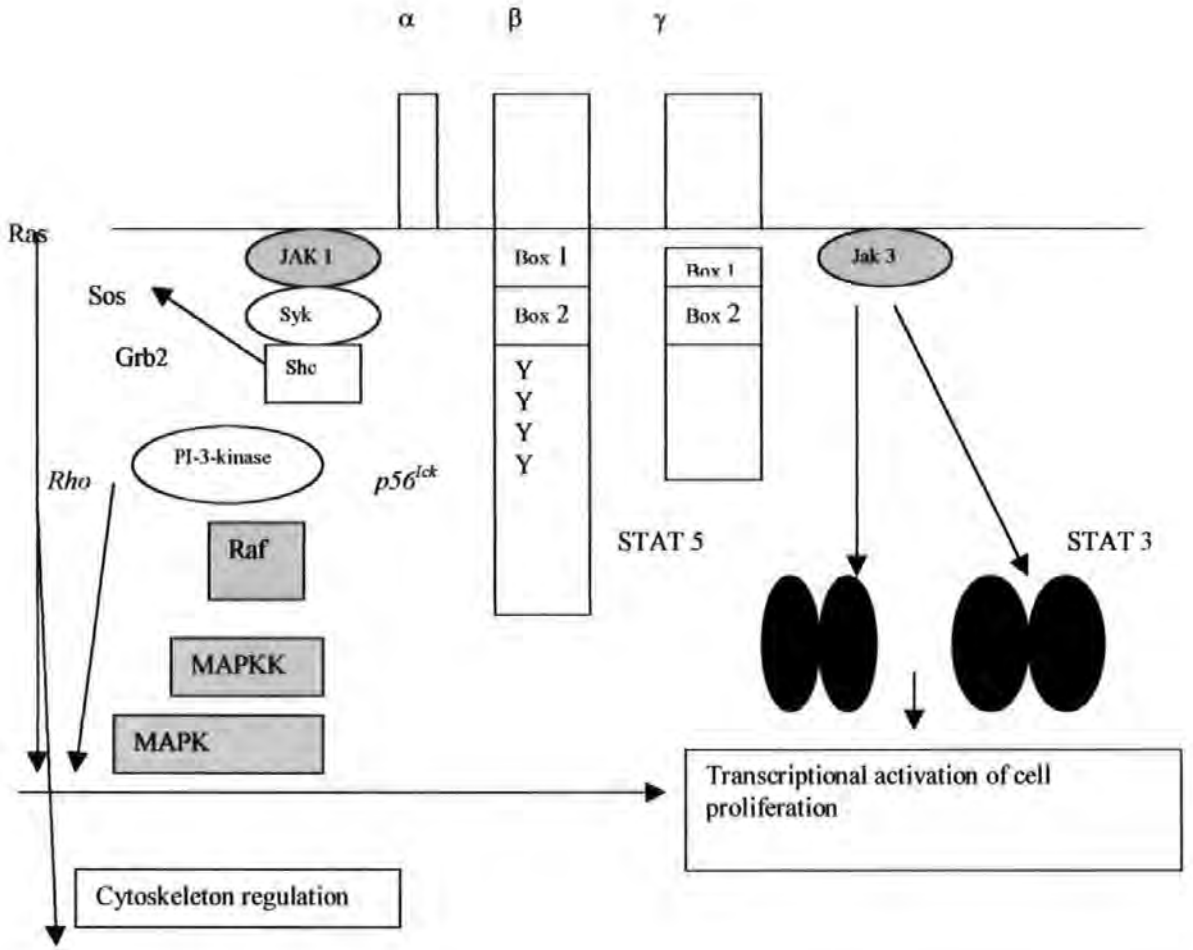
The effects of IL-2 on its target cell are mediated through specific cell surface receptors (IL-2R). The IL-2R contains three distinct subunits, a 55-kDa α chain, (Tac, p55, CD25), a 70-75kDa β chain (IL-2Rbeta, p70/p75, CD122) and a 64-kDa γ chain (IL-2R gamma, gamma c, p64). The existence of three different chains allows the formation of different isoforms of IL-2R: high, intermediate and low affinity. The three chains are noncovalently bound to form a heterotrimeric high affinity receptor (Nakamura et al. 1993, Minami et al. 1993).

The human IL-2 R α chain has been cloned and localised on chromosome 10 band 14-15 (Leonard et al. 1985). The α chain is structurally related to the IL-15R α chain and resembles members of the component receptor family (Bazan et al. 1992). The majority of the chain is

an extracellular domain and contains 11 cysteines and disruption of any of the cysteines reduces the ability of IL-2R α to bind (Rusk et al. 1988). There is also a suggestion that residues 158-160 may contribute to the interactions with the β and γc chain (Robb et al. 1988).

The human IL-2 R β chain has been mapped to chromosome 22 and the mature protein has 525 amino acids (Bazan et al. 1990). Little is known about the regulation of the IL-2R β gene. Mature unstimulated CD8+ cells are able to express the β chain (Ohashi et al. 1989) and its expression on B-lymphocytes can be induced by IL-2 or by IL-4 (Loughnan et al. 1989 and Nakanishi et al. 1996). The third chain, γc, forms part of individual receptor of several cytokines belonging to common γ chain receptor family (see chapter 1.6.6.10. Dimerisation of α, β, and γc chain induces IL-2 signal transduction (Figure 1.14).

Figure 1.6: IL-2 signalling pathway



Following stimulation IL-2R undergoes phosphorylation. Various kinases ($p56^{lck}$, Syk or JAK) phosphorylated specific substrates, triggering cascade of events organised in three major pathways; Shc activation and induction of the Ras pathway, the PI-3 kinase pathway and JAK-STAT pathway.
Abbreviations: IL-2=interleukin 2, α=alfa chain, β=beta chain, γ=gamma chain, JAK=Janus kinase, MAPK=mitogen-activated protein kinase, STAT=signal transducers and activators of transcription
Adapted from Theze et al. Immunology Today, p: 481-486,1996

1.5.7.3. Interleukin-4 (IL-4)

IL-4 was first shown to induce activation and immunoglobulin production by activated mouse B-lymphocytes (Swain et al. 1982, Sanderson et al. 1986). Molecular cloning of human IL-4 cDNA demonstrated a single open reading frame of 153 amino acids (aa) yielding a secreted glycoprotein of 129 aa with a molecular weight of 15-19 kDa. The IL-4 gene is located on the long arm of chromosome 5 on band q23-31 and is composed of 4 exons and 3 introns (Yokota et al. 1986). In close proximity are the genes for other related cytokines IL-3, IL-5, IL-13 and GM-CSF (Morgan et al. 1992). X-ray diffraction of IL-4 crystals and magnetic resonance spectroscopy of IL-4 in solution revealed a left-handed boundle of four α-helices with short stretches of β sheets. It belongs to the short chain 4-helix bundle superfamily along with IL-2 and IL-5 (Walter et al. 1992, Powers et al. 1992) (Figure 1.5). The existence of alternative IL-4 mRNA has been reported. This novel IL-4 spliced variant has been named IL-4 delta2 (IL-4 δ2), and is characterised by a lack of exon 2. This variant appears to be found naturally and is expressed at a higher level in thymocytes and bronchoalveolar lavage cells. The mechanism of action of IL-4 δ2 is not as a costimulator of T-lymphocyte differentiation but rather as an inhibitor of T-lymphocyte dependent differentiation (Atamas et al. 1996).

IL-4 can be produced by mast cells, basophils, a subpopulation of T-lymphocytes bearing NK1.1 markers and naive CD4, as well as CD8 T-lymphocytes and some IL-3 dependent bone marrow cell lines (Howard et al. 1982, Brown et al. 1987, Rodriques-Traduce et al. 1992 and Seder et al. 1992). The effect of IL-4 on the immune system is shown in Table 1.8.

Table 1.8: IL-4 effect on the immune system

On B-lymphocytes

- Induces MHC Class II expression (de Vries et al 1998).
- Induces proliferation in the presence of anti-IgM (Gordon et al. 1988)
- Induces expression and release of low affinity IgE receptor (CD23) (Vercelli et al. 1988)
- Stimulates IgE and IgG1 synthesis (Snapper et al. 1988)

On T-lymphocytes

- Induces proliferation of thymocytes (Murphy et al. 1992)
- Enhances cytotoxic activity of CTLs (Spitz et al 1988)
- Inhibits IL-2-induced CTL and LAK activity (Jin et al. 1989)

On other cells

- Stimulates mast cell growth (Wills-Karp, 1999)
- Induces MHC Class I and II expression and tumoricidal activity of macrophages (Thomson A, Cytokine handbook, Academic Press, 1998)
- Blocks IL-1, IL-6, IL-8, IL-10, IL-12 and TNFSF2 production by monocytes (Haro et al. 1989)

1.5.7.3.1. IL-4 receptor (IL-4R)

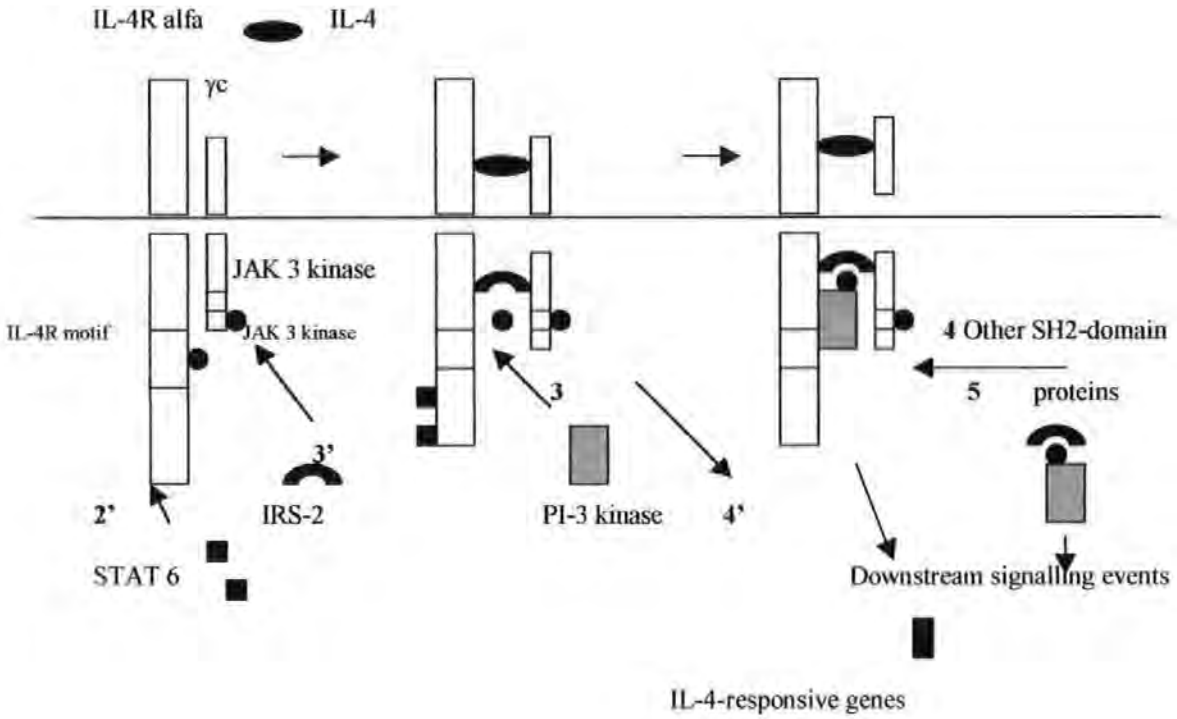
IL-4 acts via high affinity receptors expressed in low numbers on a wide range of cell types. They include B- and T-lymphocytes, granulocytes, monocytes, fibroblasts, epithelial and endothelial cells (Chomarat et al. 1997). The mature receptor represents a complex composed of at least two different proteins, the common γ chain shared by several interleukin receptors (Idzerda et al. 1990) and a 140 kDa high affinity binding chain (IL-4Rα) (Russell et al. 1993) (Table 1.9). IL-4R α is a glycoprotein composed of 800 amino acids (aa), with an extracellular domain of 207 containing two motifs. One is a single 24aa transmembrane domain, and the other a 569aa intracellular portion. The coding gene has been localised on the short arm of chromosome 16 (16p12.1) (Prichard et al. 1991). The common γc chain is a 65kDa protein, which after dimerisation with the IL-4Rα chain, increases the affinity of the

IL-4R by 2-3 fold. This Dimerisation is also essential for IL-4 induced signal transduction (Figure 1.7).

Table 1.9: Properties of the human IL-4 and IL-4Rα chain

	IL-4	IL-4Rα
Mature protein:		
amino-acids	129	800
Molecular weight kDa)	15-19	140
Disulphide bonds	3 (6 Cys)	3 (6 Cys)
Gene:		
Gene size (kilobase pairs)	10	3.6
Introns	3	
Chromosome location	5q23-31	16 (16p12.1)

Figure 1.7: IL-4 signalling pathway



IL-4 signalling cascade through the gp 140/γc IL-4R complex. (1) Binding of IL-4 to 140-kDa chain induces dimerisation of the IL-4Rα chain with γc chain. Tyrosine kinase associated to the receptor becomes activated. IL-4 binding allows also interaction of JAK-1 with box1 and box2 sequences located near the cellular membrane. (2) The IRS-2 protein interacts with the IL-4Rα and then becomes phosphorylated. (2') Monomers of STAT-6 binds to IL-4Rα chain. (3) Phosphorylated IRS-2 displays high affinity binding for SH2 domains of the p85 subunit of the phosphoinositol-3 kinase (PI-3 kinase). (3') STAT-6 is tyrosine phosphorylated by JAK-1, allowing its dimerisation and its release from the IL-4Rα chain. (4) IRS-2 protein may interact also with other SH-2 domain. (4') STAT-6 dimers are moved to nucleus leading to the activation of IL-4 regulated genes. (5) IRS-2-PI-3 kinase complex is released from the IL-4Rα.
Adapted from: Choromat P, The cytokine handbook, p 139, 1998

1.5.7.4. Interleukin-7 (IL-7)

IL-7 was originally identified as a factor secreted by bone marrow (Welch et al. 1989) and contains six exons over 33 kilobases (Lupton et al. 1990). Exon 5 of the human gene encodes the 18 amino-acid insert found only in human IL-7 (Goodwin et al. 1989). The molecular weight of IL-7 is 17.4 kDa. It is a single copy gene located on chromosome 8q12-13 (Southerland et al. 1989) in proximity to the $p53/p56^{Lyn}$ gene. IL-7 cDNA is composed of 534 nucleotides encoding a protein of 177 amino-acid residues with a signal sequence of 25 amino-acid residues and three potential N-linked glycosylation sites (Goodwin et al. 1989). Alternatively spliced human IL-7 mRNA lacking the entire exon 4 (44-amino-acid residues) has been isolated from SK-HeP-1 line (Korte et al. 1999).

Both immune and non-immune cells secrete IL-7. Bone marrow stromal cells are able to produce IL-7 as well as thymic cells and keratinocytes (Goodwin et al. 1990) intestinal epithelial cells and follicular dendritic cells (Kroencke et al. 1996). The effect of IL-7 on the immune system is shown in Table 1.10.

Table 1.10: IL-7 effect on the immune system

On B-lymphocytes

- It is able to induce the growth and differentiation of precursor B-lymphocytes.
- IL-7 most likely acts at some point after commitment to B cell lineage and exerts growth effects primarily on CD220+ pre-B-lymphocytes (Namen et al. 1990 and Hayashi et al. 1990).
- A number of genes involved in B-lymphocytes development may be upregulated by IL-7, including n-myc, c-myc (Morrow et al. 1992).

On T-lymphocytes

- T cell growth factor activity. IL-7 can influence both the proliferation and differentiation of T-lymphocytes. It may act directly on CD8+ve T-lymphocytes to augment cytotoxicity and may be a potent differentiation factor for the development of CTL. (Hickman et al. 1990)

On other cells

- Induction of pro-inflammatory cytokine production such as IL-1 and IL-6 from monocytes (Costello et al. 1992)
- Downregulates the expression of TGF-β from macrophages (Dubinett et al. 1993)

1.5.7.4.1. IL-7 receptor (IL-7R)

The IL-7 receptor (CD127) is composed of at least two subunits including a IL-7R α chain and the common γ chain (Kondo et al. 1993). The IL-7R α chain was identified by direct expression cloning (Goodwin et al. 1990) and is located on chromosome 5p13 (Lynch et al. 1992. The IL-7Rα forms a heterodimer with the γc chain, which is required for high affinity IL-7 binding (Noguchi et al. 1993). IL-7Rα has been detected on pre-B-lymphocytes, human intestinal cells, and bone marrow macrophages but not on mature B-lymphocytes (Foxwell et al. 1992). It seems that in the B cell lineage, IL-7R α expression is tightly regulated. In the thymus, double negative (CD4-ve, CD8-ve) and single positive (CD4+ve or CD8+ve) lymphocytes express this receptor. In contrast to the tightly regulated expression of the IL-7α chain, the γ chain is constitutively expressed on mature B-lymphocytes, fetal and neonatal thymocytes and mature T-lymphocytes.

Binding of IL-7 to its receptor involves activation of the Janus tyrosine kinase proteins (JAK), JAK-1 and JAK-3 (Foxwell et al. 1995). The transduction of the signal into the nucleus is then mediated by the STAT proteins (STAT-1 and STAT-5) (Zeng et al. 1994).

1.5.7.5. Interleukin-9 (IL-9)

IL-9 is a 30-40 kd glycoprotein. Its single copy gene is located on chromosome 5 near the loci for IL-3, IL-4, IL-5, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-13 (Modi et al. 1991). It contains 5 exons and 4 introns stretching over 4 kd (Renauld et al. 1990). IL-9 is produced preferentially by Th2 lymphocytes. Unstimulated freshly isolated PBMC do not express IL-9 mRNA but stimulation with PHA or anti-CD3 antibody induced substantial expression of IL-9 in CD4 T-lymphocytes (Renauld et al. 1990). After stimulation with CD3 and CD28 expression of IL-9 was restricted to CD45RO+ve T-lymphocyte subset (memory cells) (Houssiau et al. 1995). IL-9 expression is associated with HTLV-I, a retrovirus involved in adult T cell leukaemia (Kelleher et al. 1991). The effect of IL-9 on the immune system is shown in Table 1.11.

Table 1.11: IL-9 effect on the immune system

On B-lymphocytes

- IL-9 synergises with IL-4 for IgE and IgG production but not for IgM production. Generally IL-9 favors humoral autoimmunity but inhibits cell-mediated autoimmune processes. (Dugas et al. 1993)

On T-lymphocytes

- IL-9 exhibits growth-promoting activity for T-cell tumors (patients with Hodgkin and large cell anaplastic lymphoma constitutively produce IL-9) (Merz et al. 1991)

On other cells

- In murine model IL-9 promotes growth and differentiation of bone marrow derived mast cell lines (Moeller et al. 1989). It also increases IL -6 secretion by mast cell lines (Hutner et al. 1990).
- IL-9 promotes some granulocytic as well as monocytic colony growth from CD34+CD2- progenitors (Schaafsma et al. 1993)
- Addition of IL-9 was found to increase proliferation of human myeloid leukaemic cells suggesting preferential activity on transformed cells as compared to their normal progenitors (Lemoli et al. 1996).

1.5.7.5.1. IL-9 receptor (IL-9R)

The human IL-9 receptor cDNA encodes a 522 amino acid protein. IL-9R is a member of the haematopoietin receptor superfamily. It interacts with the γ chain of the IL-2 receptor, which is required for signal transduction but not for IL-9 binding (Demoulin et al. 1998).

1.5.7.6. Interleukin-15 (IL-15)

The human IL-15 gene maps to chromosome 4q31 (Anderson et al. 1995). The proximity to the IL-2 gene suggests a common ancestry within the helical cytokine family. IL-15 is a cytokine of between 14 and 15 kDa. IL-15 and IL-2 share little sequence homology, and it is possible to align their sequences on the basis of the helical structure of IL-2. There are 21 amino-acid identities between IL-15 and IL-2 (Pettit et al. 1997).

IL-15 mRNA is expressed constitutively in a wide variety of cells (monocytes and macrophages) and in many tissues (skeletal muscle, placenta, heart, lung, liver kidney and dermal layer of the skin), but is not expressed in normal T, B or natural killer (NK) cells (Grabstein et al. 1994, Doherty et al. 1996). The effect of IL-15 on the immune system is shown in table 1.12.

Table 1.12: IL-15 effect on the immune system

On B lymphocytes

- IL-15 induces proliferation following preactivation with PMA or IgM but not in resting B-lymphocytes. It is involved in the differentiation of normal B-lymphocytes and induces secretion of immunoglobulins in combination with anti-CD40 stimulation (Armitage et al.1995).
- IL-15 regulates growth of B-CLL cells regardless of their preactivation (Trentin et al. 1996).

On T-lymphocytes

- IL-15 is able to stimulate the growth of activated T lymphocytes, CD4+, CD8+ and γδ T cells. (Grabstein et al. 1994)
- IL-15 inhibits apoptosis of cytokine-deprived; activated T-lymphocytes (Akbar et al. 1997).
- It induces cytokine production by T-lymphocytes (Mori et al. 1996).

On other cells

- IL-15 may be a growth and differentiation factor for NK cells as well as inducing cytokine production by these cells (Mrozek et al. 1996).
- It activates neutrophils and mast cells proliferate in response to IL-15 (Tagaya et al. 1996).

1.5.7.6.1. IL-15 receptor (IL-15R)

The IL-15 receptor is composed of at least three subunits: an IL-15Rα chain, the IL-2Rβ chain and the γc chain. This combination has similarities with the IL-2-IL-2R system, in which the generation of the high affinity receptor for IL-2 requires the presence of all three subunits.

Antibodies recognizing the IL-2Rβ and γ chains inhibit IL-15 mediated responses. The IL-15Rα chain is a type I membrane protein of 263 amino acids and it belongs to a larger family of proteins that contain protein-binding motifs known as 'sushi domains' (Giri et al. 1994). The gene coding the human IL-15R α chain is located on chromosome 10p14-p15. The α chain is required for high-affinity binding, but not for signaling by IL-15 (Anderson et al. 1995). IL-15R α chain mRNA is expressed in a wide variety of cells and tissues, including fibroblasts, epithelial cells, B- and T-lymphocytes, monocytes, prostate, liver, testis and ovary. The IL-2Rβ-γc heterodimer forms the core signalling complex, wchich associetes with JAK1 and JAK3 and activates STAT3 and STAT5. The downstream events that follow ligand binding include the activation of nuclear proteins, such as myc and fos and the upregulation of bcl-2 and bcl-xL levels, leading to resistance to apoptotic cell death leading to enhanced survival or proliferation (DiSanto, 1997).

1.5.8. Immunoregulation of B-CLL cells

In vitro experiments have indicated that many cytokines may be involved in the induction or regulation of B-CLL B-lymphocytes proliferation (Cordingley et al. 1988, Nerl et al. 1988, Touw et al 1992 and Mainou-Fowler et al. 1995) (Figure 1.8). These include TNFSF2, IL-2, IFN-α, IL-4 and IL-7. It is still unclear which cytokines or cell-cell contacts are important to the survival of B-CLL cells in vivo, and whether the cytokines are produced by the tumour cells themselves or by accessory cells. However, it is now apparent that some cytokines play a role in the growth and differentiation of B-lymphocytes, while others may intervene in apoptosis. During immunological responses, normal B-lymphocytes secrete a number of cytokines such as GM-CSF, IFN-γ, TGF-β, IL-1α/β, IL-2 IL-7, IL-8 and IL-10 (Gause et al. 1996). Production of IL-2 by normal B-lymphocytes is limited to the proliferating cells that also have increased expression of IL-2R. This suggests autocrine production of IL-2 (Schena et al. 1992b).

An autocrine/paracrine role in malignant B-lymphocytes has only been suggested for TNFα (Cordingley et al. 1988). TNF-α has been shown to induce cell proliferation in the majority of B-CLL patients, with or without prior stimulation. Soluble TNF-receptors have been demonstrated in the serum of B-CLL patients (van Kooten et al. 1992 and Waage et al. 1992). IL-4 and IL-6 can inhibit TNF-α-induced proliferation *in vitro* (van Kooten, 1992). There is also evidence for the constitutive expression of IL-6 genes in B-CLL (Freeman et al. 1989) but it is not clear whether IL-6 is a growth or differentiation factor for B-CLL. Serum levels of IL-6 measured by ELISA are increased in B-CLL and IL-6 can inhibit apoptosis of malignant cells (Reittie et al. 1992 and 1996).

Freshly isolated B-CLL cells or cells cultured in the presence of phorbol esters or anti-μ, express the high affinity IL-2 receptor, and purified B-CLL cells respond to IL-2 with increased DNA synthesis and differentiation (Emilie et al. 1988). There is also evidence that IL-2 is able to prevent apoptosis (Huang et al. 1993a).

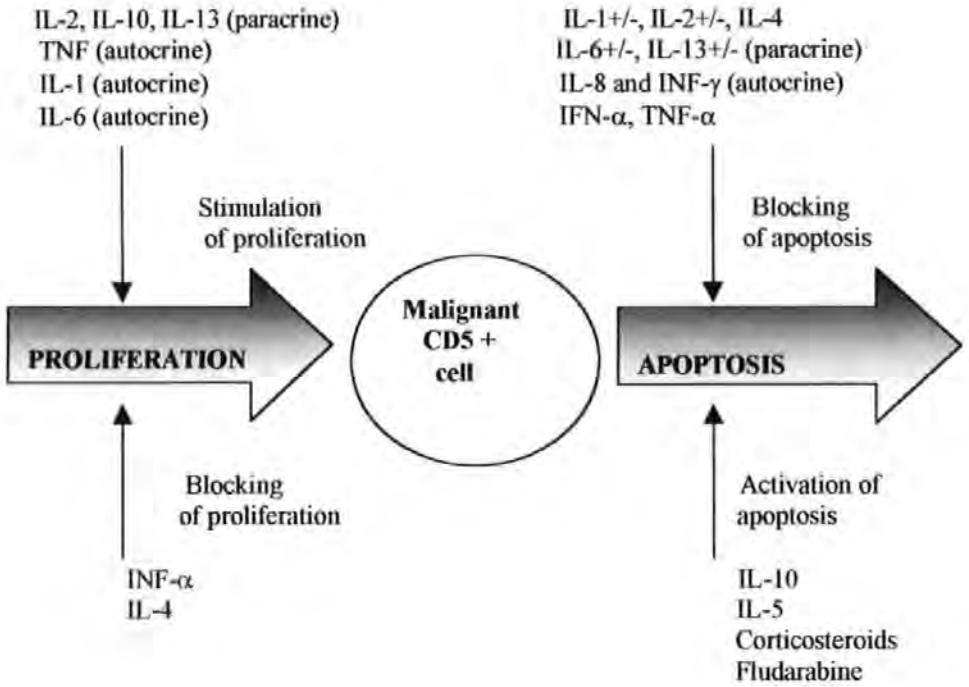
B-lymphocytes from patients with B-CLL also secrete IL-1, but its production, as assessed by mRNA and protein analysis, does not correlate with disease progression (Aquilar-Santelines et al. 1989 and 1991). However, IL-1 alone does not induce DNA synthesis in B-CLL B-lymphocytes, therefore it is unlikely to be an important autocrine growth factor.

Apoptosis is an important process through which the ontogeny of both B- and T-lymphocytes can be regulated. Disruption of this process may lead to malignancy. There is evidence that the presence of certain cytokines may be responsible for the long life *in vivo* of B-lymphocytes from B-CLL patients. These cytokines include IFN-γ and IL-4. IFN-γ prolongs the life span of B-CLL cells and is present in sera of B-CLL patients (Buschle et al. 1993). The mechanism by which INF-γ inhibits apoptosis is unclear but some data suggests inhibition of the loss of bcl-2 protein in malignant B-lymphocytes (Buschle et al. 1993).

The effect of IL-4 on B-lymphocytes from patients with B-CLL depends on which co-stimulatory signals are used *in vitro* experiments. IL-4 increases DNA synthesis in B-CLL B-

lymphocytes when they are stimulated with PMA or anti-CD40 monoclonal antibodies (Carsson et al. 1989), but inhibits DNA synthesis when the B-lymphocytes are stimulated with TNF or IL-2 (Karray et al. 1988). Most importantly, however, it has been shown that IL-4 inhibits spontaneous apoptosis, thus arresting the malignant B-lymphocytes in Go phase. This rescue from apoptosis is associated with an increase in bcl-2 levels (Panayiotidis et al. 1993 and Dancescu et al. 1992). It has also been demonstrated that IL-8, IL-13 and IFNα can suppress apoptosis of B-CLL cells (di Celle et al. 1996a,b and Chaouchi et al. 1996). A delay in down-regulation of bcl-2 *in vitro* has been proposed as a common mechanism for the suppression of apoptosis by IFNα, IL-4 IL-6 IL-13, TNFSA2 (Tanquye et al. 1997). In addition IL-5 increases apoptosis of B-CLL cells (Mainou-Fowler et al. 1994). There is an inverse correlation between IL-10 mRNA expression and disease progression in B-CLL, but this is not so apparent when serum levels and B-CLL status are compared (Sjoberg et al. 1996 and Eagle et al. 1996).

Figure 1.8: Regulation of apoptosis in B-CLL



Many factors that regulate proliferation and apoptosis of B-CLL cells are cytokines. They act on cell in autocrine and paracrine fashion and their action involves stimulation or blocking of proliferation and blocking or activation of apoptosis.

1.6. APOPTOSIS OF MALIGNANT B-LYMPHOCYTES

1.6.1. Introduction

B-cell chronic lymphocytic leukaemia (B-CLL) results from a clonal accumulation of CD5+ve B-lymphocytes. This expansion of B-lymphocytes is due to increased proliferation and extended survival due to decreased cell death (Collins et al. 1989).

Cell death can occur by two quite distinct mechanisms, namely necrosis and apoptosis (Wyllie et al. 1980 and Duvall et al. 1986). **Necrosis** occurs when cells are exposed to external conditions such as inflammation, ischaemia, hypoxia or toxic injury. It is a pathological process and begins with impairment of the cells ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles such as mitochondria and the entire cell swell and rupture (cell lysis). The cytoplasmic contents, including lysosomal enzymes, are released into the extracellular fluid. Therefore *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Van Furth et al. 1988).

The term **apoptosis** (programmed cell death-PCD) is derived from the Greek word meaning 'falling off' and was first introduced in 1972 by Kerr (Kerr et al. 1972). Although it was described many decades ago, the significance of apoptosis has been largely overlooked until recent years. Apoptosis is essential for the development and homeostasis of multicellular organisms. It is an active, metabolic, genetically determined and evolutionary selective death pathway (Wyllie et al. 1992), in which cell death can be influenced by external and internal factors (Hale et al. 1996). The scanning electron microscope has allowed more detailed images of apoptosis. An irradiated erythroleukemia model was used to demonstrate the importance of the redistribution of the nuclear membrane pores at the start of apoptosis (Pietro et al. 1994 and Falcieri et al. 1994). The main ultrastructural features of the apoptotic cell are condensation of nuclear chromatin, distinct crescent forming and undergoing of nucleolar segregation (Figure 1.9). The cell shrinks, becomes dense and cytoplasmic

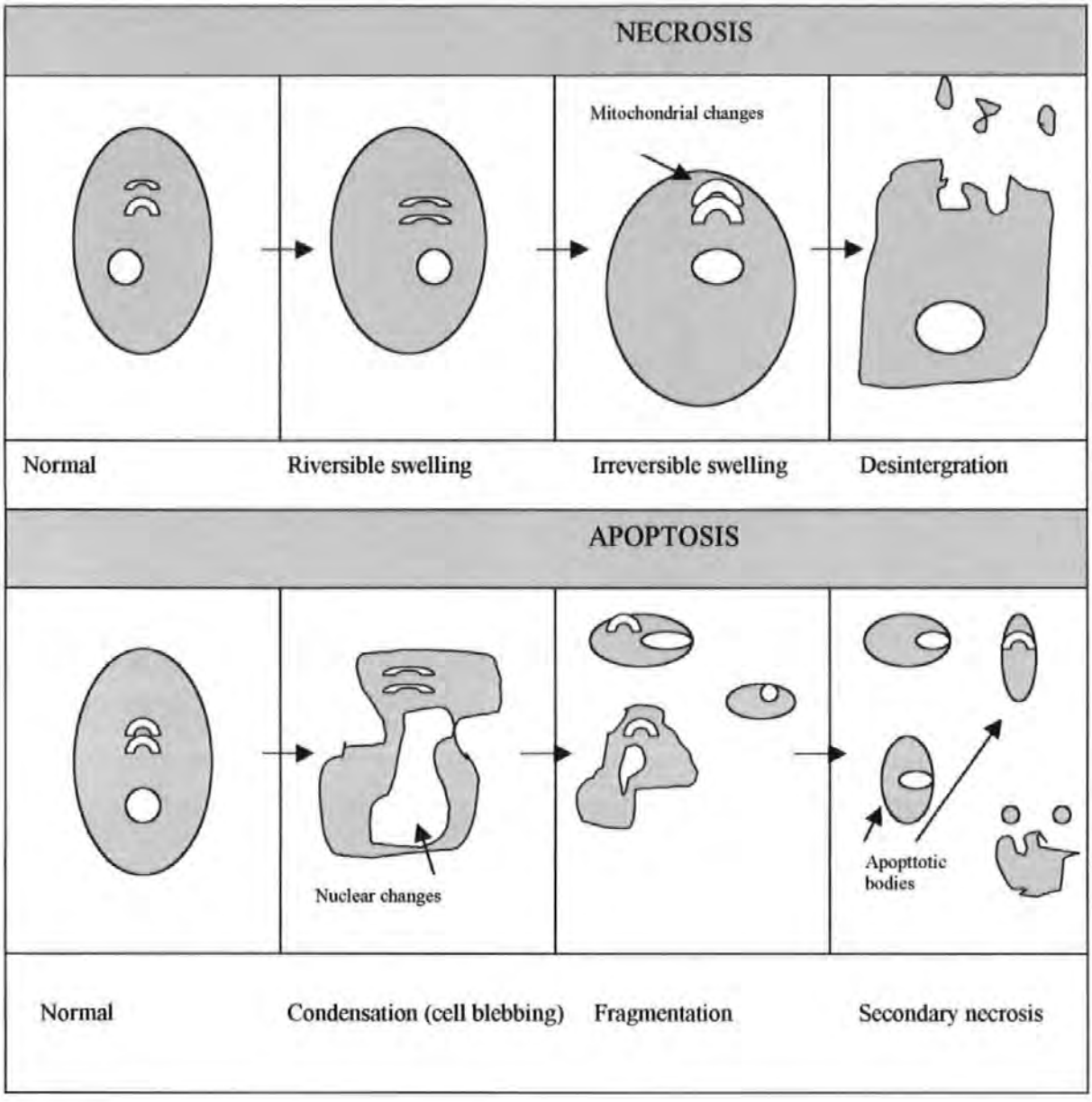
vacuolisation occurs. The nucleus fragments into membrane-bound segments and the cell surface undergoes a budding process, resulting in the formation of apoptotic bodies, which are targets for phagocytes (Samaha et al. 1995). This process allowed removal of the apoptotic cells without the cytoplasmic contents coming into contact with the neighbouring cells. The biochemical features of apoptosis are endogenous endonuclease activation, resulting in fragmentation of double stranded DNA. This cleavage of DNA at the internucleosomal linker regions results in a 'ladder' of 180-200 base pairs fragments that are easily observed after gel electrophoresis. Laddering is considered to be a hallmark of apoptosis (Compton, 1992).

The entire process of apoptosis is very rapid, the morphological changes appearing in 5 to 30 minutes. It is also so precisely executed that, for example, in the development of nematode *Caenorhabditis elegans,* exactly 131 cells die according to a well-regulated genetic programme (Hergartner et al. 1994). To ensure homeostasis as many as 10^{11} cells die by apoptosis in the adult human daily. But apoptosis occurs under physiological and pathological conditions. For example it is essential for deleting autoreactive cells during lymphoid development (Rothstein, 2000, Nilsson et al. 2000). It is a significant physiological mechanism for establishing B-lymphocytes tolerance and shaping B-lymphocyte repertoire. B-lymphocytes are most probably eliminated within the germinal centres. Mature B-lymphocytes also undergo apoptosis by signalling through CD95/APO 1/Fas. CD95 is a 48-kd transmembrane glycoprotein, which belongs to the tumour necrosis factor/nerve growth factor receptor family and it is expressed on a variety of normal human tissue and tumours (Trauth et al. 1989). Similarly apoptosis in thymocytes is associated with the elimination of self-reactive clones of developing T cells following their interaction with antigens in the thymus. Incubation of naïve T-lymphocytes with antibodies against T-cell receptor (TCR) or anti-CD3 antibodies, leads to their activation, proliferation, and cytokine production. On the

other hand, the same stimulation of activated T-lymphocytes, causes their death by apoptosis (Brunner et al. 1995).

Increased proliferation as well as reduced apoptosis may lead to disease (Akbar et al. 1997 and Graig et al. 1995).

Figure 1.9: Morphology of necrotic and apoptotic cells



Necrosis accurs when cells are exposed to extreme conditions resulting in damage of the plasma membranes. Cells ability to maintain homeostasis leads to an influx of water and extracellular ions. Intracellular organelles, most notably mitochondria and entire cell swells and ruptures.
Apoptotic cells show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation and formation of apoptotic bodies that contain mitochondria and nuclear material. Adapted from Trauth et al. 1997.

1.6.2. Regulators of apoptosis

The whole process of apoptosis can be divided into three groups: induction, execution and degradation. The induction phase is characterised by changes in the cellular environment leading to the cell activating the mechanism of apoptosis. In the execution phase, processes within the cell that result in committal to apoptotic cell death take place (Kroemer et al. 1997). In the degradation phase, the final disposal of the cell takes place. Our knowledge of the components of the regulatory machinery, which include the bcl-2 family members and caspases or cysteine proteinases related to IL-1β converting enzyme (ICE), continues to develop. Apoptotic regulators such as FasL and TNF, induce apoptosis, a fact that is largely confined to the development and regulation of the immune system.

Triggers	**Regulators**	**Executors**
DNA damage	p 53	Apaf-1
Cytokines	bcl-2	Caspases
Hypoxia	Myc/oncogenes	
Temperature	Cytochrome c	
Death receptors		

A number of pathological and physiological triggers can activate apoptosis. Regulators include factors which can stimulate or suppress apoptosis and executioners represent a point of no return in the life of the cell.

Apoptosis is regulated by a large number of internal and external factors (Staunton et al. 1998). Some play a role as initiators or inducers of apoptosis; oncogenes and tumour suppressor genes (c-Myc, p53, Bax, Bak), tumour necrosis factor (TNF), hyperthermia, cytokines, bacterial toxins, viral infections, Fas ligand, glucocorticoids, oxidants, radiation

therapy and cytotoxic drugs. Others act as inhibitors of apoptosis: growth factors, CD40 ligand, interleukins, estrogens and androgens, pharmacological inhibitors and oncogenes and tumour suppresser genes (Bcl-2, Bcl-x_L, Mcl-1) (Staunton et al. 1998). These gene products constantly interact with each other, which suggests that the process of proliferation and cell death is closely linked. Generally, there are two central pathways that can lead to apoptosis: i) positive induction by ligand binding to the plasma membrane receptor and ii) negative induction by loss of suppressor activity. Negative induction of apoptosis involves the mitochondria (Figure 1.6). The release of cytochrome c from mitochondria into the cytosol serves as a trigger to activate caspases (Green et al. 1998 and Liu et al. 1996). Each leads to the activation of cysteine protease, which has homology to the IL-1β converting enzyme (ICE) (Thornberry et al. 1998).

1.6.2.1. Inducers of apoptosis

Fas ligand, tumour suppressor genes (bax, bak, c-Myc and p53) as well as TNF, hyperthermia, cytokines, bacterial toxins and viral infections can act as initiators or inducers of apoptosis.

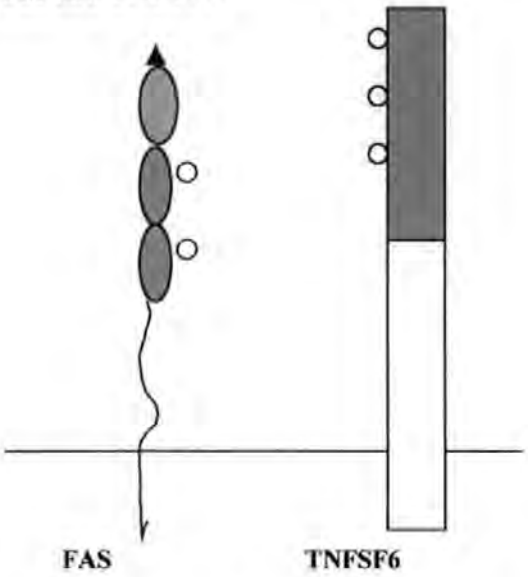
1.6.2.1.1. Fas/CD95/APO-1

The Fas receptor (CD95, Apo-1, TNFRSF6) belongs to a family of receptors including the tumour necrosis factor (TNF) and nerve growth factor (NGF) (Nagata et al. 1995). Fas, is a cell surface receptor for a ligand FasL and cross-linking of FasL (TNFSF6) triggers apoptosis on the target cells. FasL trimerises when it binds to its cell surface receptor (Ashkenazi et al. 1998). The intracellular portion of these receptors contains an 80 amino acid death domain (DD) (Figure 1.10A). The DD domain binds to MORT1/FADD, which interacts with caspase-8, a member of ICE/Ced-3 protease family. After caspase-8 activation, the cascade of caspase-mediated disassembly proceeds. It is expressed in abundance in activated mature lymphocytes, lymphocytes transformed with human immunodeficiency virus (HIV) or human

leukaemia virus (HTLV-I) and also on certain tumour cells. The ability of primary B-lymphocytes to Fas-mediated apoptosis is modulated by additional signals. CD40 engagement produces up-regulation of Fas expression and sensitivity to Fas-induced death, whereas antigen receptor or IL-4R engagement inhibits Fas induced killing (Rothstain et al. 2000).

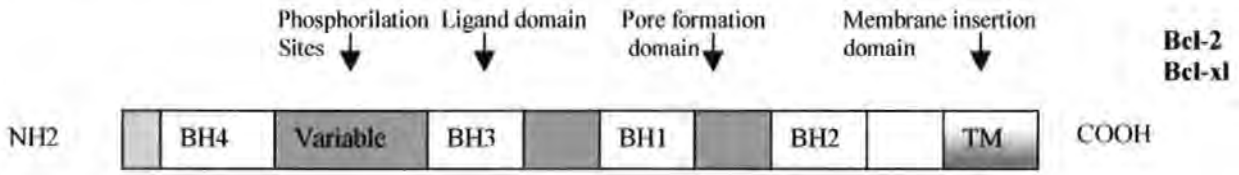
Figure 1.10: Structure of Fas, TNFSF6 and bcl-2 family

A) Structure of Fas and TNFSF6



Schematic diagram of the structure of Fas and TNFSF6. The extracellular domain of Fas contains three cysteine-rich subdomains (shaded areas). The intracellular domains are shown as bold lines. The arrow indicates N-glycosylation sites. The TNFSF6 is a type II membrane protein. The shaded area is the extracellular domain. Adapted from Kabelitz et al. 1997

B) Structure of some of the members of Bcl-2 family



All members of Bcl-2 family (except Bad and Bid) contain a hydrophobic C-terminus transmembrane (TM) domain, which serves to anchor protein to membranes. Bad lacks this sequence. BH1 and BH2 in death antagonists (Bcl-1, Bcl-xL) allow heterodimerisation with Bax to repress cell death. BH3 in death agonists (Bax, Bak) allows heterodimerisation with Bcl-xL and Bcl-2 to promote cell death. BH4 domain is conserved in death antagonists (Bcl-xL). It allows interaction with death regulatory proteins such as Raf-1, Bad and Ced-4. Adapted from Adams et al. 1989

1.6.2.1.2. Bax, Bak and Bad

These are members of the pro-apoptotic bcl-2 family. They do not induce apoptosis because their activity is maintained in a latent form. After an appropriate signal, Bax undergoes a conformational change and moves to the mitochondrial membrane where it induces release of the mitochondrial cytochrome c into the cytosol (Wolter et al. 1997). Similarly Bak appears to undergo conformational changes and Bad is sequestered in the cytosol when cytokines are present (Datta et al. 1997). An activation cascade of pro-apoptotic proteins from bid to bak or bax integrates the pathway from surface death receptors to the irreversible efflux of cytochrome c (Korsmeyer et al. 2000).

1.6.2.1.3. C-myc

C-myc is a proto-oncogene whose dysregulated expression promotes cell proliferation and neoplastic transformation. It is localized in the nucleus and has a short half-life. When factors which suppress c-myc, such as bcl-2, insulin-like growth factor or platelet-derived growth factor are missing, c-myc is able to induce apoptosis (Evan et al. 1992).

1.6.2.1.4. p53

P53 is a tumour suppressor that inhibits or reduces transformation by viral and cellular oncogenes or other genotoxic stresses like DNA damage and hypoxia (Levine et al. 1997, Miyashita et al. 1994). P53 is capable of inducing apoptosis in several physiological conditions and, above all, following radio- or chemo-induced DNA lesions (Lane, 1992). It appears to act as a component of feedback control mechanisms, which regulate entry of the cell from the G_1 phase of the cell cycle into S phase and passage through the S phase to G_2 and mitosis. Cells with inactivated p53 might therefore survive abnormally. After exposure to stressful stimuli, p53 is activated through post-translational mechanisms. One of the important target genes transcriptionally activated by p53 is the cyclin-dependent kinase (CDK) inhibitor (Milne et al. 1994).

Mutations or deletion of p53 are the most frequent genetic abnormalities found in human tumours. P53 is a short-lived protein that is maintained at very low or undetectable levels. Many human p53 mutants have been described. As far as apoptosis is concerned, p53 target genes such as bax and Fas/APO1 have been implicated. Translocation of bax to the mitochondria and release of cytochrome c trigger activation of caspase 9 and the downstream caspase cascade (Somasundaram, 2000).

1.6.2.2. Inhibitors of apoptosis

1.6.2.2.1. Bcl-2

The main anti-apoptotic protein is bcl-2. Bcl-2 is a member of the family of over a dozen proteins including suppressors of apoptosis (bcl-2 or Bcl-x_L) or promoters of apoptosis (bak and bax) (Korsmeyer et al. 2000) (Figure 1.10B). These proapoptotic proteins interact antagonistically with anti-apoptotic proteins via their BH3 (Bcl-2 homology domain) domain that is determined by the ratio of the level of expression of each protein (Han et al. 1996 and Zha et al. 1996). BH3-dependent heterodimerisation between bcl-2 family members appears to be the key function of the anti-apoptotic bcl-2 family members (Degterev et al. 2001).

The bcl-2 gene was first discovered as a proto-oncogene found at the break points of t (14:18) chromosomal translocations in low-grade B cell lymphoma (Tsujimoto et al. 1984, Cleary et al. 1986). This translocation was found to increase expression of the bcl-2 gene by placing the coding region under the control of the immunoglobulin heavy chain enhancer (Tsujimoto and Croce, 1986). The bcl-2 protein is 239 amino acids in length. It is localised in the outer mitochondrial membrane and the nuclear envelope, with a portion of the bcl-2 protein in the endoplasmic reticulum (Krajewski al. 1993). The level of bcl-2 expression is regulated during normal lymphoid development. An example of this is up-regulation of bcl-2 in the earliest stages of lymphoid maturation in pro-B-cells and CD4 and CD8-thymocytes and down-regulation at the later stage in pre-B/IgM IgD- and CD8+CD4+ lymphocytes. This is

followed by up-regulation of bcl-2 in mature B- and T-lymphocytes (Pezzella et al. 1990). An additional role for bcl-2 in survival of the high-affinity B-lymphocytes in the germinal centres has also been proposed (Liu et al. 1991).

Over-expression of bcl-2 is capable of antagonising apoptosis triggered by c-myc or p53. However, bcl-2 rescues apoptosis without affecting the mitogenic function of c-myc nor reversing growth arrest by p53. This suggests that bcl-2 may be a downstream regulator of c-myc or p53. Bcl-X is a member of the bcl-2 family. Bcl-X transcripts are alternatively spliced into two products: bcl-X_L, the long form and bcl-X_S, the short form. Bcl-X_L acts to inhibit apoptosis in a similar fashion to bcl-2, whereas bcl-X_S antagonises its action (Dietrich, 1997). Many questions remain concerning the molecular mechanisms by which these membrane bound bcl-2 family members exert their control over caspase activation. Recent reports demonstrate that the mitochondrial protein cytochrome c can promote caspase activation in a cell-free apoptosis system (Liu et al. 1996). It is released into the cytosol following the induction of apoptosis by many different stimuli, including CD95, TNF and chemotherapeutic DNA-damaging agents (Reed et al. 1997). Cytochrome c is released from mitochondria in cells undergoing apoptosis and activation of caspase-3 is prevented by bcl-2 or bcl-X_L expression (Yang et al. 1997 and Kharbanda et al. 1997). In B-CLL, bcl-2 is over-expressed and may prolonged survival of the malignant B-CLL clone (Aviram et al. 2000) and over-expression of bcl-2 may correlate with the stage of the disease (Niewiadomska et al. 2000).

1.6.2.3. Executioners of apoptosis

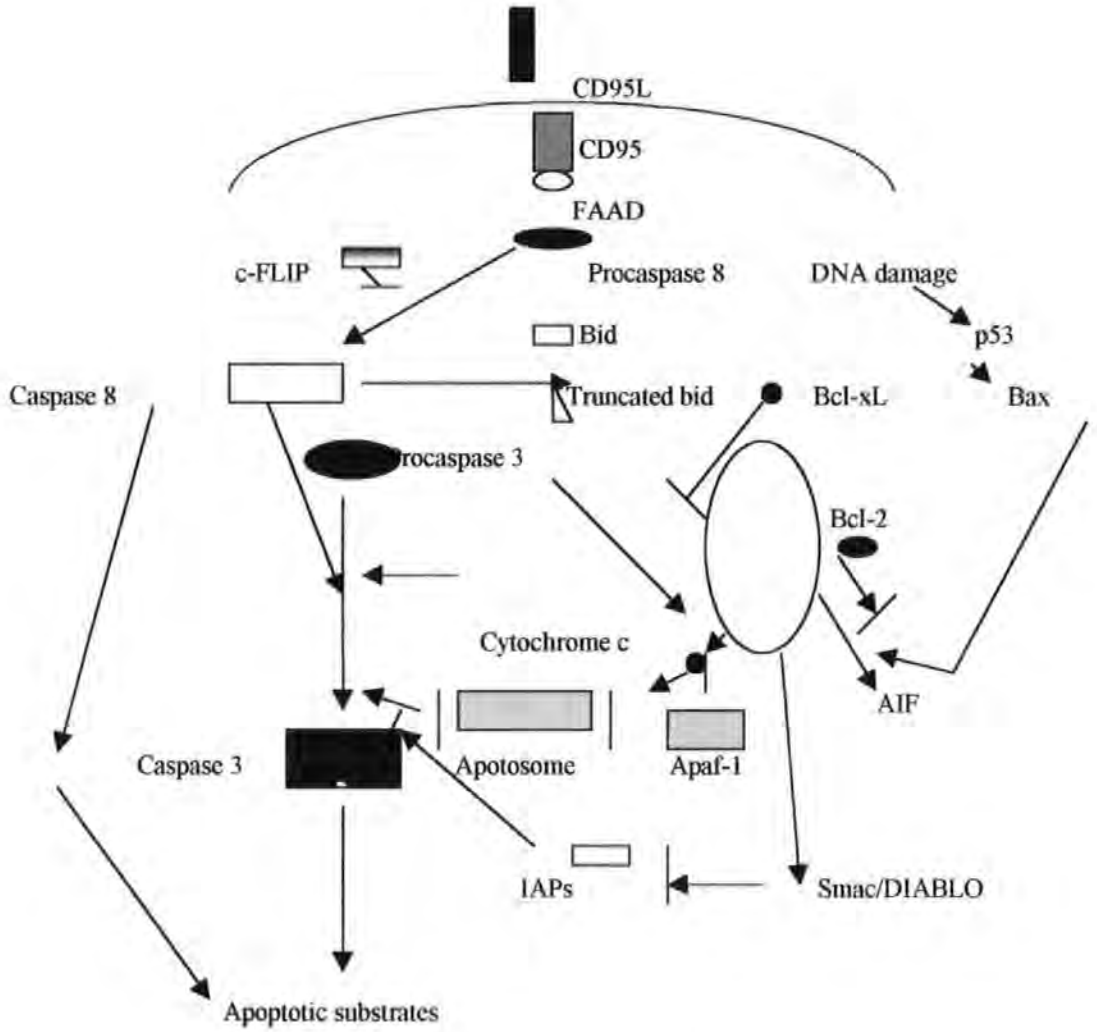
1.6.2.3.1. Caspases

The basic effectors of apoptosis are death proteases, referred to as caspases. Cysteine Aspartate Specific Proteases (Caspases) are synthesized as inactive "proenzymes" that are processed by proteolitic cleavage to form an active enzyme. Within apoptotic cell, caspases trigger a cascade of proteolitic cleavage events and are thought to participate in apoptosis by

disabling important cellular processes and breaking down structural components of the cells (Nicholson and Thornberry, 1997).

The caspases are a family of cysteine proteases that play a critical role in the execution phase of apoptosis. They are responsible for many biochemical and morphological changes associated with apoptosis (Cohen, 1997 and Cryns et al. 1998). Caspases are related to mammalian interleukin 1β-converting enzyme (ICE) and to the nematode apoptotic gene product Ced-3 (Salveson et al. 1997). Since their discovery in 1993 at least 10 different caspases have been identified. It has been proposed that 'initiator' caspases with long pro-domains, such as caspase-8, either directly or indirectly activate 'effector' caspases such as caspases 3, 6, and 7 (Cohen, 1997, Fraser et al. 1996). These effector caspases then cleave intracellular substrates, such as poly polymerase (PARP) and lamins, during the execution phase of apoptosis. Caspase-8 is though to be an initiator caspase in many forms of apoptosis in addition to CD95-induced apoptosis. Procaspase-9 has been also proposed to be an initiator caspase. In the presence of dATP and cytochrome c, its long N-terminal domain interacts with Apaf-1 resulting in the activation of caspases –3, -6 and –7 (Li et al. 1997, Zou et al. 1997 and Srinivasula et al. 1998).

Figure 1.11: Death receptor pathway



The death-receptor pathway is triggered by CD95. Binding of CD95 ligand (CD95L) induced receptor clustering and formation of a death inducing signalling complex. This complex recruits via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase 8 activation. This action can be blocked by recruitment of the degenerate caspase homologue c-FLIP (FLICE-like inhibitory protein). The mitochondrial pathway is used in response to extracellular cues and internal insults such as DNA damage. Pro and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they complete to regulate cytochrome c by a mechanism, which is still unknown. If a pro-apoptotic member wins, an array of molecules is released from the mitochondrial compartment. The main of these is cytochrome c, which associates with APAF-1 (apoptotic protease-activating factor-1) and then procaspase-9 to form the apoptosome. The death-receptor and mitochondrial pathways converge at the level of caspase 3 activation. Caspase-3 activation and activity is antagonised by the IAP proteins (inhibitor of apoptosis), which themselves are antagonised by the Smac/DIABLO protein (mitochondrial protein, Smac=DIABLO) released from mitochondria. Cross talk between death-receptor and mitochondrial pathway is provided by Bid, a pro-apoptotic BCL-2 family member. Caspase 8-mediated cleavage of Bid greatly increases its pro-apoptotic activity.
Adapted from Hergartner, 2000.

1.6.3. Apoptosis in B-CLL

Apoptosis is the physiological process in the homeostasis of cells, including normal B-lymphocytes (Krammer et al. 1994). B-CLL is characterised by a progressive accumulation of clonal malignant B-lymphocytes, increased proliferative capacity and diminished cell death. It has been suggested that apoptosis is in some way 'defective' or inhibited in cancer (Hoffman et al.1994). Malignant B-lymphocytes when cultured *in vitro* die by apoptosis suggesting the influence of humoral factors and/or cellular interactions (Collins et al. 1989). Spontaneous apoptosis (SP), which shows inter-patient variation, affects 3% to 65% of cells at 72hr. It is not found in normal B and T lymphocytes under the same culture conditions. During SP, the level of bcl-2 protein decreases, while the expression of c-myc and wild type p53 protein increases (Panayiotidis et al. 1995). Cytokines such as, IFNα, IFNγ, IL-1, IL-2, IL-4, IL-6, IL-8 and IL-13 are able to suppress apoptosis of malignant B-lymphocytes. In these situations, apoptosis is decreased by the preservation of bcl-2 expression (Tangye et al. 1997, Panayoiditis et al. 1993 and Jewell et al. 1995). On the other hand B-lymphocytes can be induced to undergo apoptosis by in vitro treatment with IL-5 and IL-10 (Mainou-Fowler et al. 1994, Osorio et al. 1998).

Apoptosis of leukaemic cells in vitro is associated with down-regulation of expression of the bcl-2 oncogene (Tangye et al. 1996). Oncogenes are mutated form of proto-oncogenes, whose functions are to promote normal growth and division of cells. The term oncogene itself is derived from the Greek ward 'oncos' meaning tumor. Elevated levels of bcl-2 protein have been observed in B-CLL patients (Schena, 1992 and Hanada et al. 1993) but this is not associated with the clinical stage of the disease (Robertson et al. 1996 and McConkey et al. 1996). Evidence that bcl-2 may play a central role in preventing malignant B-lymphocytes from apoptosis come from several studies. The induction of anti-APO-1 mediated apoptosis in activated B-CLL cells appears to correlate with reduced expression of bcl-2 mRNA (Mapara et al. 1993).

There are conflicting reports about the correlation of bcl-2 and apoptosis in drug induced cell death. Some authors found a decrease in bcl-2 protein after treatment with chlorambucil, theophiline and nucleoside analogues (Mentz et al. 1996, Peterson et al. 1996), while others found no correlation at all (Consoli et al. 1998).

Mutations in the tumour suppressor gene p53 occur in 20 to 30% of all cases of B-CLL. This percentage becomes more apparent with disease progression. Inactivation of the p53 gene can occur as the consequence of a mutation or possibly from functional inactivation of the protein product by the mdm2 protein (Bannerji et al. 2000). This protein is upregulated by p53 and then binds to p53 to inactivate it as part of an intracellular negative feedback and as a consequence an accumulation of the malignant cells takes place.

Receptor mediated signaling through receptors such as CD6, CD40 (TNFRSF5) and CD23 are also responsible for the regulation of B-CLL apoptosis. Anti-IgM induced apoptosis is counteracted by cross-linking of the CD6 receptor. This in turn is related to the increase in the bcl-2/bax ratio (Osorio et al. 1997). Another signaling molecule, TNFRSF5, is a transmembrane glycoprotein belonging to the TNF family. CD40 ligand (CD40L, TNFSF5) is expressed on activated T-lymphocytes. Interaction between CD40 and its ligand leads to inhibition of apoptosis of malignant B-lymphocytes (Buske et al. 1997). The mechanism of TNFSF5 action is not yet fully understood, but it is possible that stimulation with TNFSF5 affects cell proliferation rather than being directly involved in the apoptotic process. Triggering via CD40 correlates with an increase in the nuclear levels of NFκB / Rel complexes (Romano et al. 1998).

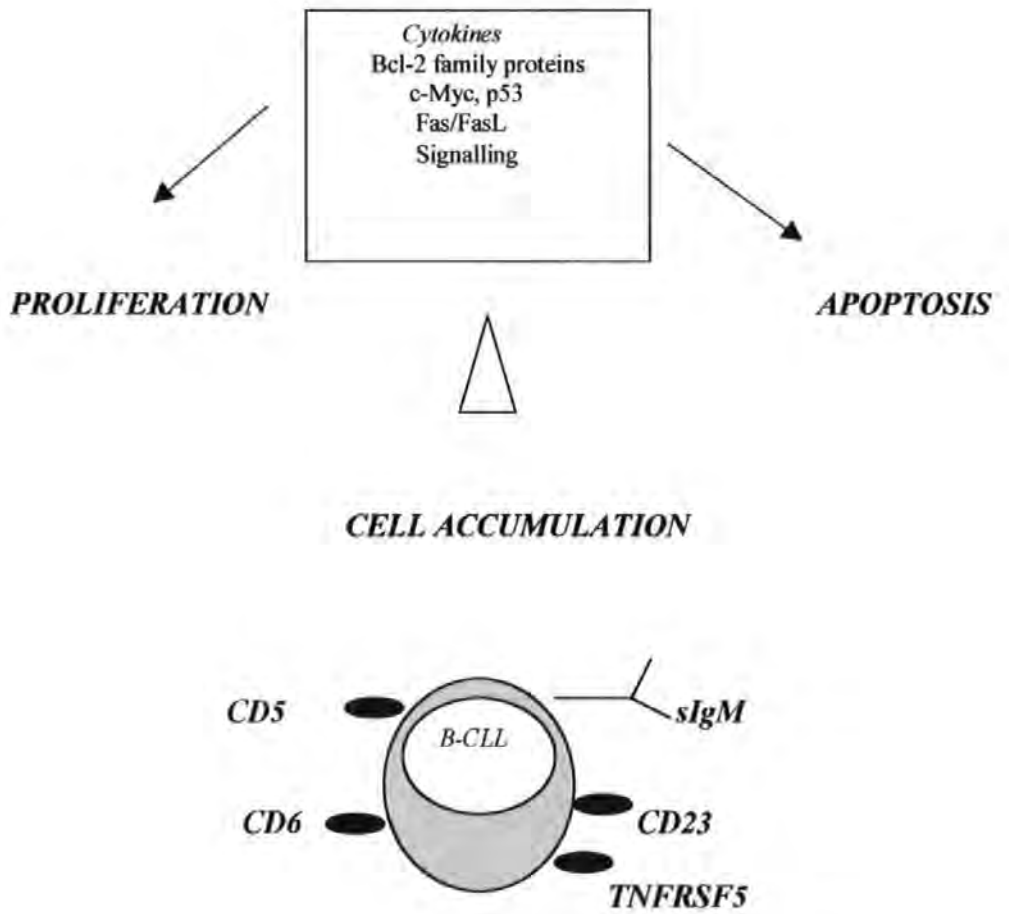
CD23 is a molecule expressed in the majority of B-CLL cells. Cross-linking of the CD23 antigen also results in an anti-apoptotic effect (Zhao et al. 1998).

Cross-linking of the Fas receptor can induce B-lymphocyte apoptosis (Nagata et al. 1995). Fas (APO-1, CD95 or TNFSF6) is a member of the tumour necrosis factor/nerve growth factor family. However malignant B-lymphocytes are either Fas negative or have very weak

expression of Fas and are resistant to anti-Fas induced apoptosis (Panayiotidis et al. 1995). On normal B-lymphocytes, CD95 is expressed in 11to 30% of cells. Interferon gamma and anti TNFSF5 are able to increase Fas expression on normal and malignant B-lymphocytes, whilst IL-2, IL-4, IL-10 and TNFSF2 have no effect on Fas expression (Cantwell at al. 1995). IL-2 and IL-7 were able to increase Fas expression in B-CLL with a parallel increase in bcl-2 and bcl-X_L, a decrease in ICE and a stable level of bax (El-Tounsi et al. 1995).

Various drugs are able to induce apoptosis of malignant B-lymphocytes. Irradiation, corticosteroids, fludarabine, chlorambucil, 2-chlorodeoxyadenosine, mitoxantrone, etoposide, vincristine and vinblastine all mediate apoptosis (Binet et al. 1995). Furthermore, some data suggest that high bcl-2/bax ratios may be predictive of a drug resistant phenotype in B-CLL cells and that modulation of these proteins is essential for the induction of cell death (Pepper et al. 1999). Theophiline may also induce apoptosis, and this action can be inhibited by IL-4 (Mentz et al. 1996). There is also some evidence that telomere length, as well as telomerase activity, exerts a strong impact on the survival of B–CLL patients (Bechter et al. 1998) All the internal and external factors described here may extend the life of B-CLL cells, increase their proliferative capacity and diminish cell death (Figure 1.11).

Figure 1.12: Proliferation and apoptosis in B-CLL



An altered balance between proliferation and apoptosis can lead to the progressive accumulation of CD5 positive B-CLL cells. Cytokines, proliferation/apoptosis-related genes and co-receptor signaling (e.g. TNFRSF6, CD6), are important factors in the regulation of the balance. Adapted from Osorio et al. 1998

1.6.4. Identification of apoptosis

There are a number of methods for measuring apoptosis. Classical histological features of apoptosis are seen by light microscopy using nucleic acid binding dyes such as acridine orange or propidium iodine. However, this method is often not objective. The characteristic features of the apoptotic cell are reduced size due to cell shrinkage and cytoplasmic condensation, plasma membrane undulation (blebbing), condensation of chromatin, beginning at the periphery of the nucleus followed by nuclear fragmentation (karyorrhexis), dilatation of endoplasmic reticulum and finally formation of apoptotic bodies (Arends et al. 1990 and Arends and Wyllie 1991). Nuclear swelling and patchy chromatin condensation can distinguish necrotic cells. Swelling of the mitochondria, vacuolization of cytoplasm and plasma membrane rupture leads to the formation of 'ghost like cells' and finally dissolution of DNA (karyolisis) (Kerr et al. 1972). Changes in the membrane of apoptotic cell and loss of transport function across the membrane are used to assay cell viability. Cationic dyes such as trypan blue, propidium iodine or 7 amino-actinomycin-D are able, after a short incubation, to distinguish between necrotic and late apoptotic cells. Another dye, H0342 stains apoptotic cells strongly and live cells weakly.

Flow cytometric identification of apoptotic or necrotic cell death is based on the analysis of those biochemical and morphological changes that enable us to distinguish between apoptotic and necrotic cell (Gorczyca, 1999). Apoptotic cells differ from necrotic cells in their ability to deflect light. The intensity of the light scatter in forward direction provides information on the cell size and structure while the intensity of the light scattered at the right angles to the laser (side scatter) correlates with granularity, refractiveness and the presence of intracellular structures that can reflect light. In apoptotic cells, forward light scatter decreases due to cell shrinkage while side scatter may increase transiently due to condensed chromatin and fragmented apoptotic bodies (Salzman et al. 1990, Ormerod et al. 1995, Cotter, 1998).

Other methods are based on detection of apoptosis-associated changes in distribution of plasma membrane phospholipids or transport functions of the membrane. Annexin V-FITC staining determines the percentage of cells undergoing apoptosis (Kopmann et al. 1994). Annexin V relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a calcium-dependent, reversible, phospholipid-binding anti-coagulant protein with a high affinity for PS. About 50 exposed phospholipid monomers bind per Annexin V molecule. Propidium iodide (PI) is used to distinguish viable from nonviable cells in conjunction with Annexin V. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to this agent. Cells undergoing apoptosis stain with Annexin V and cells that stain negative for both Annexin V and PI are either at the end stage of apoptosis, undergoing necrosis, or are already dead.

The major advantage of flow cytometry is that it offers a multiparametric analysis of several cell attributes, including cell cycle position. It can provide a rapid, simple, quantitative and objective assay for the enumeration of cell viability and death. Flow cytometric data correlate with classical DNA fragmentation assays (Nicoletti et al. 1991, Darzynkiewicz et al. 1992 and Carbonari et al. 1995).

The most commonly applied flow cytometric methods are based on the detection of endonucleotic DNA degradation. The degradation occurs first as multiple nicks are made along the DNA between nucleosomes (Linch, 1998). Certain enzymes can add biotin or digoxigenin labeled nucleotides to the DNA ends. The method, which is often used to detect fragmented DNA, utilizes a reaction catalyzed by exogenous TdT (terminal deoxytidyl transferase), often referred as 'end-labeling' or TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) (Gold et al. 1994).

1.7. ANTISENSE OLIGONUCLEOTIDES

1.7.1. General introduction

One way of controlling the expression of cytokines or other molecules that are upregulated in particular diseases is by the use of antisense oligonucleotides (ONs). The following is an outline of the way in which ONs function.

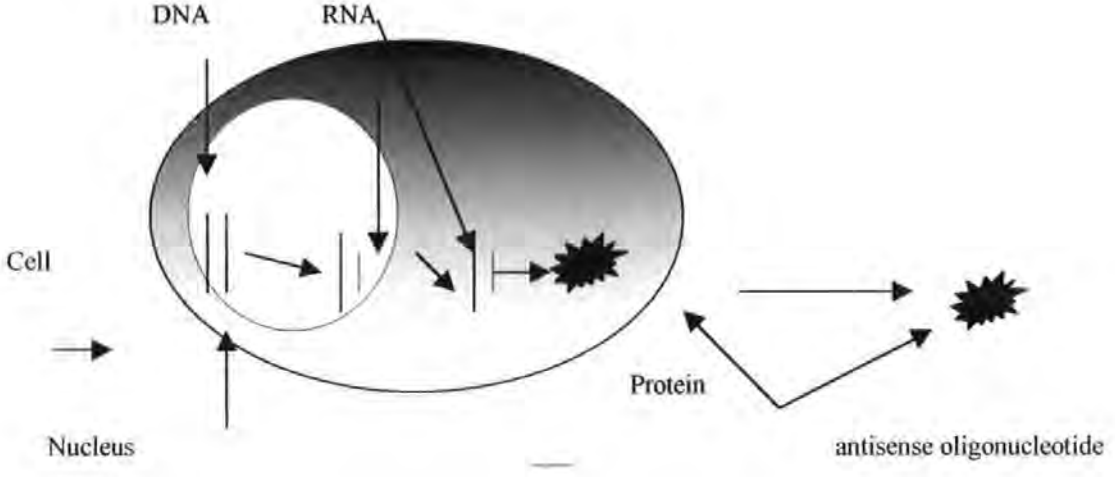
The use of antisense or complementary single-stranded DNA able to inhibit translation of corresponding RNA, in cell-free systems has been considered for some time (Paterson et al. 1977). Subsequently, in 1978 the Rous sarcoma virus was reported to interfere with viral production and cell transformation when exposed to oligonucleotides complementary to the 3'-reiterated terminal sequences in chick embryo fibroblasts (Zamecnik, 1978). Antisense oligodeoxynucleotides (ONs) are short synthetic, chemically modified stretches of DNA (13-30 nucleotides in length) that are complementary to a target sequence (DNA or RNA). ONs are added extracellularly to a cell culture system and are expected to enter the cell and selectively hybridise with corresponding RNA or DNA, forming a duplex using Watson-Crick base pairing rules which blocks transcription, translation or splicing.

The base cytosine forms hydrogen bonds exclusively with guanosine (C-G) and adenine with thymine or uracil (A-T or A-U). Non-homologous or mismatched sequences of other genes do not form stable hybrids. There are several possible mechanisms by which antisense oligonucleotides disrupt protein synthesis. The most plausible is an active mechanism, which allows for binding of RNase H. RNase H is an endonuclease that recognises the RNA-DNA duplex and destroys the RNA but leaves the DNA oligonucleotide intact to hybridise with yet another mRNA target (Minhull et al. 1986, Dash et al. 1987, Walder, 1988) (Figure 1.13).

Another possible way of inhibiting protein synthesis is steric blockade of ribosomal subunit attachment to mRNA at the 5' cap site or interference with proper mRNA splicing through antisense binding to splice donor or splice acceptor sites (Meyers et al. 2000).

The cell takes up oligonucleotides via adsorptive endocytosis or pinocytosis or both (Gao et al. 1993, Crooke et al, 1995). The existence of oligonucleotide receptors has also been postulated (Hanss et al. 1998). A series of experiments was devised to evaluate the use of oligonucleotides as hybridisation competitors and future therapeutic agents. However it soon become apparent that although the concept of antisense technology is simple, the development as a therapeutic agent has been slow and difficult (Stain et al. 1993). Most mammalian cells appear to be able to internalise oligonucleotides but this process requires long incubation times and high doses of antisense (Crooke et al. 1993). Cell cultures *in vitro* provide the most convenient way to investigate antisense oligonucleotides.

Figure 1.13: Mechanism of action of antisense oligonucleotides



Antisense oligonucleotides (ONs) can bind to the targeted RNA in the nucleus or cytoplasm. Binding in the nucleus results in degradation of RNA-ON duplex by RNase H. In the cytoplasm, the bound antisense inhibits expression of the mRNA by interfering with protein translation. In effect, the protein of interest fails to be synthesised.

1.7.2. Oligonucleotide modulation of cytokine expression and their value in hematological malignancies

Naturally occurring antisense RNAs in prokaryotes play a role in regulating expression of their corresponding genes (Mizuno et al. 1984, Simmons et al. 1988). This phenomenon is also present in eukaryotic cells (Izant et al. 1984, Izant et al. 1985). Therefore one can assume that ONs may be therapeutic in malignancy. However, the development of a reliable gene disruption strategy and its application in living cells has been very difficult, particularly in a clinical setting. The number of potential gene targets for haematological disorders is very wide and there are many reports of experiments demonstrating antisense effects (Bishop et al. 1994, Cotter, 2000). One of the most important is using antisense oligonucleotides to inhibit the function of the tumour suppressor gene p53 and the bcl-2 oncogene. In some forms of neoplasia, such as acute leukaemia and CML, p53 is mutated, and targeting p53 by oligoantisense *in vitro* results in inhibition of colony growth (Bishop et al. 1994). Probably more important is the suppression of bcl-2 overproduction by antisense oligonucleotides (Cotter, 2000). Overproduction of bcl-2 protein prevents apoptosis so blocking bcl-2 expression may reverse this.

Since many cytokines prevent apoptosis of malignant B-CLL B-lymphocytes, it seems reasonable to speculate that regulation of cytokine production by antisense oligonucleotides may be a logical approach to prevention of disease.

Human IL-2 has been the target of one antisense approach resulting in up to 80% inhibition of PBMC proliferation, a decrease in T-cell receptor expression and G_0/G_1 arrest of PBMC (Kato et al. 1994). Similarly oligonucleotide antisense targeting different regions of murine IL-4 mRNA resulted in the inhibition of IL-4 production in murine B-lymphocytes or inhibition of B-cell lymphoma clones (Haruna et al. 1993, Louie et al. 1993). Other cytokines such as IFN-γ, IL-1-α, IL-6, TNFSF2 and TNFSF1 have also been modulated by antisense approaches (Abken et al. 1992, Boeve et al. 1994). Many cancer-associated genes are

alternatively spliced. Modification of splicing using antisense therapy may be yet another way of introducing gene therapy (Mercatante et al. 2000).

1.8. AIMS OF THE PROJECT

B-CLL is a clonal expansion of B-lymphocytes characterised by immunophenotypic and genetic changes that influence their behaviour. There are also changes in the B-CLL T-lymphocyte population that may effect the malignant B-lymphocytes. Cytokines that act as intra and inter-cellular messengers are known to have anti-apoptotic affects on malignant B-lymphocytes. However this may be further complicated by the presence of cytokine splice variants that act as a self-regulatory system that may also afect malignant B-lymphocytes. Thus the balance between expression of different cytokines and also between expression of cytokine wild type and splice variant(s) may affect cell homeostasis. Using antisense oligonucleotides that interfere with transcriptional control of cytokine genes, the influence of cytokines on apoptosis of malignant and normal B-lymphocytes can be studied.

Therefore the aims of this project were:

1. To establish whether malignant B-lymphocytes express intracellular IL-4, a cytokine not normally associated with B-lymphocytes, to determine surface IL-4R expression and to compare these results with their normal counterparts.
2. To measure mRNA transcript expression of the IL-2 γ chain receptor family (IL-2, IL-4, IL-7, IL-9 and IL-15) in B- and T-lymphocytes of B-CLL patients and compare the results with the expression in normal cells.
3. To modulate cytokines and their spliced mRNA variants and protein expression by antisense oligonucleotides and to measure the effect of this modulation on apoptosis of malignant as well as normal B-lymphocytes.
4. To measure the effect of cytokine specific antisense oligonucleotides on intracellular expression of these cytkines in malignant as well as normal B-lymphocytes.

CHAPTER 2: MATERIALS AND METHODS

2.1. PATIENTS

A group 34 of patients with B-CLL, stage A(0), who were untreated or had not received any treatment in the last 6 months was studied. Diagnosis was based on a sustained lymphocytosis of more than 5.0 x 10^9 / l accompanied by the presence of more than 40 % lymphocytosis in the bone marrow. All patients were of Caucasian origin. For practical reasons (patients dying, commencing treatment or refusing to give subsequent samples), different patients were used for each part of the project. Some of the patient characteristics, such as the percentage of CD5+ve lymphocytes and total lymphocyte count, were obtained from the diagnostic laboratory results. Local Research Ethics Committee approval was obtained for this study.

2.2. CONTROLS

The controls used in this study were normal healthy Caucasian volunteers from within the Derriford Combined Laboratory. The same group was used throughout the study.

2.3. BASIC CULTURE MEDIUM PREPARATION

Culture medium was prepared using RPMI 1640 (Gibco Ltd, UK) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco Ltd, UK), 2 mM glutamine (Sigma Chemicals, UK), 0.2 % sodium bicarbonate and fetal calf serum 10% FCS (Gibco Ltd, UK).

2.4. PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION

For the cytokine protein studies, venous blood was collected in sodium heparin vacutainer tubes (Becton Dickinson, UK), transferred to universal tubes (Philip Harris Scientific, UK) and peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation using LymphoPrep (Nycomed, UK) at 2000 rpm for 20 min. For the mRNA study, peripheral blood was collected in 5% disodium ethylene diamine tetra-acetic acid (Na_2EDTA) and PBMCs were isolated as described above. The layer containing PBMCs was then transferred

to fresh universal tubes and washed three times with RPMI 1640 (Gibco Ltd, UK). Following centrifugation, the supernatant was removed and the pellet of cells resuspended in basic culture medium (1 ml). The number of cells present in 1 ml of this suspension was determined using a Neubauer Haemocytometer (Weber Scientific International, UK). Briefly cells were resuspended 1:1 in trypan blue solution (Sigma, UK) and counted under the microscope. The number of cells was calculated according to the following formula:

Number of lymphocytes in 1 ml = n x 4 x 2 x 10^4

n= number of cells present in 4 small squares of haemocytometer chamber

2.5. ISOLATION OF LYMPHOCYTES USING DYNABEADS

Peripheral blood was obtained by venepuncture in 5 % Na_2EDTA and PBMCs were obtained and washed as described in 2.4. Using Dynabeads M-45-CD19 (Pan B) and CD2 (Pan T) (Dynal, UK), CD19+ve and CD2+ve cells were positively depleted from the PBMC suspension according to the manufacturer's instructions. Attempts were made to count the obtained lymphocytes but this proved to be difficult due to Dynabeads still attached to the cells. The cells were then pelleted and frozen in liquid nitrogen until required for Western Blotting, Northern Blotting and nested RT-PCR.

2.6. PREPARATION OF RNA

Pure CD19+ve and CD2+ve cell populations were isolated using Dynabeads M-450 (Dynal, UK), according to the manufacturers instructions (section 2.6). Total cellular RNA was extracted from CD19+ve and CD2+ve lymphocytes using RNA STAT-60 (Biogenesis Ltd, UK) according to the manufacturer's instructions. Briefly, cells were homogenised using 1 ml of RNA STAT-60 (1 ml per 5-10x10^6 cells) by repetitive pipetting. Following homogenisation, homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of RNA-

STAT-60 was added to the homogenate and the mixture was shaken vigorously for 15 seconds and incubated at room temperature for further 2-3 min. Homogenate was centrifuged at 12,000 g (max) for 15 min at 4^0C. Following centrifugation, the upper aqueous phase was removed to a fresh tube and mixed with 0.5 ml of isopropranolol. After 5-10 min of incubation at room temperate the mixture was centrifuged at 12,000 g for 10 min at 4^0C. The RNA precipitate was washed with 75% ethanol. The RNA pellet was dried in a vacuum for 5-10 min and dissolved in RNase free water. RNA was diluted in 50 µl of pure water and frozen at -70^0C. The purity and concentration of the cellular RNA was confirmed using a Cecil 5500 spectrophotometer (Cecil Instruments, UK).

The concentration of RNA was calculated according to the formula recommended in RNA-STAT-60 manuals:

1 unit at 260 nm corresponds to 40 µg of RNA per ml

A_{260} = 1= 40 µg/ml

Pure RNA has a ratio A_{260} / A_{280} of 1.7-2.00

Concentration of RNA = 40 x A_{260} x dilution factor = concentration µg/ml

Total yield = concentration x volume of stock in ml

2.7. STATISTICAL ANALYSIS

2.7.1. T-test

Pairwise evaluation of means was performed with the Students t test. The Students t test is used to determine whether two samples are likely to have come from two underlying populations, which have the same mean. Mackintosh software was used to evaluate the Students t-test. P values less than 0.05 were considered to indicate statistical significance.

2.7.2. Spearmans' Rank Correlation Test

The Spearmans' Rank Correlation Test measures the degree of relatedness between two continuous variables. It is used to compare rankings of cases of a variable in two different conditions (before and after treatment with antisense oligonucleotides). Mackintosh software was used to evaluate the results. P values less than 0.05 were considered to indicate statistical significance.

2.8. FLOW CYTOMETRIC ANALYSIS OF IL-4 AND IL-4 RECEPTOR (IL-4R) EXPRESSION

Flow cytometry is a technique that allows measurements of single cells or particles as they flow in a fluid stream one by one through the sensing point (collimated laser beam). In the first part of the study flow cytometry was used to measure the percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4 and surface IL-4R. The PBMCs used in this part of the study were unstimulated. The reasons for using unstimulated cells was that mitogens addition may artificially interfere with actual state of the malignant cells.

2.8.1. Measurement of surface IL-4 receptor expression by flow cytometry

The protocol used below is a modification of the protocol used in the Derriford Combined Laboratory. PBMCs, isolated according to the procedure described in section 2.1.4, were washed and resuspended in PBS (phosphate buffered saline) (Sigma, UK) at a concentration of $10x10^{6}$/ml. 100 µl of PBMCs suspension was then pipetted into labelled tubes and the appropriate antibody added. For single CD19 or CD3 staining, 10 µl of mouse anti-human IgG1 fluorochrome conjugated (FITC) antibody (Dako, UK) was used. For determination of surface IL-4R expression, cells were incubated with primary mouse anti-human IL-4R IgG1 antibody (Genzyme, UK) at a concentration of 5 µg/ml. After 30 min of incubation at 4^{0}C, the cells were washed three times in cold PBS supplemented with 0.2 % bovine serum albumin

(BSA) (Gibco Ltd, UK). After washing, 10 µl of secondary, sheep anti-mouse R-Phycoerythrin (PE) conjugated antibody (Sigma, UK) was added. After a further 30 min of incubation at 4^0C, the cells were washed 3 times and each sample was stained for the presence of CD19 or CD3 antigen respectively. 10 µl of mouse anti-human CD19 and CD3 (FITC) IgG1 monoclonal antibody (Dako, UK) was added. After 30 min of incubation at 4^0C and washing with PBS buffer, the cells were resuspended in 1 ml of PBS and analysed immediately by flow cytometry (Becton Dickinson-FACScan, UK).

2.8.2. Measurement of intracellular IL-4 by flow cytometry

The protocol used below is a modification of the protocol described in Serotec Catalogue (1996). A washed PBMCs suspension, isolated according to the protocol described in section 2.1.4, was diluted to a concentration of $1x10^6$/ml in culture medium consisting of RPMI 1640 (Gibco Ltd, UK) supplemented with 10 % FCS (Gibco, Ltd, UK) and 2 mM monensin (Sigma, UK). The PBMCs were plated out in a 24 well tissue culture plate and incubated for 6 hr in a 5 % CO_2 incubator at 37^0C. After 6 hours of incubation, the cells were washed in PBS containing 0.2 % BSA (Gibco Ltd, UK) and 0.02 % sodium azide (Sigma, UK) and adjusted to a concentration of 0.5 $x10^6$/ml. 50 µl of Fixation medium (Serotec UK) was added. After 15 min incubation and one wash, 50 µl of Permeabilisation Medium (Cytoperm, Serotec, UK) together with the appropriate antibody (negative control-mouse IgG), anti-CD19 FITC, anti-CD3 FITC and/or mouse IgG1 anti-human IL-4 PE (Pharmingen, USA) was added and further incubated for 30 min at room temperature. The cells were then washed once in PBS buffer and resuspended in 0.25 ml of 0.5 % paraformaldehyde (Sigma, UK) and store at 4^0C until acquisition on the flow cytometer.

2.8.3. Preincubation of PBMCs with anti-IL-4R monoclonal antibody

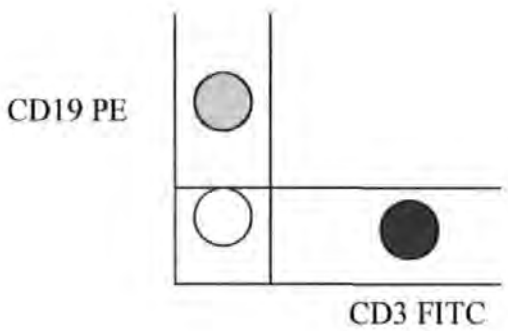
PBMCs were isolated from two patients with B-CLL. The cells were resuspended in basic culture medium (section 2.3) at a concentration of $1x10^6$/ml. To 3 ml of resuspended cells, 10 µl of anti-IL-4R monoclonal antibody was added and the mixture incubated for 1 hr at 37^0C. After incubation, thePBMCs were washed twice ready to be used in section 2.8.2.

2.8.4. Analysis of flow cytometric data

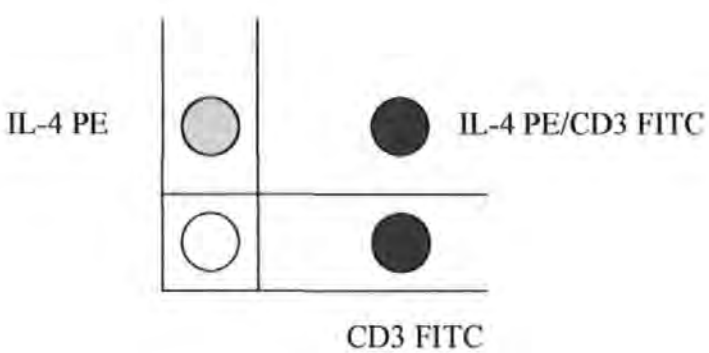
Analysis on the FACScan (Becton Dickinson, UK) was performed using an argon ion laser. Instrument calibration / standardisation was performed using the manufacturer instructions. Live gating of the forward (FSC) and side scatter (SSC) channels was used to exclude debris and to selectively acquire lymphocyte events. All values presented are based on the percentage of lymphocytes as determined by light scatter. Individual fluorescence populations were determined by the use of acquisition and quadrant analysis software (Cellquest, Becton Dickinson). Results are shown as the percentage of CD3+ve, CD19+ve lymphocytes expressing intracellular IL-4 and surface IL-4R in PBMC suspensions corrected for non-specific binding using non-specific controls. Joining dots of the same density derived the individual contour plots (Figure 2.1). The number of events acquired for each sample was 10,000 whenever possible.

Figure 2.1: Flow cytometric analysis of different cell populations

A)



B)



Plot of CD3 FITC (T-lymphocytes) versus CD19 PE (B-lymphocytes) showing three discrete populations (A). Plot of CD3 FITC versus IL-4 PE CD3+ve lymphocytes stained for IL-4 (B)

2.8.5. Measurement of secreted IL-4 protein by ELISA

Cells were set up as follows. Washed PBMCs prepared according to the procedure described in section 2.4 were adjusted to a concentration of $1x10^6$/ml in culture medium consisting of RPMI 1640 (Gibco Ltd, UK) supplemented with 10% FCS (Gibco Ltd, UK). The PBMCs were plated out in a 24 well tissue culture plate and incubated overnight in a 5% CO_2 incubator at 37^0C. Cells were stimulated with phytohemagglutynin (PHA) (Sigma, UK) at a of concentration 1μg/ml or pokeweed mitogen (PWM) at a concentration of 5 μg/ml (Sigma, UK) and incubated for 48 hours at 37^0C. PHA is a specific T cell mitogen while, PWM is able to stimulate both B and T-lymphocyte population to release cytokines. Since IL-4 is not easily detected in supernatants of unstimulated cells by ELISA addition of mitogens increase cells capacity to produce IL-4. Collected supernatant were frozen at –20 ^{0}C. Thawed supernatants were then assayed in duplicate for the concentration of IL-4 using a commercial ELISA kit (Amersham, UK). The ELISA plates were read at 450 nm and the samples calculated using a Dias Microplate Reader (Dunatech Laboratory, UK)

2.9. NESTED REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) mRNA ANALYSIS

The intracellular staining results have shown an increased percentage of malignant B-lymphocytes as well as normal T-lymphocytes expressing intracellular IL-4. In view of this more specific tests needed to confirm the presence or absence of specific mRNA transcripts in B and T-lymhocytes separated from patients and normal controls. There are many transcript assays able to quantitatevely or semi-quantitatevely detect mRNA transcripts (e.g. competetive RT-PCR, real time PCR, expression microarrays, RNA protection assay). However in view of the previews inconclusive foundings of the IL-4 mRNA transcripts in the malignant B-cell populationand the low aboundance of this transcripts a modificationof RT-PCR method was chosen. A nested reverse transcriptase polymerase chain reaction (RT-PCR) was used to amplify mRNA

sequences of the cytokines that use the common gamma chain (γc) as part of their individual receptor as well as IL-2 δ2, IL-2 δ3, IL-4 δ2, IL-4 δ3, IL-4R, IL-5 and IL-13. In the first instance this assay was used check the presence or absence of individual transcripts. However with the purchase of Multi-Imager the semi-quantitive measurements of each individual band were obtained. Due to limited amounts of cDNA each experiment was repeated only once.

The amplification of beta-actin gene was performed as a single RT-PCR reaction.

2.9.1. Preparation of cDNA

Total RNA was prepared according to the protocol described in section 2.6 and frozen at –80^{0}C until further use. First strand complementary DNA (cDNA) was prepared using the SUPERSCRIPT^{TM} Preamplification System for the First Strand cDNA Synthesis (GIBCO Ltd, UK) according to the manufacturer's specifications. Briefly, 1μl of oligo dT_{12-18} (0.5 μg/ml) was added to RNA at a final concentration of 2 μg/ml. DCPE water was then added to achieve a final volume of 12 μl. The mixture was first incubated for 10 min at 70^{0}C and then on ice for 1 min. 7 μl of the reaction mixture (2 μl of 10 x buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of 10nM dNTPs and 2 μl of 0.1 M DTT) was added to the RNA mixture and incubated for 5 min at 42^{0}C. After incubation, 1 μl (200 U) of Superscript II RT was added to each tube and incubated at 42^{0}C for 50 min. The reaction was terminated at 70^{0}C for 15 min. 1 μl of RNase H was added to each tube and the mixture incubated for 20 min at 37^{0}C.

2.9.2. Nested RT-PCR protocol

The PCR mixture at a final concentration of 25 μl, contained 2 μl of cDNA, 2.5 μl of 10x buffer (Tris-HCL-10 mM, KCL-50 mM, $MgCL_2$-1.5 mM) (HT Biotechnologies, UK), 2 μl of dNTPs mixture (0.125 mM each) (Bioscience, UK), 1 μl of each primer (100ng/ul=10 pmol) (MWG-Biotech, Germany), 0.5 μl of super Taq (0.125 U) (HT Biotechnology, UK) and

finally water. The primers used in the first and second round of amplification are listed in table 2.2. The PCR mixture conditions are described in Table 2.2. For the second round of amplification, 1µl of first round product was used together with other elements of the mixture. After the second round of amplification, the PCR products were subjected to electrophoresis on a 1.4 % UltraPure agarose gel (Gibco, UK). Ethidium bromide (2,7-diamino-10-ethyl-9phenyl-pheanthridinium) (Sigma, UK) staining was used to detect individual bands. A molecular weight marker 100 to 2642 bp (Boehringer Mannheim, UK) was used with each gel. The gel was run in 0.5x TBE buffer at 200 V for 30 min to 1 hour. The gel was visualised using a UVP computer software package (UVP International, UK) and UVP UV transiluminator (UVP International, UK).

The following stock solutions were used:

Tris/borate electrophoresis buffer (TBE)

5 X concentrated solution = 54 g Tris base + 27.5 g boric acid + 20 ml of 0.5M EDTA added to 1L of water (pH 8.0)

Working solution = 0.5 x

Ethidium bromide

10 mg/ml^{-1} in H_2O

Xylene cyanol loading buffer

0.25% v/v Xylene cyanol, 10% v/v Glycerol in 10 x TBE

Table 2.1: Primers used in nested RT-PCR reaction

Cytokine	Sense primer (5'-3')	Anti-sense primer (5'-3')
β-actin	Exon 5'-GTG GGG CGC CCC AGG CAC C-3'	Exon 5'-CGC GCT CGG TGA GGA TCT CT-3'
IL-2 outer primer	Exon 1-5'-CCT CAA CTC CTG CCA CAA TG-3'	Exon 4-5'-GAG CCC CTA GGG CAC AAA AAG AAT C-3'
IL-2 inner primer	Exon 1:-5'-CAC TAA GTC TTG CAC TTG TCA C-3'	Exon 4-5'-GTG TTG AGA TGA TGC TTT GAC-3'.
IL-2 δ2 inner primer	Exon 1: 5'-CAC TAA GTC TTG CAC TTG TCA C-3'	Exon 3: 5'-CTG ATT AAG TCC CTG GGT CTT AAG-3'
IL-4 outer primer	Exon 1: 5'-CTC ACC TCC CAA CTG CTT CCC CC-3'	Exon 4: 5'-GAT CGT CTT TAG CCT TTC CAA G-3'
IL-4 inner primer	Exon 1: 5'-GGC AAC TTT GTC CAC GGA CAC AAG-3'	Exon 4: 5'-GTT GGC TTC CTT CAC AGG AGA GG-3'
IL-4δ2 outer primer	Exon 1: 5'-CAG CAT TGC ATC GTT AGC TTC TCC TG	Exon 3:5'-GGT TCC TGT CGA GCC GTT TCA GTT AT-3'
IL-4 δ2 inner primer	Exon 1: 5'-GGC AAC TTT GTC CAG GGA CAC AAG-3'	Exon 3:5'-GCA GCC CTG CAG AAG GTT TCC TT-3'
IL-7 outer primer	Exon 1: 5'-GCC ACG CCG TAG TGT GTG CCG C-3'	Exon 6:5'-CTT GGA GGA TGC AGC TAA AGT TC-3'
IL-7 inner primer	Exon 1: 5'-CAC AGA CTC GGC AAC TCC GCG GAA G-3'	Exon 6: 5'-CAG TGT TCT TTA GTG CCC ATC-3'
IL-9 outer primer	Exon 1-5'-GGT CCT TAC CTC TGC CCT GCT CC-3'	Exon 4-5'-CTT GCC TCT CAT CCC TCT CAT C-3'
IL-9 inner primer	Exon 1-5'-CTT CCT CAT CAA CAA GAT GC-3'	Exon 4-5'-GGT TGC ATG GCT GTT CAC AG-3'
IL-15 outer primer	Exon 1-5'-GGA TGG ATG GCT GCT GGA AAC-3'	Exon 6-5'-GAG TTC ATC TGA TCC AAG GTC-3'
IL-15 inner primer	Exon 1-5'-GTG GCT TTG AGT AAT GAG AAT T-3'	Exon 6-5'-GTC TAA GCA GCA GAG TGA TG-3
γc outer primer	Exon 1-5'-CAT CAT TAC CAT TCA CAT CCC TC-3'	Exon 8-5'-CTG TAG TCT GGC TGC AGA CTC TC-3'

γc inner primer	Exon 1-5'-CTG CTG GGA GTG GGG CTG AAC AC-3'	Exon 8-3'-CCC TTA GAC ACA CCA CTC CAG G
IL-5 outer primer	Exon 1: 5'-GAG GAT GCT TCT GCA TTT GAG-3'	Exon 4-5'-CTA TTA TCC ACT CGG TGT TC-3'
IL-5 inner primer	Exon 1: 5'-CCT TGG CAC TGC TTT CTA CTC-3'	Exon 4:5'-CTT GCA GGT AGT CTA GGA ATT GG-3'
IL-13 outer primer	Exon 1: 5'-GGC GCT TTT GTT GAC CAC GGT C-3'	Exon 4:5'-GAA CCG TCC CTC GCG AAA AAG-3'
IL-13 inner primer	Exon 1: 5'-CAT TGA GGA GCT GGT CAA CAT C-3'	Exon 4:5'-GCC ACC TCG ATT TTG GTG TCT CG-3'
IL-4R inner primer	Exon 1: 5'-GCG AAA TGT CCT CCA GCA TG-3'	Exon: 5'-CCA GTC CAA AGG TGA ACAAGG GG-3'

Table 2.2: Condition for the first and second round of nested RT-PCR amplification

Cytokine	Nr. of cycles	Denaturation (temp/time)	Annealing (temp)	Extension (temp)	Band size (base pair)
Beta actin	30	94^0C-2 min	63^0C	72^0C	487 bp
Gamma c 1st	35	94^0C-2 min	59^0C	72^0C	961 bp
Gamma c 2nd	35	94^0C-2 min	63^0C	72^0C	889 bp
IL-2 1st	35	94^0C-2 min	59^0C	72^0C	644 bp
IL-2 2nd	35	94^0C-2 min	55^0C	72^0C	420 bp
IL-2delta2 1st	35	94^0C-2 min	57^0C	72^0C	259 bp
IL-2delta2 2nd	35	94^0C-2 min	60^0C	72^0C	162 bp
IL-4 delta2 1st	35	94^0C-2 min	62^0C	72^0C	443 bp
IL-4 delta2 2nd	35	94^0C-2 min	62^0C	72^0C	115 bp
IL-4R 1st	35	94^0C-2 min	62^0C	72^0C	1515 bp
IL-4R 2nd	35	94^0C-2 min	62^0C	72^0C	308 bp
IL-7 1st	35	94^0C-2 min	61^0C	72^0C	798 bp
IL-7 2nd	35	94^0C-2 min	61^0C	72^0C	659 bp
IL-9 1st	35	94^0C-2 min	62^0C	72^0C	411 bp
IL-9 2nd	35	94^0C-2 min	55^0C	72^0C	246 bp
IL-15 1st	35	94^0C-2 min	57^0C	72^0C	708 bp
IL-15 2nd	35	94^0C-2 min	54^0C	72^0C	558 bp
IL-5 1st	35	94 C^0-2 min	54 C^0	72 C^0	391 bp
IL-5 2nd	36	94 C^0-2 min	57 C^0	72 C^0	260 bp
IL-13 1st	35	94 C^0-2 min	60 C^0	72C^0	385 bp
IL-13 2nd	36	94 C^0-2min	60 C^0	72 C^0	234bp

2.9.3. Data analysis

The density of bands was measured using Fluor-S^{TM} Multi-Imager (BioRad Laboratories, UK) by drowing individual and the same size quadrants around each band. The data analysed using the Student t-test and Spearmans Rank Correlation test described in section 2.8.

2.10. ANTISENSE STUDY

2.10.1. Introduction

The techniques described above were used for detecting protein and mRNA of cytokines that may play a role in the pathogenesis of B-CLL. Antisense oligonucleotides (ONs) were used to try to modify the transcription and protein expression of IL-2 and IL-4 cytokines.

However, since oligonucleotide technology is relatively new, several general considerations have to be applied in order to obtain optimal results. The first is the choice of cell type. Cells that are known to express the gene of interest and preferably at a high level are the best choice. The state of the cells as well as the type of cell lines is also important.

The others are modification, length and sequence selection as well as purity and the delivery system. Several chemical modifications have been proposed. In phosphorothioate oligodeoxynucleotides (S-ODN), one of the non-bridging oxygen atoms in the nucleotide backbone is replaced with sulphur. This increases the protection from both endonucleases and exonucleases and supports RNase H activity (Campbell et al. 1990 and Aghtar et al. 1991). S-ODN have a half-life greater than 18 hr in pure fetal calf serum and more than a week in cell culture medium containing 10% FCS (Shaw et al. 1991). Other modifications give rise to 2'-O-methyl oligonucleotides. In this modification there is an addition of a methoxy group to the C2' position of the sugar. This modification enhances nucleases resistance but does not support RNase H activity. In order to secure RNase H activity this modification must be incorporated into a gapped or chimeric structure (McKay et al. 1999). However they have lower water solubility but are highly resistant to nuclease degradation (Tidd et al. 1989). To

further increase nuclease resistance, to improve the affinity to the target RNA and to facilitate cellular uptake, new backbone modifications have been designed (Figure 1.14). They include hydroxymethyl phosphonates, carbonates or aminoalkyl derivatives (Agraval et al. 1995). Chimeric oligos have also been created. Oligos with phosphorothioate core and methylphosphonate caps at both ends have favourable characteristics of *in vivo* stability and distribution (Agraval et al. 2000).

In the process of selecting the best oligonucleotides, the length of the antisense molecule is very important. It has been calculated that the minimum length of an oligonucleotide has to be between 15-20 bases (Branch et al. 1998). It has been shown that antisense longer than 30 bases can trigger a genetic cascade of cellular antiviral response (Manche et al. 1992)

Another important consideration is sequence selection. One successful strategy has been to adopt a 'gene-walk' approach. Another approach, which helps in the selection of the best oligonucleotides, is to use DNA chip technology. In this case the antisense sequence is bonded to a silicone chip. The target RNA is labelled with radioactive nucleotide and hybridised to the chip. The 'hot spots' created by areas of strongest radioactive signals correlate with accessible sites (Milner et al. 1997 and Kuss and Cotter, 1999). One of the most popular sites is the region surrounding the start codon (AUG), followed by mismatch sites. Targeting splicing sites in order to inhibit the mRNA processing mechanism, as opposed to the message (Sierakowska et al. 1996), is also plausible. Four contiguous G residues are known to exhibit non-specific anti-proliferative action independent of the overall sequence of the oligos (Yamamoto et al. 1994). Palindromes such as GACGTC, AGCGCT and AACGTT are known to induce interferon production (Yamamoto et al. 1994), whereas GT rich oligos inhibit action of IFN-γ (Yaswen et al. 1993). Finally, the use of certain CpG motifs will result in B lymphocyte and natural killer cell stimulation with the release of cytokines such as tumour necrosis factor and interferon (Krieg et al. 1995).

Purification by reverse phase HPLC is often the best choice, although not all toxic by-products are removed.

Transfection agents such as liposomes, peptides and poly-cations greatly facilitate oligonucleotide activity (Bennet et al. 1992, Bongartz et al. 1995, Kukowaska-Latallo et al. 1996, Boussif et al. 1995). One of the most effective delivery systems is the use of cationic lipids. They can enhance cellular uptake by 1000-fold (Bennett et al. 1992) at the same time promoting nuclear accumulation. If a delivery system is not used, isolated spots are visible only in the cytoplasm (Shoji et al. 1991).

2.10.2. Testing for antisense entering the cells

IL-2a rodamine-red conjugated antisense ON was tested for its ability to enter PBMCs by a process of endocytosis. B-CLL and normal control PBMCs prepared according to the protocol described in section 2.4, were washed and resuspended at a concentration of $1x10^5$/ml in culture medium described in section 2.3 and plated into a 96 well plate. Rodamine-red conjugated IL-2a antisense ON was added at concentrations of 0.5 μM, 1 μm, 1,75 μM, 2.5 μM, 5 μM, and 10 μM. The lymphocytes were then viewed under an UV microscope after 30 min, 1, 2, 3 and 4 hours. No carrier was used.

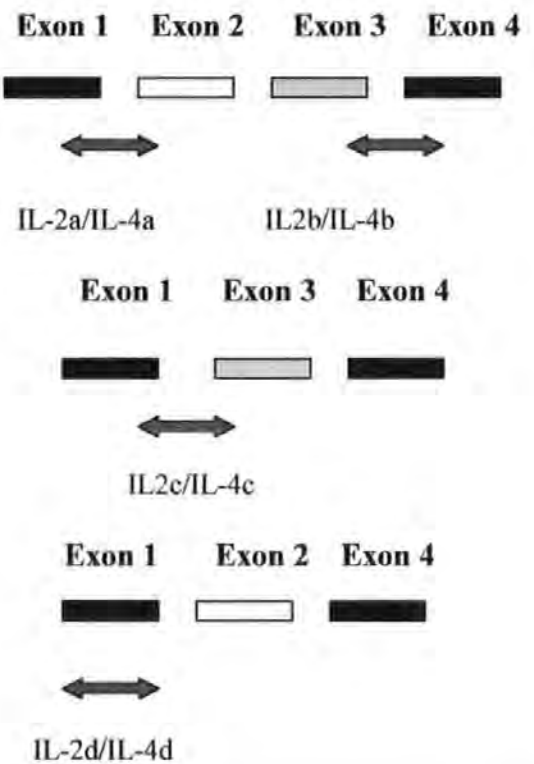
2.10.3. MTT cytotoxicity assay

The MTT cytotoxicity assay is based on the capacity of active mitochondrial dehydrogenase in living cells to convert 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT) by the cleavage of the tetrazolium ring, into a water-insoluble dark blue formazane product. The MTT assay has been developed as an alternative system to conventional ^{3}H-thymidine uptake for measurements of cell proliferation. In this study the TACS™ MTT Assay Kit (R&D) was used to measure cell proliferation and viability of B-CLL cells and normal controls PBMCs after incubation with various antisense ONs.

Sequences of all the antisense oligonucleotides used are listed in Table 2.3. Figure 2.1a and b describes the location of appropriate antisense oligonucleotides. The number of antisense ONs were specially designed to interfere with the transcription of wild type and spliced variants of IL-2 and IL-4.

Figure 2.2: Individual antisense oligodeoxynucleotide (ONs) positions

a)



IL-2a, IL-4a, IL-2b, IL-4b, IL-2c, and IL-4c antisense ONs were designed at the junction of appropriate exons. IL-2d and IL-4d antisense ONs were situated within exon one.

b)

Antisense	Blocking effect
IL-2a or IL-4a	IL-2 or IL-4 wild type and δ3
IL-2b or IL-4b	IL-2δ3 or IL-4δ3
IL-2c or IL-4c	IL-2δ2 or IL-4δ2
IL-2d or IL-4d	IL-2 or IL-4 wild type and δ2 and δ3 spliced variants

Table 2.3: Sequences of the antisense oligonucleotides

Name	Oligonucleotide antisense
Control antisense (1) Control antisense (2)	5'- AGT AGT TTT GGA AA-3' 5'-TTT TTA AAA AAA ATT T-3'
IL-2a	5'-CTT GTA ATA TTA ATT-3'
IL-2b	5'-TCA GAT CCC TTT AGT-3'
IL-2c	5'-TTC TGT GGA TTA ATT-3'
IL-2d	5'-AGT GCA AGA CTT AG-3'
IL-4a	5'-GCA CAG AGC TTC TAC T-3'
IL-4b	5'-CAG GAA TTC AAG CCC-3'
IL-4c	5'-GTT GTG TCT TCT GCT-3'
IL-4d	5'-AAA GGT TTT GAT GA-3'

2.10.3.1. Protocol

PBMCs from patients with B-CLL were separated according to the protocol described in section 2.4. Following separation, PBMCs were washed in 5 ml of sterile PBS (Gibco, UK) centrifuged at 1200 rpm for 5 min, resuspended in culture medium (section 2.3) and serially diluted from 5×10^6 to 5×10^3 cells/ml. This was undertaken to determine the appropriate B-CLL cell number for the use in the MTT assay. PBMCs suspention was plated out at 100 µl per well. Three control wells of culture medium were included. The cells were then incubated for 48 hours at 37^0C in a CO_2 incubator.

The MTT assay was performed according to the manufacturer's protocol. Briefly, 10 µl of MTT reagent (TACS^{TM} MTT Assay Kit, R&D, UK) were added to each well and the plate incubated for 3 hours at 37 ^{0}C. When the cells were viewed using an inverted microscope, the appearances of punctuate intracellular precipitate was visible.

When a purple precipitate was visible, 100 µl of Detergent Regent was added to all the cells. The plate was left in the dark at 18-24^{0}C for at least 2 hours. The absorbency was measured at 570 nm with a reference wavelength of 650 nm and the results calculated using a Dias microplate reader Dynatech Laboratory, UK).

2.10.4. Annexin V staining

Annexin V-FITC staining was used to determine further the percentage of cells undergoing apoptosis after addition of various antisense ONs. Annexin V relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis.

2.10.4.1. Protocol

PBMCs isolated according to the protocol described in section 2.4 were resuspended to a concentration of 0.5x10^6 and put into in a 24 well plate. Different antisense ONs (Table 2.4) were added at a final concentration of 6 µM (3x2 µM). The cells were incubated for 48 hours at 37^{0}C in a CO_2 incubator, collected into separate tubes, centrifuged at approximately 1200 rpm for 10 min. and washed. Annexin V staining was performed according to the manufacturers protocol. Briefly, the cells were washed in 1x cold PBS buffer containing calcium (Gibco, UK), and then gently resuspended in Annexin V Incubation Reagent (R&D System, UK) (buffer contained 10 µl of 10x b Binding buffer, 10 µl Propidium iodide, 0.5 µl of Annexin V FITC and 79 µl of H_2O) together with 10 µl of appropriate monoclonal (Table 2.4). The mixture was incubated in the dark for 15 min at room temperature. After incubation, the cells were washed once in 300 µl of Binding buffer (R&D System, UK) and resuspended in 400µl of 1x Binding Buffer (R&D System, UK) ready for flow cytometric analysis.

Table 2.4: Tube labelling

Nr	Content	Antibody	Amount
1	negative control	Mouse IgG_1 FITC, mouse IgG_1 PE (Becton Dickinson, UK)	10 µl each
2	leucogate	Anti human CD45/CD14 (Becton Dickinson, UK)	10 µl
3	single staining	Mouse anti human CD19 PE (Becton Dickinson, UK)	5 µl
4	single staining	Mouse anti human CD3 PE (Becton Dickinson, UK)	5 µl
5	single staining	Annexin V FITC	0.5 µl
6	single staining	Propidium iodide	10 µl
7	double staining	Annexin V FITC plus mouse anti-human CD19 PE	0.5 µl 5 µl
8	double staining	Annexin V FITC plus mouse anti-human CD3 PE	0.5 µl 5 µl

2.10.4.2. Flow cytometric analysis of Annexin V stainning.

Cells were studied in an Epics Elite flocytometer (Coulter, UK). Alignment of the instrument was performed daily according to the manufacturer's protocol. Annexin V conjugated to FITC, and PI, were used to analyse multiple antigenic determinants within wanted cell population. This was accomplished with a single excitation wavelength (488nm), using a 15m Watt argon ion laser. FITC emission was measured as a green signal (530nm-peak fluorescence) by the FL1 detector and R-PE was measured as an orange signal (575nm-peak fluorescence) by the FL2 detector. FL1 is a green fluorescence signal received by the photomultiplier tube (PMT) and FL2 is an orange-red fluorescence signal received by the PMT. An electronic calculation to remove signal overlap was also performed through compensation. In this case the fluorescence measurements of the cell sample stained with one fluorochrome was electronically forced to be identical to that of the unstained cells. In each

individual experiment, PBMCs were also stained with CD45 FITC/CD14 PE conjugated antibody to allow cells to be identified as lymphocytes only.

Results are shown as the percentage of CD3+ve, CD19+ve lymphocytes double stained with Annexin V FITC or Propidium iodine. Joining dots of the same density derived the individual contour plots (Figure 2.1). The number of events acquired for each sample was 10,000 whenever possible.

2.11. FLOW CYTOMETRIC ANALYSIS OF INTRACELLULAR IL--2 AND IL-4 AFTER INCUBATION WITH ANTISENSE ONs

2.11.1. Cell incubation

PBMCs from patients with B-CLL and from normal controls were prepared according to the protocol described in section 2.4. Cells were resuspended in appropriate culture medium (section 2.3), seeded out in 24-culture plate at a concentration of $0.5x10^6$ and incubated at 37^0C for 6 hours. The cells were stimulated with PMA at a final concentration of 5ng/ml. Ionomycin was added at a concentration of 0.5 μg/ml. Antisense ONs at a total concentration of 6 μM (3x2 μM) were added to the appropriate wells together with Golgi Plug™ (Pharmingen, UK), which contains Brefeldin A (Pharmingen, UK), a fungal metabolite and a protein transport inhibitor which interferes with vesicular transport from the rough endoplasmic reticulum to the Golgi complex. Addition of protein transport inhibitor is necessary since it prevents cytokines been used by the cell or being released into supernatants. For every 1 ml of cell culture 1μl of Golgi Plug mixture was added.

2.11.2. Flow cytometric analysis of intracellular IL-2 and IL-4 after incubation with antisense ONs

After 6 hours incubation, the PBMCs incubated as described in section 2.14.1, were washed in Dulbecco's PBS without Mg^{2+} or Ca^{2+} (Gibco BRL, UK), 1% heat inactivated FCS and 0.09% sodium azide (Staining Buffer) and resuspended in 50 µl of the above buffer. About 5µl of CD45FITC /CD14PE, CD19 or CD3 conjugated monoclonal antibody (Becton Dickinson, UK) was added. The PBMCs were incubated for 30 min at 4^0C. After incubation, the cells were washed twice in staining buffer (1ml/wash per tube) and pelleted by centrifugation (250 x g). The cells were then resuspended in 250 µl per tube of Cytofix/Cytoperm solution (Pharmingen, UK) for 20 min at 4^0C, washed twice in 1 x Perm/Wash solution (Pharmingen, UK) (1 ml per tube) and pelleted. Fixed and permeabilised cells were then resuspended in 50 µl of Perm/Wash solution containing IL-2 or IL-4 specific PE conjugated antibody (Beckton Dickinson, UK). The cells were then incubated at 4^0C for 30 min in the dark. After incubation, the cells were washed twice in 1x Perm/Wash (1 ml per tube) and resuspended in Staining Buffer prior to flow cytometry (described in section 2.12.3.2).

2.11.3. Flow cytometric analysis of intracellular IL-2 and IL-4 after incubation with antisense ONs and Lipofectin

Separated PBMCs (section 2.4) were washed with serum free growth medium without antibacterial agents (section 2.3). Cells were seeded in 6 well culture plate at a concentration of 2-3 x 10^6 per well in 0.8 ml serum free growth culture medium (OPTI-MEM Gibco, UK). This experiment was performed according to the manufacturer's protocol. Briefly, 100 µl of 1µM antisense ONs solution in OPTI-MEM (no serum here) was prepared. Different concentration (1µl, 2 µl, 5 µl, 10 µl, 15 µl and 25 µl) of of Lipofectin (Gibco BRL, UK) were added to 100 µl of OPTI-MEM reduced serum medium and allowed to stand for 30-45 min.

The two solutions were mixed together gently and incubated at room temperature for 10-15 min. 200 µl of Lipofectin/oligonucleotide solution were added and the cell suspension was incubated for 5-24 hr at 37^0C in a CO_2 incubator. Then 4 mls of culture medium were added and the cells incubated for 48 hours at 37^0C in 5 % $C0_2$ incubator. At the end of the incubation period, the cells were collected by centrifugation at 1200 rpm for 10 min, resuspended and analysed immediately by flow cytometry. Flow cytometric analysis of the cell samples was as described in section 2.13.3.2.

2.11.4. IL-2 and IL-4 protein measurements by ELISA after incubation with antisense ONs

The washed PBMCs suspension (described in section 2.4) was adjusted to a concentration of $1x10^6$/ml cells in culture medium consisting of RPMI 1640 supplemented with 5% FCS. Various antisense ONs were added at a concentration of 6 µM (total concentration) (first dose 1hour before PHA stimulation, second at stimulation time and third after 24 hours of incubation). The PBMC suspension was plated out in a 24 well tissue culture plate. Some samples were stimulated with PHA at a concentration of 1µg/ml. The cells were incubated for 24 hours at 37^0C in a 5% CO^2 incubator. The cells were then harvested and the supernatants stored at -20^0C until required. The supernatants were thawed and assayed in duplicate using a commercial ELISA kit (R&D Systems) for the concentration of IL-2 and IL-4 protein. The ELISA plates were read at 450nm and 490nm respectively and the results calculated using Dias Microplate Reader (Dynatech Laboratory, UK)

CHAPTER 3: RESULTS OF FLOW CYTOMETRIC ANALYSIS OF IL-4 AND IL-4 RECEPTOR (IL-4R) EXPRESSION IN B-CLL AND NORMAL CONTROL CELLS

3.1. Introduction

Flow cytometry, using fluorescent probes that bind to specific cell-associated antigens, enables the measurement of different phenotypic, biochemical and molecular characteristics of individual cells (Jason et al. 1997, Prussin et al. 1995). In this study, flow cytometry was used to enumerate percentages of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4 and its surface receptor (IL-4R). IL-4 displays anti-apoptotic activity on malignant B-lymphocytes in B-CLL. Thus the intracellular presence of IL-4 as well as the IL-4R in B and T-lymphocytes of B-CLL patients and normal healthy controls was investigated and the results were analysed using the Student t-test.

3.1.1. Patients and controls

Patient characteristics, including phenotyping, were obtained by the method described in section 2.1, and are listed in Table 3.1. Some of the patient characteristics, such as the percentage of CD5+ve lymphocytes and the total lymphocyte count were obtained from the Derriford Combined Laboraotory.

The controls used in this study consisted of normal healthy Caucasoid volunteers from within the Derriford Combined Laboratory. The same controls were used throughout the study (Table 3.2).

Table 3.1: Clinical and immunophenotypic features of the B-CLL patients used in the IL-4 intracellular study

Patients Nr.	Gender	Age (Years)	% CD3+ve cells	% CD19+ve cells	Absolute lymphocyte count (x10^9/l)	Stage (Binet/Rai)
1	F	55	14.0	70.1	18.6	A(O)
2	F	45	2.5	92.6	66.2	A(O)
3	M	80	7.5	60.9	38.7	A(O)
4	F	85	11.3	49.4	19.2	A(O)
5	M	60	9.5	88.3	40.2	A(O)
6	M	64	10.5	27.3	8.5	A(O)
7	M	67	21.8	45.5	13.2	A(O)
8	M	66	11.6	72.2	90.0	A(O)
9	M	69	5.3	53.6	52.1	A(O)
10	M	77	9.3	75.0	163.3	A(O)
Mean value			**10.1 SD+/-5.2**	**63.5 SD+/-20.1**		

The table shows flow cytometric analysis of the percentage of CD19+ve and CD3+ve lymphocytes present in the PBMC population of patients with B-CLL. PBMCs were stained with anti-CD19 FITC and anti-CD3 PE. The absolute lymphocyte count was obtained from the diagnostic laboratory. The staging system used at the time of the diagnosis is that described by Binet and Rai.
F=Female, M=Male, %=Percentage, SD=Standard deviation

Table 3.2: Immunophenotypic features of normal healthy controls

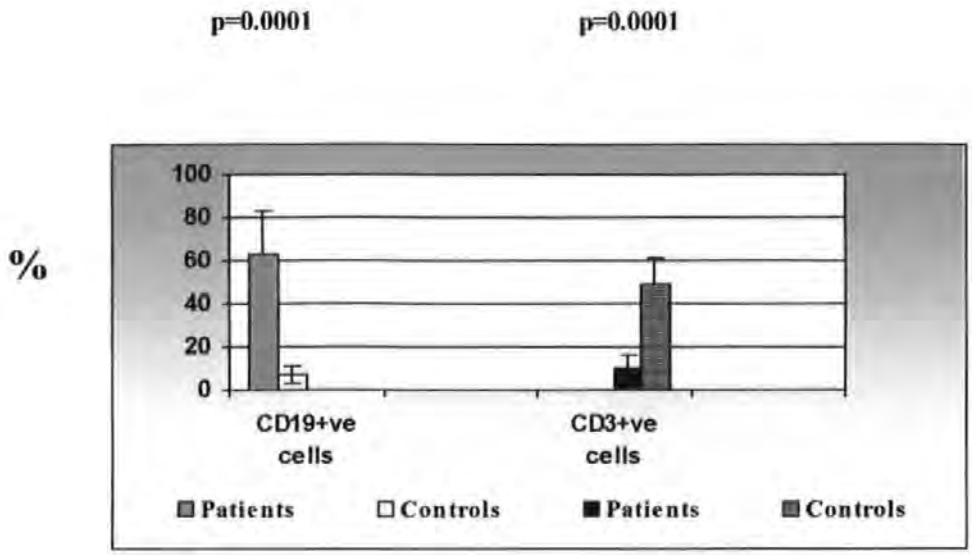
Controls Nr.	Age (Years)	Gender	% CD3 +ve cells	% CD19 +ve cells
1	42	F	61.5	4.9
2	45	F	41.8	4.2
3	35	F	34.8	3.6
4	55	M	38.5	8.9
5	61	F	54.8	10.1
6	51	M	31.1	1.4
7	50	F	55.4	6.8
8	48	M	52.5	8.2
9	55	F	62.1	12.0
10	60	M	58.5	6.0
Mean value			**49.0 SD+/-11.5**	**6.6 SD+/-3.2**

The table shows flow cytometric analysis of the percentage of CD19+ve and CD3+ve lymphocytes in the PBMC population of normal controls. PBMCs were stained with anti-CD19 FITC and anti-CD3 PE conjugated monoclonal antibodies.
F=Female, M=Male, %=Percentage, SD=Standard deviation

3.1.2. Flow cytometric analysis of the percentage of CD19+ve and CD3+ve lymphocytes in B-CLL patients and normal controls

B-CLL is characterised by an increased percentage of CD19+ lymphocytes. Flow cytometry was used to measure the percentage of cells expressing surface CD19 and CD3 antigens in patients with B-CLL and in normal controls. Separated PBMCs (section 2.4) were stained with anti-CD19 FITC and anti-CD3 FITC conjugated monoclonal antibodies. Each experiment was performed only once. The percentage of CD19+ve cells was significantly higher in B-CLL patients than normal controls (mean value: **63.5%,** SD+/-20.1 [range: 27.3-92.5] and **6.6%,** SD+/-3.2 [range: 1.4-11.9] respectively, p=0.001). Conversely the percentage of CD3+ve cells was significantly higher in normal controls than in patient samples (mean value: **49.0%,** SD+/-11.5 [range: 31.1-62.1] and **10.1%,** SD+/-5.2 [range: 2.4-21.8] respectively, p=0.001) (Table 3.1 and 3.2, Figure 3.1). Normal control PBMCs had a percentage of CD19+ve and CD3+ve lymphocytes at a ratio of 1:7.4. B-CLL patient PBMCs had a percentage of CD19+ve and CD3+ve lymphocytes at an inverted ratio of 6:1. The result shows diversity in the percentage of the CD3+ lymphocytes in patients. Patient 2 has 2.5% CD3+ lymphocytes while patients 7 has 21.8% CD3+ lymphocytes. The same percentage in normal controls remained relatively stable (Table 3.2). Using Spearmans' Correlation Test no significant correlation between the percentage of CD19+ve and CD3+ve lymphocytes was found in patients and controls (r=-0.43, p=ns and r=0.06, p=ns respectively).

Figure 3.1: Percentage of CD19+ve and CD3+ve lymphocytes in PBMCs of B-CLL patients and normal controls



The graph shows the mean values as well as p-values of the percentage of CD19+ve and CD3+ve lymphocytes in patients with B-CLL and normal controls. A Student t-test was performed. The percentage of CD19+ve lymphocytes was significantly higher in patients with B-CLL than in controls (mean value: 63.5%, [range: 27.3-92.6] and 6.6%, [range: 1.4-11.9] respectively, p=0.001). Conversely, the percentage of CD3+ve cells was significantly higher in normal controls than in patients with B-CLL (mean value: 49.0%, [range: 31.1-62.1] and 10.1% [range: 2.5-21.8] respectively, p=0.001).
%=Percentage

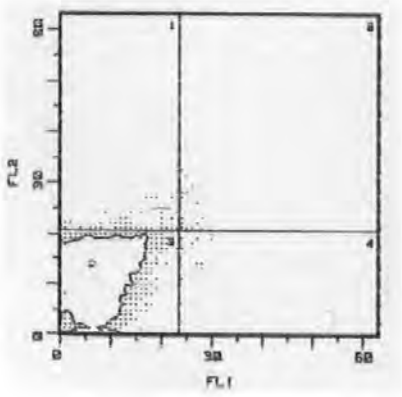
3.1.3. Measurement of intracellular IL-4 expression in CD19+ve and CD3+ve lymphocytes in B-CLL patients and normal controls

In order to study the intracellular expression of IL-4, PBMCs from ten patients with B-CLL and from ten normal healthy controls were double-stained with anti-CD3 FITC or anti-CD19 FITC and IL-4 PE-conjugated monoclonal antibodies, and analysed by flow cytometry. Figure 3.2 is a representation of the flow cytometric results obtained using a Becton Dickinson flow cytometer. Each individual experiment was performed only once. The results represent mean values and the percentage of double stained CD19+/IL-4+ or CD3+/IL-4+ lymphocytes (Figure 3.3, Appendix 1). It shows a significantly higher proportion of CD19+ve lymphocytes expressing intracellular IL-4 in patients with B-CLL than in controls (means: **22.4%,** SD+/-13.1 [range: 6.4-53.8] and **7.2%,** SD+/-3.1 [range: 1.5-11.9] respectively, p=0.03). The proportion of CD3+ve cells containing IL-4 was also significantly higher in patients with B-CLL compared to controls (means: **9.6%,** SD+/-8.5 [range: 2.2-26.1] and **2.2 %,** SD+/-1.3 [range: 0.41-4.2] respectively, p=0.01) (Figure 3.3).

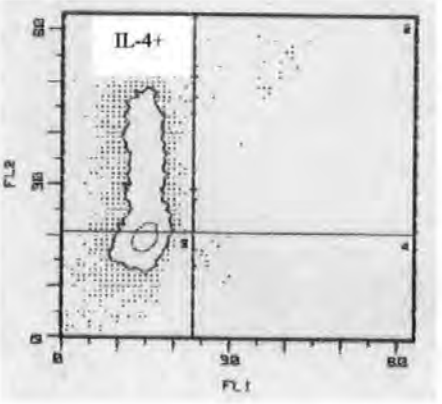
In addition, PBMCs obtained from two patients with B-CLL were pre-incubated with anti-IL-4R antibody and the percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4 was measured. As show in Table 3.3, pre-incubation of PBMCs with anti-IL-4R antibody did not change the percentage of cells expressing intracellular IL-4.

Figure 3.2: Flow cytometric analysis of patient PBMCs stained with anti-CD19 and anti-IL-4 monoclonal antibody

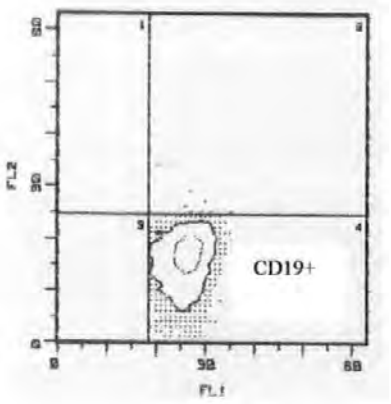
A) Negative control



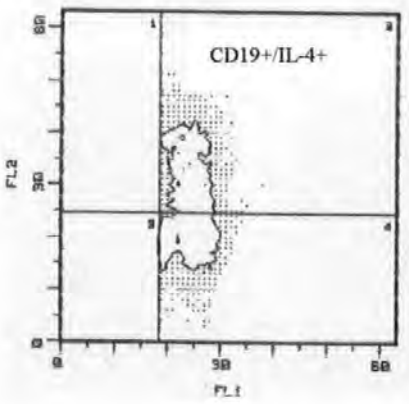
B) IL-4 PE+



B) CD19 FITC +

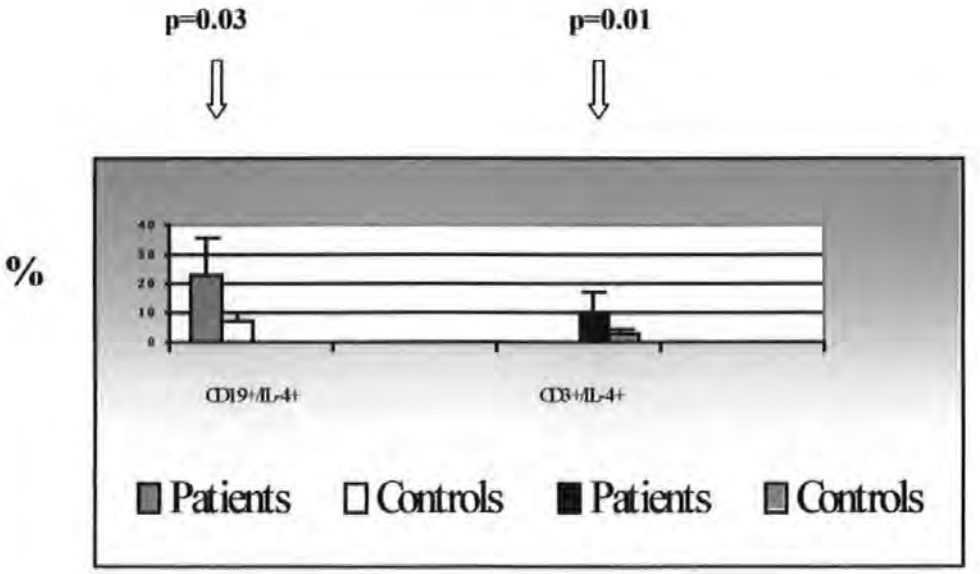


C) CD19 FITC+/IL-4 PE+



PBMC from patients with B-CLL was stained with anti-CD19 FITC and anti-IL-4 PE monoclonal antibodies. Figure-A shows negative control. Figure-B shows the results after intracellular staining with anti-IL-4 PE antibodies. Figure-C shows staining with anti-CD19 FITC antibody but the gate was set on quadrants 2 and 4 only. Figure-D is a representation of the cells stained with surface anti-CD19 FITC and intracellular anti-IL-4 PE conjugated antibodies. Double stained cells appear in quadrant 2. Number of events in each case was 10000.

Figure 3.3: Percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4 in patients with B-CLL and normal controls



The graph shows the mean values and the p values of the data presented in Appendix 1. The percentage of CD19+ve lymphocytes expressing intracellular IL-4 was significantly higher in B-CLL patients than in controls (mean value: 22.4% [range: 6.4-53.8] and 7.2% [range: 1.5-11.9] respectively, p=0.03). The percentage of CD3+ve lymphocytes expressing intracellular IL-4 was also significantly higher in patients than in controls mean value: 9.6%. [Range: 2.2-26.1] and 2.2% [range: 0.4-4.2] respectively, p=0.01).

%=Percentage, IL=Interleukin

Table 3.3. The percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4 after pre-incubation with anti-IL-4R monoclonal antibody

Patient Nr	%CD19+/IL-4+	%CD19+/IL-4+ (Pre-incubated)	%CD3+/IL-4+	%CD3+/IL-4+ (Pre-incubated)
5	53.8	50.0	4.7	6.5
6	21.7	22.5	2.5	3.5

PBMCs from two patients with B-CLL were pre-incubated with anti-IL-4R monoclonal antibody and the percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4 measured by flow cytometry. In each experiment 10,000 CD19+ or CD3+ lymphocytes were collected.
IL=Interleukin, %=Percentage

3.1.4. Measurement of surface IL-4R expression in CD19+ve and CD3+ve lymphocytes in B-CLL patients and normal controls

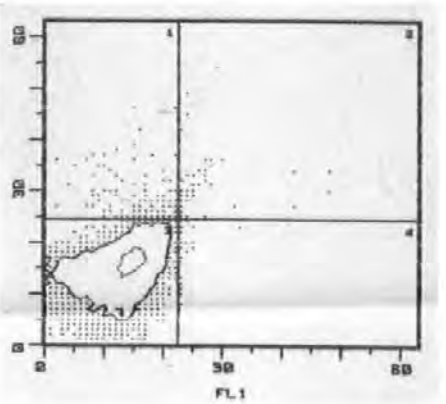
The function of cytokines is dependent on appropriate receptors expressed on target cells. The aim of this part of the project was to establish whether unstimulated CD19+ve and CD3+ve lymphocytes from patients with B-CLL and from normal controls were able to express surface IL-4R. Unstimulated PBMCs were immediately stained with anti-CD19 FITC and anti-IL-4R PE or anti-CD3 FITC and anti-IL-4R PE conjugated monoclonal antibodies and analysed by flow cytometry. In each individual experiment, whenever possible, 10,000 of CD19+ve or CD3+ve cells were collected as shown in Figure 3.4. The results were calculated using the Student t-test.

There was a marked difference in the range of CD19+ve lymphocytes expressing surface IL-4R between individual patients (range 11.22-97.13). This difference was not so marked within the normal control population (range: 41.55-88.37).

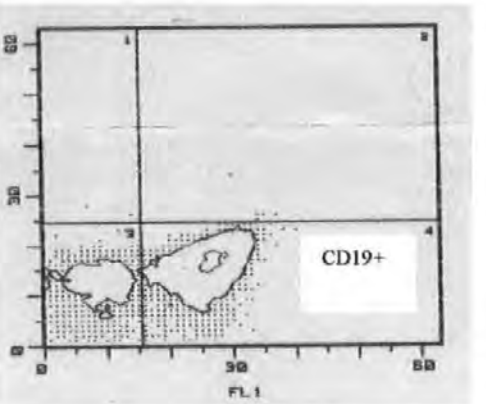
The proportion of CD19+ve lymphocytes expressing IL-4R was not significantly different between B-CLL cells and normal healthy controls (mean value: **57.7%,** SD+/-31.0 [range: 11.2-97.1] and **68.7%,** SD+/-14.3 [range: 41.6-88.4] respectively, p=ns). The proportion of CD3+ve lymphocytes expressing IL-4R in B-CLL patients was significantly higher in patients than in controls (means: **4.1%,** SD+/-2.3 [range: 0.6-7.8] and **1.7%,** SD+/-1.1 [range: 0.6-3.7] respectively, p=0.001), (Figure 3.5, Appendix 2).

Figure 3.4: Flow cytometric analysis of patients PBMCs stained with anti-CD19 and anti-IL-4R antibody

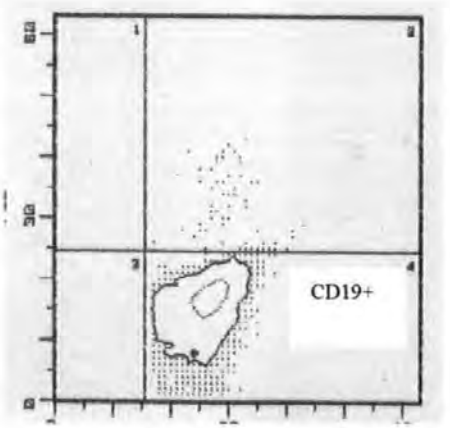
A) Control



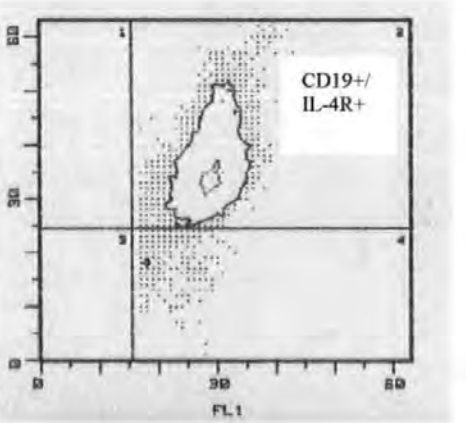
B) CD19 FITC-non-gated cells



C) CD19 FITC gated cells

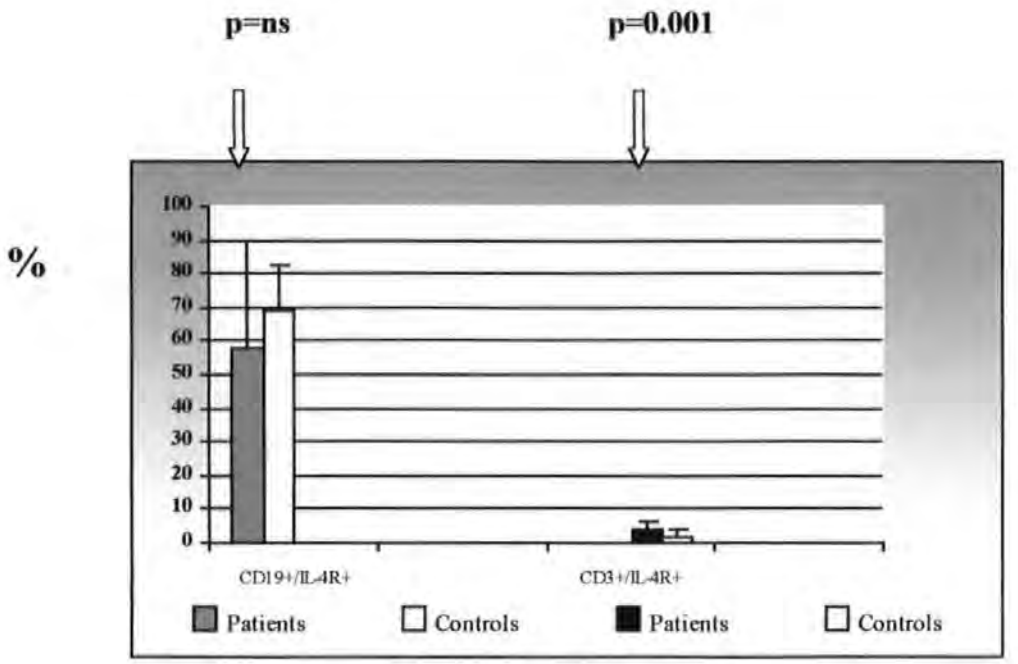


D) CD19 FITC/IL-4R PE



PBMCs from patients with B-CLL were stained with anti-CD19 FITC and anti-IL-4 receptor PE (IL-4R) monoclonal antibodies. Figure A show negative control. Figure B shows the results after staining with anti-CD19 FITC conjugated antibody. Figure C shows staining with anti-CD19 FITC antibody but the gate set on quadrants 2 and 4 only. Figure D shows the cells stained with anti-CD19 FITC and anti-IL-4R PE antibody. Double stained cells appear in quadrant 2. Number of events in each case was 10000.

Figure 3.5: Percentage of CD19+ve and CD3+ve cells expressing surface IL-4R



The graph shows the data from Appendix 2. PBMCs separated from ten patients with and ten normal controls were stained with anti-CD19 FITC and anti-IL-4R PE or anti-CD3 FITC and anti-IL-4R PE conjugated monoclonal antibodies and analysed by flow cytometry. The figure shows the mean values of the percentage of CD3+ve and CD19+ve lymphocytes expressing surface IL-4R. The percentage of the CD19+ve lymphocytes expressing IL-4R was higher in normal controls but did not reach statistical significance (mean value: 57.7% [range: 11.2-97.1} and 68.7% [range: 41.6-88.4] respectively, p=ns. The percentage of CD3+ve lymphocytes expressing surface IL-4R was significantly higher in patients than in controls (mean value: 4.1% [range: 0.6-7.8] and 1.7% [range: 0.6-3.7] respectively, p=0.001).
%=Percentage, ns=Not significant, IL-Interleukin

3.1.5. Quantification of IL-4 protein secretion by ELISA

The previous experiments (section 3.1.3) showed that patients with B-CLL have a significantly higher percentage of CD19+ve lymphocytes expressing intracellular IL-4 protein. In order to evaluate the correlation between the expression of intracellular IL-4 in B- and T-lymphocytes and the level of IL-4 protein, phytohaemagglutinin (PHA) (T cell mitogen) and pokeweed mitogen (PWM) (B and T-lymphocyte mitogen) -stimulated and unstimulated cell cultures were set up, using PBMCs separated from seven B-CLL patients and from seven normal health controls. Due to high cost of ELISA immunoassay this experiment was performed only once using one ELISA plate. PBMCs were harvested after 48 hours incubation and cell free supernatant analysed by a commercial ELISA kit. Table 3.4 shows the level of IL-4 protein secretion. The unstimulated PBMCs from patients and controls did not secrete detectable IL-4 protein. Supernatants from six control samples had an increased amount of IL-4 protein following stimulation with PHA mean value: 2.53 pg/ml (range: **0-5.74pg/ml**) or PWM mean value; 1.69 pg/ml (range: **0-4.78pg/ml**). The cell culture supernatants from only two of the seven B-CLL patients had measurable amounts of IL-4 protein after PHA stimulation; mean value: 2.04 pg/ml (range: **0-13.27pg/ml**) and none after PWM stimulation. The overall level of PHA and PWM stimulated IL-4 protein secretion in patients with B-CLL was low.

Table 3.4: IL-4 protein secretion in patients with B-CLL and in normal controls

Patient number	IL-4 spont. secretion (pg/ml)	IL-4 secretion PHA stim. (pg/ml)	IL-4 secretion PWM stim. (pg/ml)	Number of controls	IL-4 spont. secretion (pg/ml)	IL-4 secretion PHA stim. (pg/ml)	IL-4 Secretion PWM stim. (pg/ml)
1	nd	13.27	nd	1	nd	0.31	0.23
2	nd	nd	nd	2	nd	5.51	3.27
3	nd	nd	nd	3	nd	1.02	1.02
4	nd	nd	nd	4	nd	5.12	0.15
5	nd	1.02	nd	5	nd	0.00	2.37
6	nd	nd	nd	6	nd	5.74	4.78
7	nd	nd	nd	7	nd	nd	nd
Mean value	**0**	**2.04**	**0**		**0**	**2.53**	**1.69**

PBMCs isolated from patients with B-CLL and from normal controls were stimulated with either PHA or PWM and, following 48 hours incubation, IL-4 protein was measured in cell free supernatants by a commercial ELISA kit. In unstimulated PBMC from patients and controls no detectable amount of IL-4 was observed. Spont. secretion=Spontaneous secretion, PHA=Phytohemagglutinin, PWM =Pokeweed mitogen, nd = Not detectable

3.1.6. Measurement of intracellular IL-4 expression in CD19+ve B-lymphocytes in PBMCs after depletion of T-lymphocytes and monocytes

The effect of T-lymphocytes and monocytes populations on the intracellular expression of IL-4 in CD19+ve B-lymphocytes from patients with B-CLL was investigated in this part of the study. The T-lymphocyte population was depleted from PBMC preparations from five patients with B-CLL, using CD2+ve Dynabeads. Monocytes were depleted by repeated adherence to plastic. Because Dynabeads were still attached to the lymphocytes at the time of analysis, no attempt was made to establish the purity of the CD19+ve lymphocyte population. As shown below (Table 3.5), the presence of the T-lymphocyte and monocyte populations influenced the percentage of CD19+ve lymphocytes expressing intracellular IL-4. The expression of intracellular IL-4 was highest in B-lymphocytes incubated on their own compared to B-lymphocytes incubated with monocytes and T-lymphocytes together, although this did not attain significance (mean value: **32.1%**, SD+/-27.6 [range: 4.2-76.7] and **24.6%,** SD+/-27.9 [range: 5.4-70.8] respectively, p=ns). No statistically significant difference was observed between the percentage of CD19+ve lymphocytes expressing intracellular IL-4 when B- and T-lymphocytes where incubated together versus undepleted PBMCs (mean value: **25.5%,** SD+/-19.7 [range: 8.7-53.9] and **24.6%,** SD+/-27.9 [5.4-70.8] respectively, p=ns), (Table 3.5).

Table 3.5: Flow cytometric analysis of CD19+ve lymphocytes expressing intracellular IL-4 after depletion of T-lymphocytes and monocytes

Patients number	**PBMCs (I)**	**PBMCs minus monocytes (II)**	**PBMCs minus monocytes and T lymphocytes (III)**
	%CD19+/IL-4+	**%CD19+/IL-4+**	**%CD19+/IL-4+**
3	31.8	35.9	35.3
5	70.8	54.0	76.7
8	5.4	8.7	15.8
9	10.8	20.8	28.5
10	4.5	7.3	4.2
Mean value	**24.6**	**25.3**	**32.1**
SD+/-	**27.9**	**19.7**	**27.6**
Variable: I / II		**Variable: I / III**	
p=ns		**p= ns**	

The table shows flow cytometric analysis of the percentage of CD19+FITC/IL-4+ in PBMCs from five patients with B-CLL before and after the depletion of T-lymphocytes and monocytes or monocytes only. Student t-test was used to evaluate the statistical significance between percentage of CD19+/IL-4+ lymphocytes in PBMCs population (I) and PBMCs population depleted of monocytes (II) or monocytes and T-lymphocytes respectively (III). No significant difference was observed in both cases.
SD =Standard deviation, ns=Not significant, %=Percentage, IL-Interleukin

3.5. Summary

- PBMCs from patients with B-CLL had a higher percentage of CD19+ve B-lymphocytes than normal healthy controls. This is not surprising since one of the features of B-CLL is an increased lymphocyte count with the majority of B-lymphocytes being of clonal origin.
- PBMCs from patients with B-CLL contained a significantly greater percentage of CD19+ve as well as CD3+ve lymphocytes expressing intracellular IL-4 compared to normal healthy controls.
- In addition, pre-incubation of PBMCs with anti-IL-4R antibody did not change the percentage of cells expressing intracellular IL-4.
- Only patients' CD3+ve lymphocytes had a significantly higher percentage of CD3+ve lymphocytes expressing surface IL-4R compared to normal controls.
- Supernatants from unstimulated PBMCs from patients with B-CLL had no detectable IL-4 protein. However, when the cells from the patients were stimulated with either PHA or PWM alone, two patients had detectable IL-4 protein after PHA stimulation but none after PWM stimulation. In contrast, normal controls had detectable IL-4 protein after stimulation with PHA and PWM.
- Depletion of T lymphocytes and monocytes from patient PBMC increased the percentage of CD19+ve lymphocytes expressing intracellular IL-4 protein. These results show a trend towards T-lymphocytes having some influence on the B-lymphocytes expression of intracellular IL-4.

CHAPTER 4: RESULTS OF MEASUREMENT OF CYTOKINE mRNA EXPRESSION BY NESTED RT-PCR

4.1. Introduction

It was previously established that PBMCs from patients with B-CLL had a greater percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4. However it was not possible to demonstrate increased secretion of IL-4 protein (by ELISA) or the presence of mRNA (by Northern blotting) in CD19+ve lymphocytes isolated from patients with B-CLL (data not shown). In view of this it was decided to study the expression of mRNA using a more sensitive technique such as nested RT-PCR. In addition to IL-4, several other cytokines or receptors such as IL-2, IL-4, IL-7, IL-9, IL-15, IL-4Rα, γc, IL-5 and IL-13 mRNA were also analysed.

High quality RNA was isolated from the population of CD19+ve and CD2+ve lymphocytes as described in section 2.5. This RNA was run on 1% agarose gel and the presence of the characteristic 18S and 28S bands identified (Figure 4.1 A). The ratio between the density of the 28S and 18S RNA bands should be 2:1. If the 18S RNA band is more intense than 28S, this is an indication of RNA degradation. The optical density (OD) of each individual RNA sample was measured and the presence of RNA quantified (Figure 4.1 B).

However before the RT-PCR studies were performed, the PCR conditions were optimised. The magnesium concentration (Mg++), annealing temperature, cycle length and the number of cycles were checked. Proper evaluation of magnesium concentration is important since this affects several aspects of PCR including DNA polymerase activity which can affect primer annealing which in turn can affect specificity (Figure 4.1 C). Figure 4.2 shows the presence of the β-actin mRNA "housekeeping gene" in all samples used in this study. Some of the samples had to be repeated to confirm the presence of β-actin gene. Due to limited quantity of cDNA obtained from each patient and the difficulties in obtaining further samples from the same patients each experiment was performed only once. All the samples were run simultaneously on one gel. Using densitometry, the density of each band was measured by

drawing a rectangular shape. However due to a different intensity of the bands and their different size some human effort may have occurred.

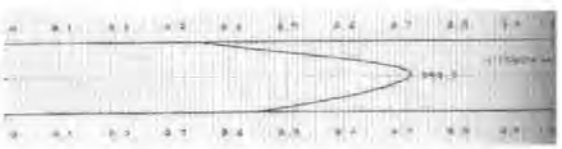
Figure 4.1: Optimisation of RT-PCR reaction

A) Total RNA was separation using RNA-zol or RNA-STAT 60

1 2 3 4 5 6 7 8

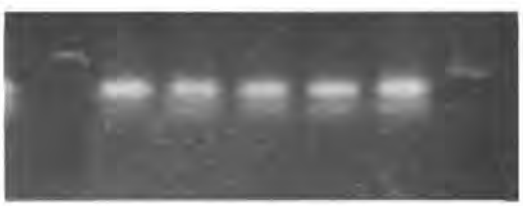
RNA in lane 1, 2 and 3 was purified using RNA-STAT 60. RNA in lane 4,5,6,7 and 8 was prepared using RNA-zol

B) RNA optical densitometry



Optical density of each of the RNA preparations was measured using Cecil 5000 series spectrophotometer. Pure RNA has an A260/A280 ratio of 1.9-2.1.
1 absorbance unit at 260nm corresponds to 40 μg of RNA per 1 ml

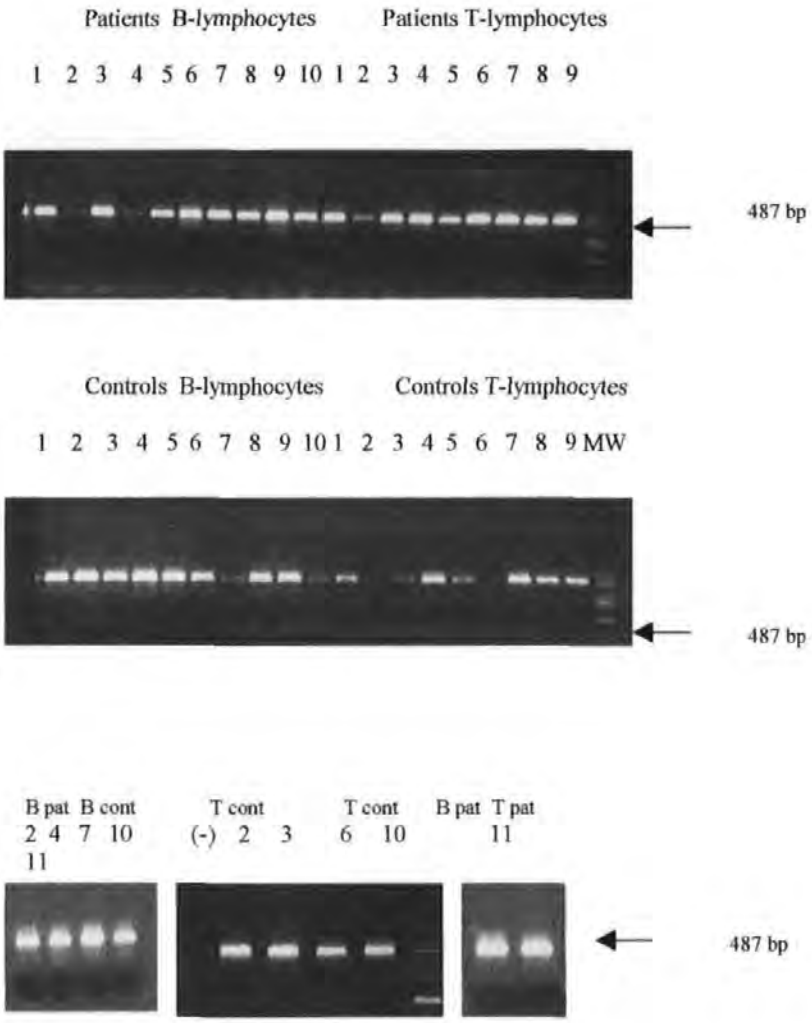
C) Optimisation of magnesium concentration



H_2O 1.5mM 2.5mM 4mM 5mM 6mM MW

PCR reaction was performed using cDNA from B-CLL patient. IL-4 specific primers were used. Different concentration of magnesium did not influence the quality of the PCR product.

Figure 4.2: Beta-actin (β-actin) RT-PCR results after first round of amplification



A nested RT-PCR technique was used to identify β-actin products in B- and T-lymphocyte samples of patients with B-CLL and normal controls. β-actin primers were used to check the quality of the cDNA samples prepared for further experiments. Some of the samples were repeated twice.
MW=molecular weight marker, (-)=negative control, B pat= patients B cells, B cont= controls B cells, T pat= patients T cells, T cont= controls T cells

1.2. Patients and controls

Details concerning normal healthy controls are listed in table 3.2 and clinical and immunophenotypic features of the patients are listed in table 4.1. However, in patients 12 and 19 data concerning the percentage of CD5+ve lymphocytes were not available from their hospital records.

Table 4.1: Clinical and immunophenotypic features of B-CLL patients

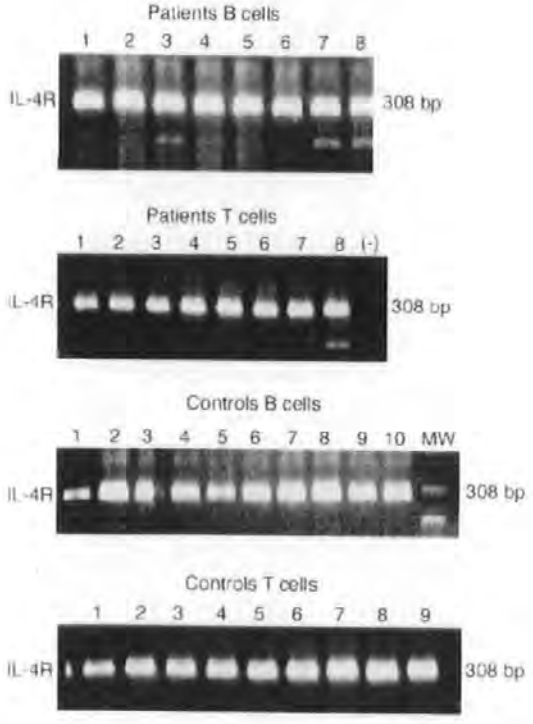
Patient number	Gender	Age (Years)	%CD5 lymphocytes	Absolute lymphocyte count ($x10^9/l$)	Stage (Binet/Rai)
11	M	77	32	92.0	A(0)
12	M	76	25	53.5	A(0)
13	M	64	84	53.3	A(0)
14	M	64	96	16.8	A(0)
15	F	60	79	46.7	A(0)
16	F	70	50	25.0	A(0)
17	F	70	15	75.0	A(0)
18	F	79	84	86.0	A(0)
19	M	65	37	48.8	A(0)
20	F	61	15	15.0	A(0)
21	F	64	63	18.4	A(0)

The percentage of CD5+ve lymphocytes and the absolute lymphocyte count were obtained from the diagnostic laboratory. The staging system used at the time of the diagnosis is that described by Binet and Rai.
F=Female, M=Male, %=Percentage, na=Not available

4.3. Analysis of IL-4 receptor (IL-4R) α chain mRNA expression by nested RT-PCR

The expression of IL-4R α chain mRNA in purified populations of B and T-lymphocytes from patients with B-CLL and from normal controls were analysed (Figure 4.3). The IL-4R α chain RT-PCR product was present in B and T-lymphocytes in patient and control samples. Statistical significance was measured using the Student t-test. There was no significant difference between the density of patient and control B-lymphocyte IL-4Rα chain RT-PCR products (mean value: **40.5** [range: 32.5-46.6] and **36.5** [range: 20.1-42.7] respectively, p=ns). In contrast there was a strong statistically significant difference between the density of patient and normal T-lymphocyte IL-4Rα chain RT-PCR products (mean value: **45.0** [range: 40.3-49.4] and **45.0** [range: 42.6-47.9] respectively, p=ns). In addition the ratio between patient B and T-lymphocyte mean density values of IL-4R α chain RT-PCR products was 1: 1.05 while the ratio in normal healthy controls was 1:1.2 (Figure 4.4, Appendix 3).

Figure 4.3: IL-4R alpha (IL-4Rα) chain RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify IL-4R mRNA transcripts in B- and T-lymphocytes in patients with B-CLL and in normal controls. The figure shows transcripts after a second round of amplification. A 100 bp molecular weight marker was used.
MW=molecular weight marker
(-)=negative control

Figure 4.4: Mean density values of IL-4R α chain nested RT-PCR products

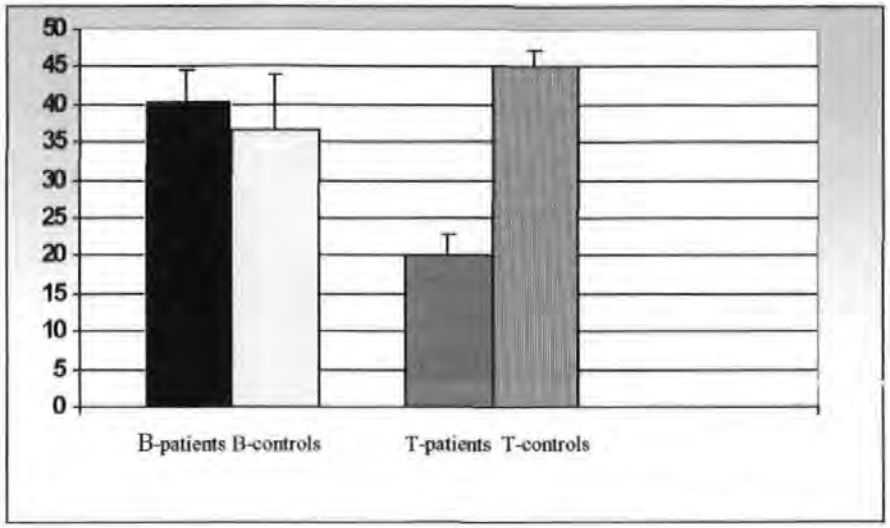
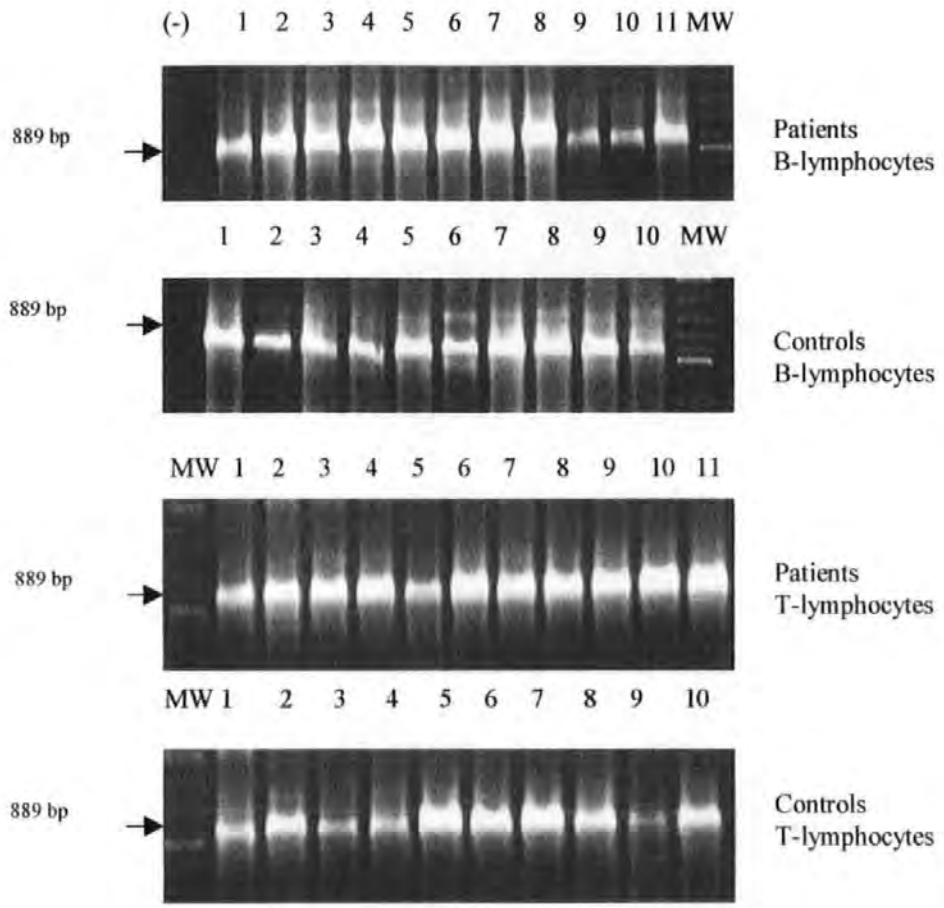


Figure 5.4 shows mean values of the densitometry results of the IL-4R α chain RT-PCR product shown in Appendix 3. The Students t test was used to measure statistical significance. No significant difference was found between the density of patient and control B-lymphocyte IL-4R α chain RT-PCR products (mean value: 40.5 [range: 32.5-46.6] and 36.5 [range: 20.1-42.7] respectively, p=ns). In contrast, there was a significant difference between the density of patient and normal control T-lymphocyte IL-4R α chain RT-PCR products (mean value: 45.0[range 15.9-23.8] and 45.0 [range: 42.6-47.9] respectively, p=ns). In addition, the ratio between patient B and T-lymphocyte mean density values of IL-4R α chain RT-PCR products was 1:05 and the same ratio in normal healthy controls was 1:1.2.

4.4. Analysis of common gamma (γc) chain mRNA expression by nested RT-PCR

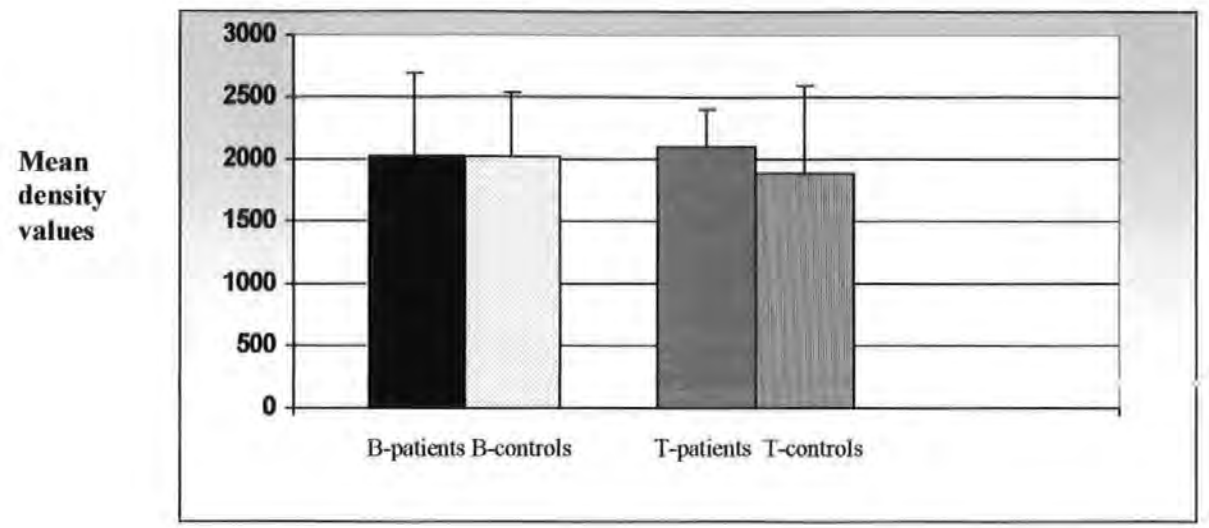
The presence of γc transcripts in B-CLL and control B- and T-lymphocytes was measured using primers spanning the entire region of the γc chain (Figure 4.5, Appendix 4). There was no significant difference between the density of patient and control B-lymphocyte γc RT-PCR products (mean value: **2039.2,** SD+/644.0 [range: 830.9-2619.5] and **2025.4,** SD+/-466.2 [range: 1381.0-2604.3] respectively, p=ns). There was no statistically significant difference between the density of patient and normal T-lymphocyte γc RT-PCR products (mean value: **2094.9,** SD+/-348.1 [range: 1353.2-2528.2] and **1893.2,** SD+/-699.7 [range: 610.0-2793.6] respectively, p=ns). The ratio between patient B and T-lymphocyte mean densities of γc chain RT-PCR products was 1: 1 and in normal healthy controls was 1: 0.9 (Figure 4.6).

Figure 4.5: Common gamma chain (γc) RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify common gamma (γc) chain products in B- and T-lymphocytes in patients with B-CLL and in normal controls. The figure shows RT-PCR products after a second round of amplification. A 100bp molecular weight ladder was used.
MW-molecular weight marker
(-)- negative control

Figure 4.6: Mean density values of common gamma (γc) chain nested RT-PCR products



The figure shows mean density values of the γc RT-PCR products presented in Appendix 4. The Students t test was used to measure statistical significance. No significant difference was demonstrated between the density of patient and control B-lymphocyte γc RT-PCR products (mean value: 2039.2 [range: 830.9-2619.5] and 2025.4 [range: 1381.0-2604.3] respectively, p=ns). Similarly, there was no significant difference between the density of patient and normal T-lymphocyte γc RT-PCR products (mean value: 2094.9 [range: 1353.2-2528.2] and 1893.2 [range: 610.0-2793.6] respectively, p=0.406). In addition the ratio between patient B and T-lymphocyte mean density values of γc RT-PCR products was 1: 1 and in normal healthy controls 1: 0.9. SD=Standard deviation, ns=Not significant

4.5. mRNA analysis of cytokines belonging to the common gamma chain (γc) receptor family by nested RT-PCR

Using primers specially designed to cover the entire region of the IL-2, IL-4, IL-7, IL-9, and IL-15 genes, the presence of individual mRNA RT-PCR products in B- and T-lymphocytes from patients and controls was investigated. These cytokines share a common subunit known as the common gamma chain (γc) receptor. Statistical significance was measured using the Students t test.

The IL-2 RT-PCR product density was higher in patient than in control B-lymphocyte samples (mean value: **521.0,** SD+/-747.0 [range: 10.1-1802.3] and **22.9,** SD+/-18.7 [range: 0-66.4] respectively, p=0.04) (Figure 4.7). There was a difference between IL-2 RT-PCR product density in patient and control T-lymphocyte samples but this did not reach statistical significance (mean value: **1220.0,** SD+/-894.5 [range: 0-2272.9] and **1717.0,** SD+/-524.3 [range: 252.8-2027.1] respectively, p=ns). In addition, the ratio between patient T and B-lymphocyte mean density values of the IL-2 RT-PCR products was 1: 2.3. More importantly, the same ratio in normal healthy controls was much higher at 1: 75 (Appendix 5, Figure 4.8).

There was no significant difference in the density of the IL-4 RT-PCR product comparing patient and control B-lymphocytes (mean value: **127.2,** SD+/-133.9 [range: 7.7-416.7] and **51.3,** SD+/-72.6 [range: 0-221.0] respectively, p=ns) or patient and normal T-lymphocytes (mean value: **252.1,** SD+/-249.6 [range: 3.8-673.8] and **337.7,** SD+/-284.5 [range: 0-743.6] respectively, p=ns). The ratio between patient T- and B-lymphocyte mean density values of the IL-4 RT-PCR products was 1: 1.9. The same ratio in normal healthy controls was much higher at 1: 6.6 (Appendix 6, Figure 4.9, 4.10).

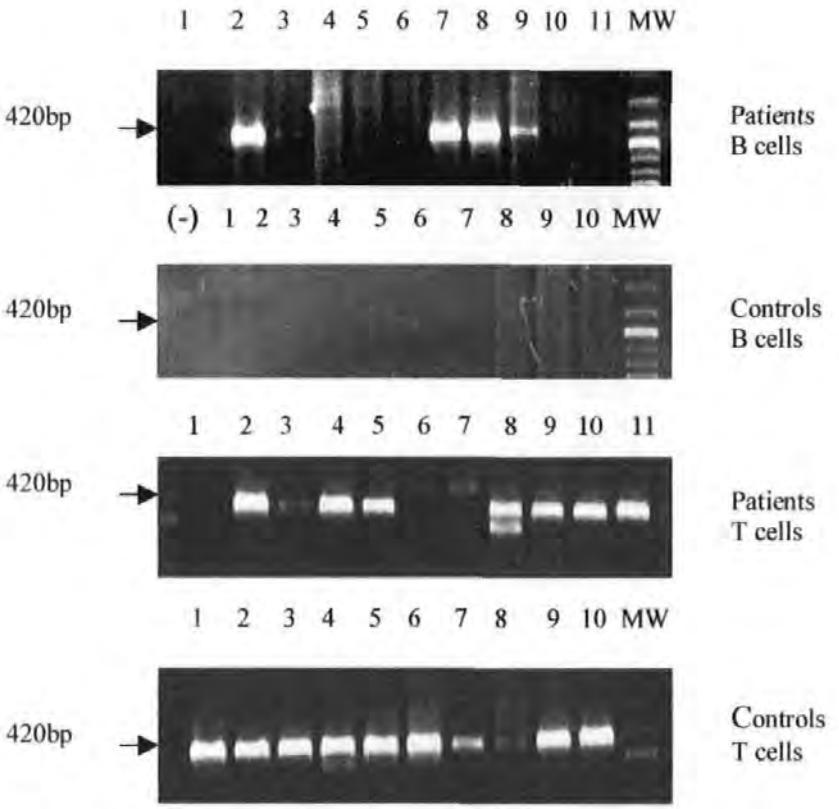
There was a significant difference between the IL-7 density of patient and control B-lymphocyte IL-7 RT-PCR products (mean value: **1600.0,** SD+/-772.8 [range: 465.1-2693.0] and **1239.9,** SD+/-925.5 [range: 0-2610.8] respectively, p=0.03). There was a significant difference between the density of patient and normal T-lymphocytes IL-7 RT-PCR products

(mean value: **2480.5,** SD+/-868.9 [range: 800.2-3229.5] and **1484.6,** SD+/-1202.6 [range: 20.1-3349.7] respectively, p=0.04). The ratio between patient B and T-lymphocyte mean density values of IL-7 RT-PCR products was 1: 1.6 and the ratio in healthy controls was 1: 1.2 (Appendix 7, Figure 4.11, 4.12). IL-9 RT-PCR products were not detected.

No significant difference between the IL-15 RT-PCR product density of patient B and control B-lymphocyte IL-15 RT-PCR products was demonstrated (mean value: **1861.7,** SD+/-201.1 [range: 1545.8-2293.6] and **1777.2,** SD+/-95.4 [range: 1626.4-1905.1] respectively, p=ns). However, a significant difference between the density of patient and normal T-lymphocytes IL-15 RT-PCR products was found (mean value: **1432.4,** SD+/-433.9 [range: 459.1-1974.9] and **2279.6,** SD+/-251.9 [range: 1698.4-2529.8] respectively, p=0.001). The ratio between patient B and T-lymphocytes mean density values of IL-15 RT-PCR products was 1: 0.8 and in normal healthy controls was 1: 1.3 (Appendix 8, Figure 4.13, 4.14). Figure 4.9 shows the mean density values of all cytokines belonging to the γc receptor family plus IL-4R and γc RT-PCR products.

In addition, IL-7 RT-PCR products were composed of many additional unidentified bands most probably representing IL-7 splice variants. However, I was unable to provide evidence about their identities (due to inconclusive sequencing). In addition, an additional band was present when the IL-15 RT-PCR product was run on a gel. The additional band had a higher molecular weight than IL-15 and was not sequenced. Automated sequencing (MWG, Germany) was used to identify the RT-PCR products (Appendices (22-28).

Figure 4.7: IL-2 wild type RT-PCR products after a second round of amplification



A nested RT-PCT technique was used to identify IL-2 wild type (primers spanning exon 1 to 4) mRNA in B and T cells of patients with B-CLL and normal controls. A 100 bp molecular weight marker was used.
MW=molecular weight ladder
(-)=negative control

Figure 4.8: Mean density values of IL-2 nested RT-PCR products

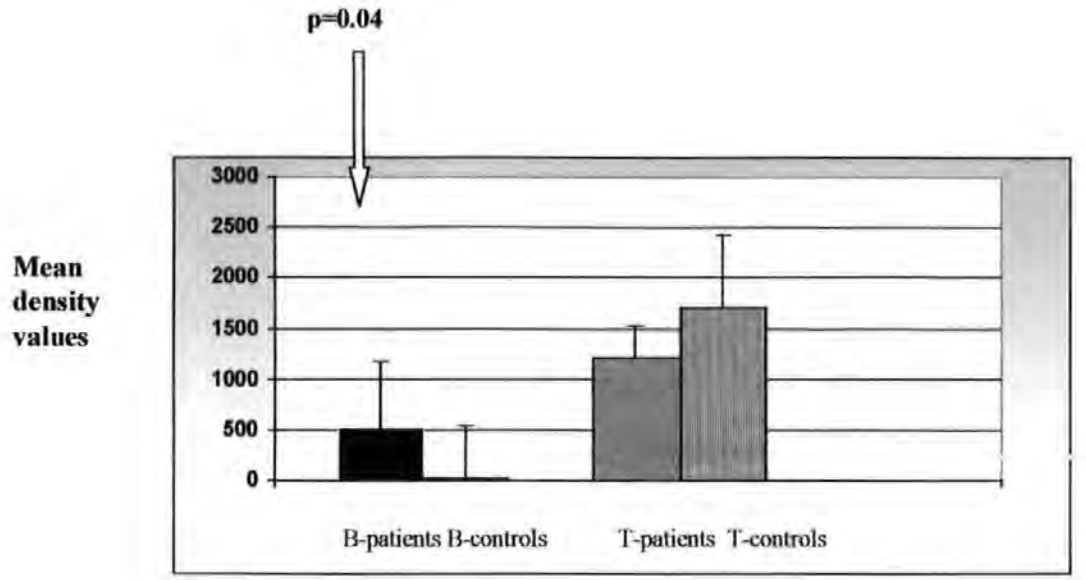
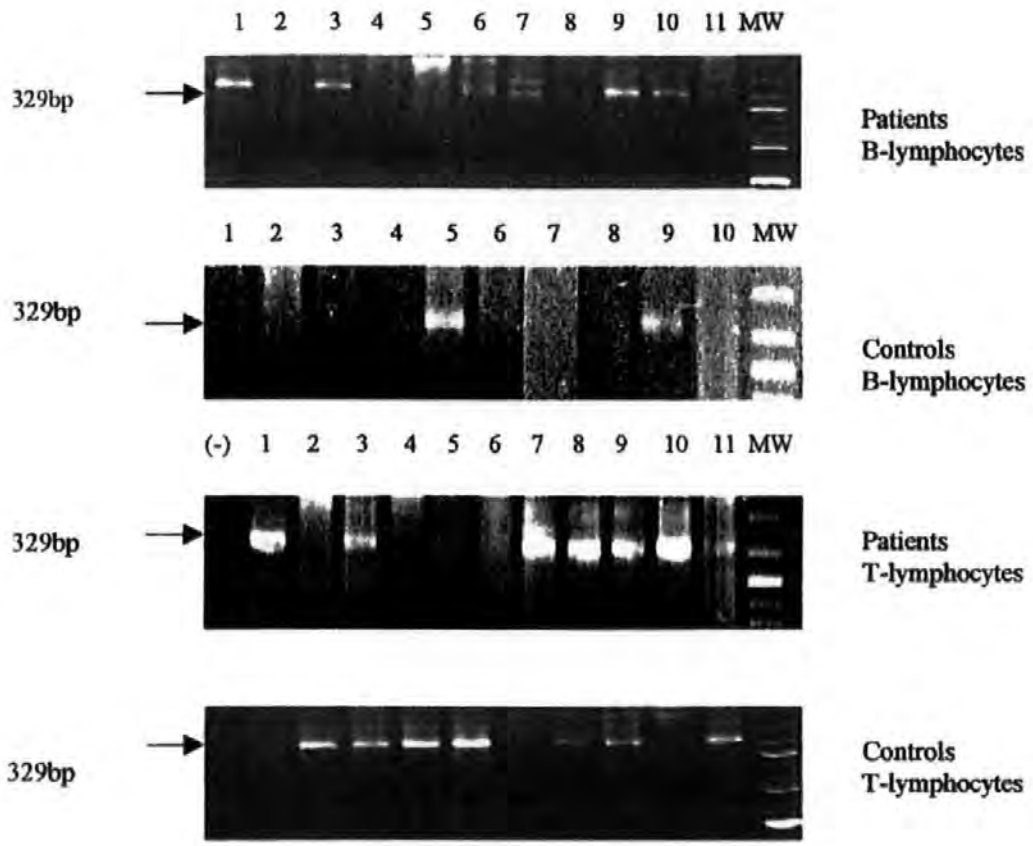


Figure 5.8 shows mean density values of the densitometry results of the IL-2 RT-PCR products presented in Appendix 5. The Students t test was used to measure statistical significance. There was a significant difference between the density of patient and control B-lymphocyte IL-2 RT-PCR products (mean value: 521.1 [range: 0-1860.5] and 22.9 [range: 0-66.4] respectively, p=0.04). There was no significant difference between the density of patient and normal T-lymphocyte IL-2 RT-PCR products (mean value: 1220.0 [range: 0-2272.9] and 1717.0 [range: 252.8-2027.1] respectively, p=ns). The ratio between mean density value of patient B and T-lymphocyt IL-2 RT-PCR products was 1: 2.3. The same ratio in normal healthy controls was much higher at 1: 75.
SD=Standard deviation, ns=Not significant

Figure 4.9: IL-4 wild type RT-PCR products after the second round of amplification



A nested RT-PCR technique was used to identify IL-4 wild type (primers spanning exon 1 to 4) PCR products in B and T-lymphocytes in patients with B-CLL and in normal controls. The figure shows IL-4 PCR product after a second round of amplification. A 100bp molecular weight ladder was used.
MW=molecular weight marker, (-)= negative control

Figure 4.10: Mean density values of IL-4 nested RT-PCR products

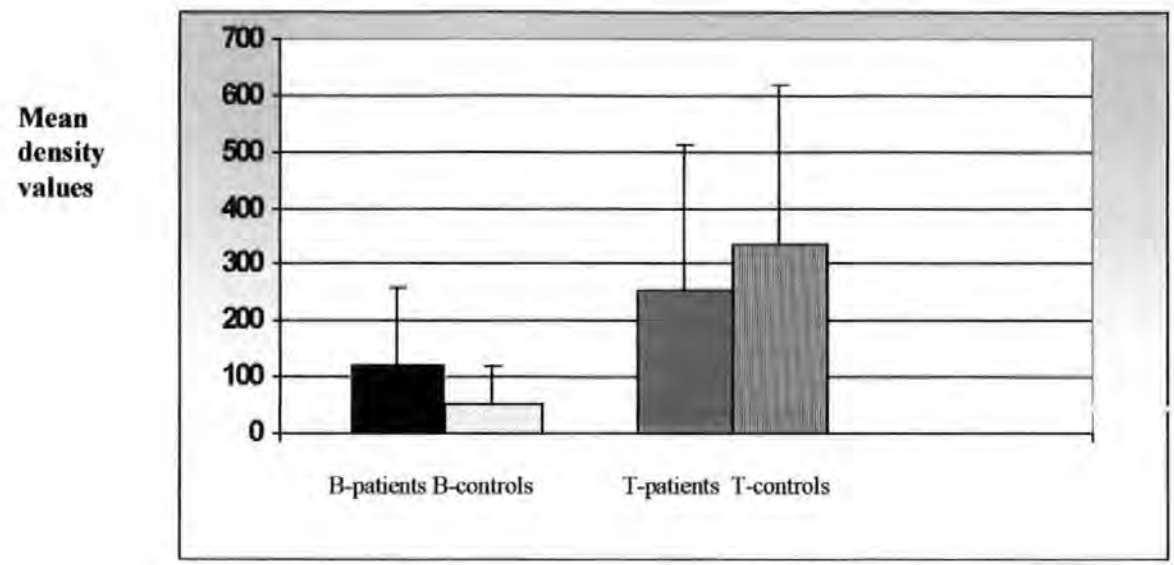
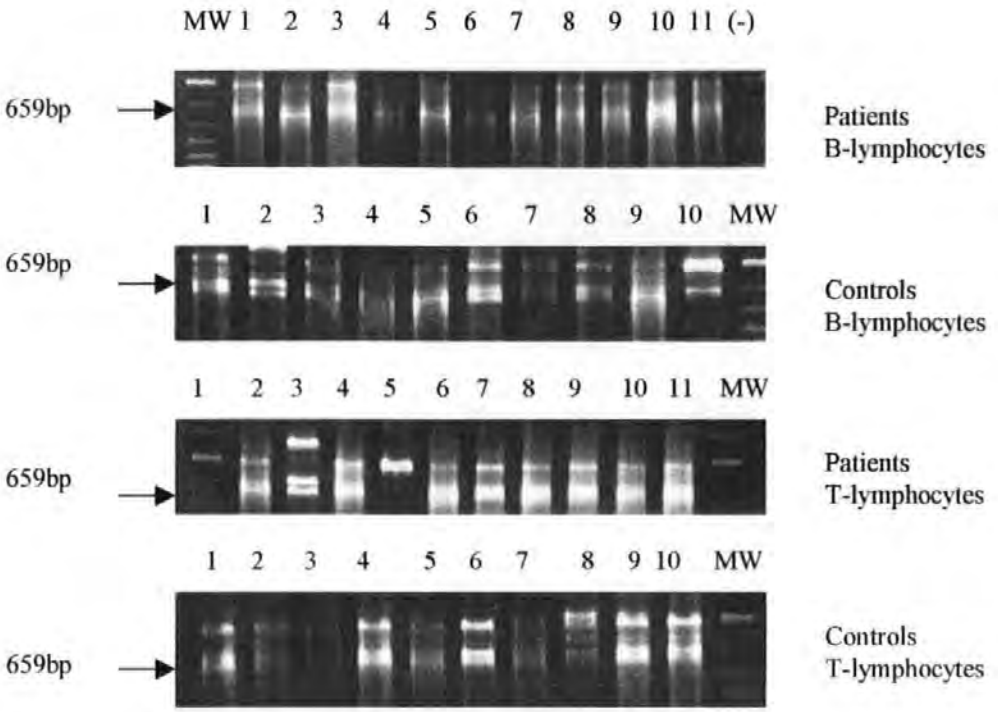


Figure 4.10 shows mean density values of the densitometry results of the IL-4 RT-PCR products presented in Appendix 6. The Students t test was used to measure statistical significance. There was no significant difference between the density of patient and control B-lymphocyte IL-4 RT-PCR products (mean value: 127.2 [range: 7.7-416.7] and 51.3 [range: 0-221.0] respectively, p=ns). There was no significant difference between the density of patient and normal T-lymphocyte IL-4 RT-PCR products (mean value: 252.1 [range: 3.8-673.8] and 337.7 [range: 0-743.6] respectively, p=ns). The ratio between mean density value of patient B and T-lymphocyte IL-4 RT-PCR products was ratio: 1: 2. More importantly, the same ratio in the normal healthy controls was much higher at 1: 6.6.
SD=Standard deviation, ns=Not significant

Figure 4.11: IL-7 wild type RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify IL-7 products in B- and T-lymphocytes in patients with B-CLL and in normal controls. The figure represents RT-PCR products after a second round of amplification. A 100 bp molecular weight marker was used.
MW=molecular weight marker
(-)=negative control

Figure 4.12: Mean density values of IL-7 nested RT-PCR products

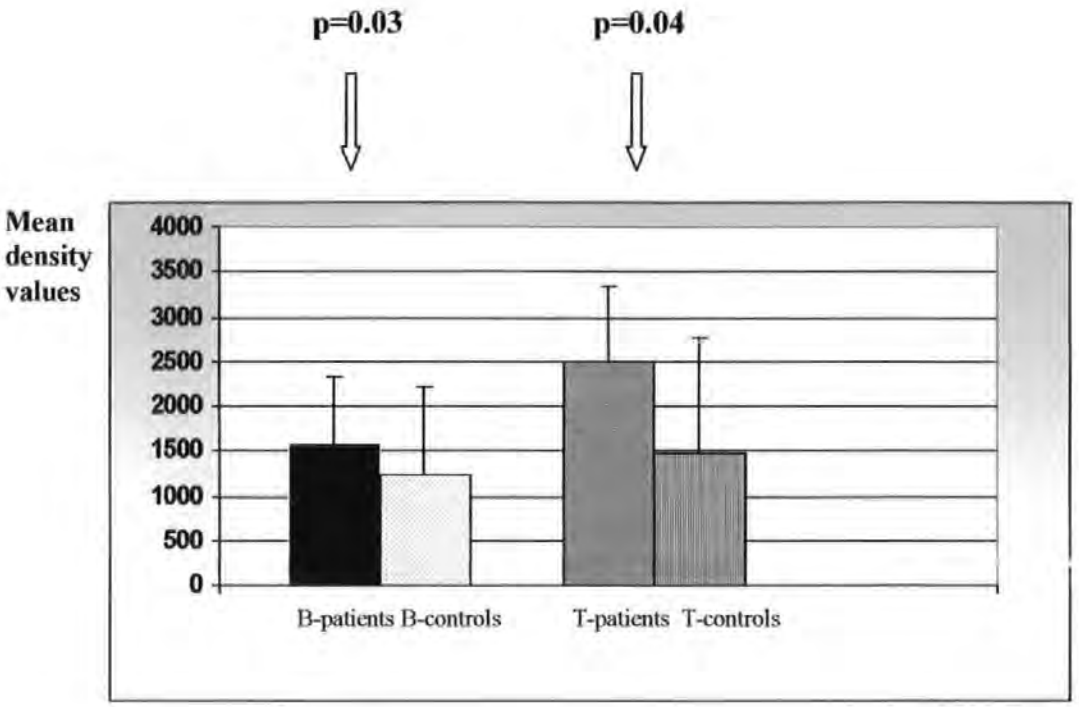
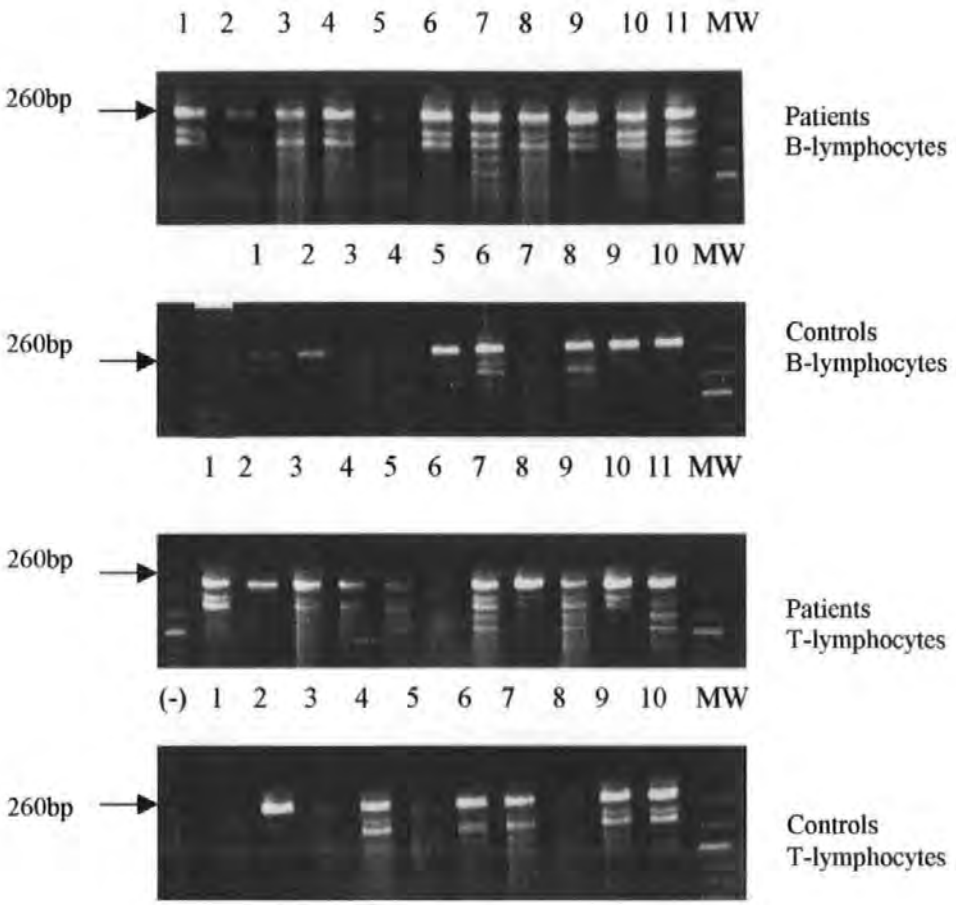


Figure 4.13 shows mean density values of the densitometry results of the IL-4 RT-PCR products presented in Appendix 7. The Students t test was used to measure statistical significance. There was a significant difference between the density of patient and control B-lymphocyte IL-7 RT-PCR products (mean value: 1600.0 [range: 465.1-2693.0] and 1239.9 [range: 0-2610.8] respectively, p=0.03). There was a significant difference between the density of patient and normal T-lymphocyte IL-7 RT-PCR products (mean value: 2480.5 [range: 800.2-3229.5] and 1484.6 [range: 20.1-3349.7] respectively, p=0.04). The ratio between patient B and T-lymphocyte IL-7 RT-PCR products was 1: 1.6 and in normal healthy controls was 1: 1.2.
SD=Standard deviation, ns=Not significant

Figure 4.13: IL-5 wild type RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify IL-5 products in B- and T-lymphocytes of patients with B-CLL and normal controls. The figure represents RT-PCR products after a second round of amplification. A 100 bp molecular weight ladder was used.
M=-molecular weight marker
(-)=negative control

Figure 4.14: Mean density values of IL-15 nested RT-PCR products

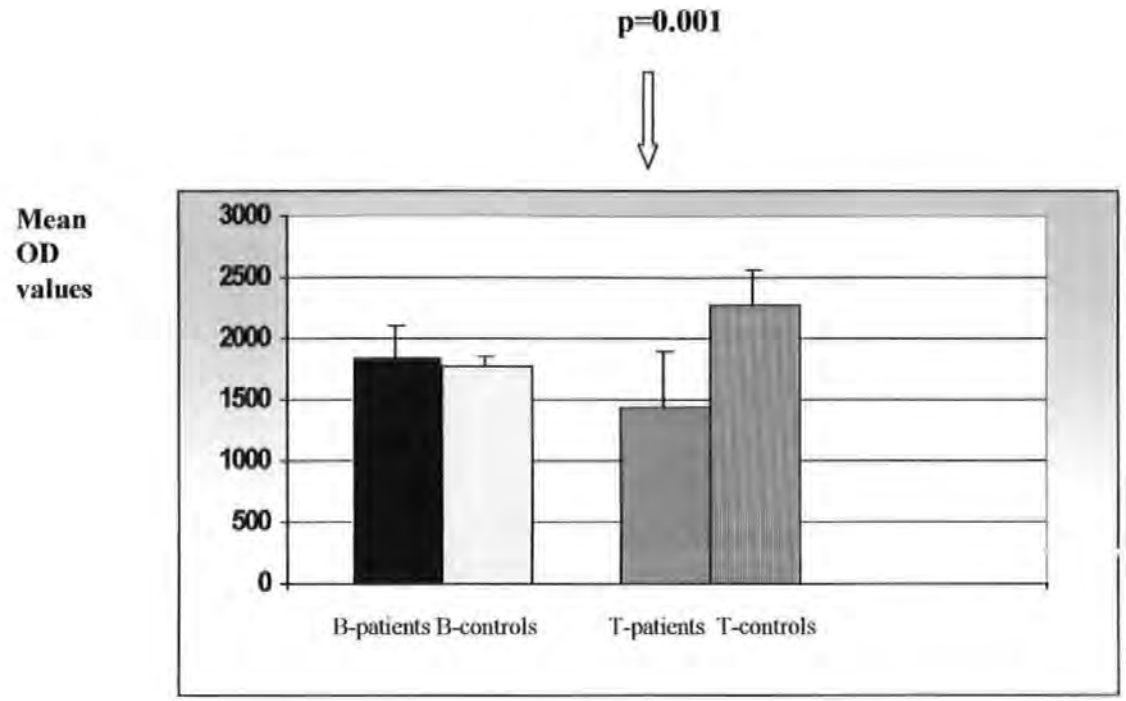


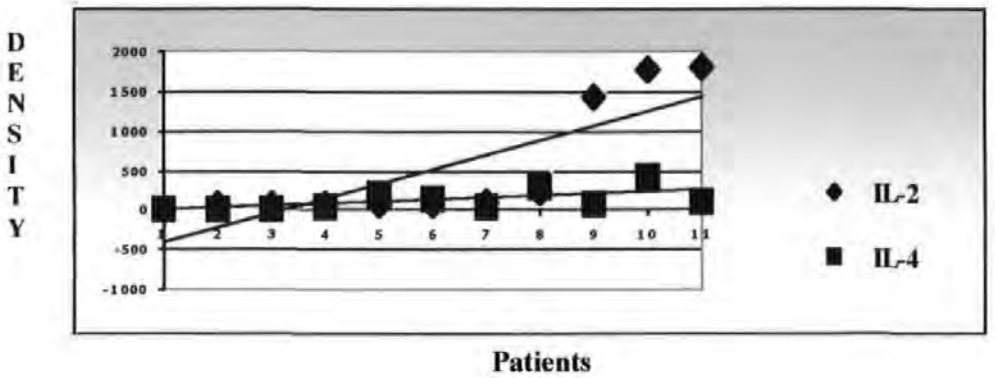
Figure 4.14 shows mean density results of the densitometry results of the IL-15 RT-PCR products presented in Appendix 8. The Students t-test was used to measure statistical significance. There was no significant difference between the density of patient and control B-lymphocyte IL-15 RT-PCR products (mean value: 1861.7 [range: 1545.8-2293.6] and 1777.2 [range: 1626.4-1905.1] respectively, p=ns). There was a significant difference between the density of patient and normal T-lymphocyte IL-15 RT-PCR products (mean value: 1432.4 [range: 459.1-1974.9] and 2279.6 [range: 1698.4-2529.8] respectively, p=0.001). The ratio between mean density values of patient B and T-lymphocyte IL-15 RT-PCR products was 1: 0.8 and in normal healthy controls 1: 1.3.
SD=Standard deviation, ns=Not significant

4.6. Correlation between gamma chain related cytokine RT-PCR product densities

Since it is known that IL-2, IL-4, IL-7 and IL-15 use the γc chain as part of their individual receptor complexes, inter-cytokine regulation may occur. In order to determine whether a correlation existed between the OD of RT-PCR products of the above cytokines, the data was analysed using Spearman's Rank Correlation. In B-CLL B-lymphocyte samples, a strong positive correlation was found (0.74, $p=0.008$) between the densities of the IL-2 and IL-4 RT-PCR products. In B-CLL T-lymphocyte samples, a positive correlation was found between IL-2 and IL-4 RT-PCR products and between IL-4 and IL-7 RT-PCR products ($r=0.62$, $p=0.03$ and $r=0.06$, $p=0.05$ respectively). Finally in control T-lymphocytes samples, a strong negative correlation was found between IL-2 and IL-4 RT-PCR products ($r=-0.72$, $p=0.01$) (Figures 4.15-4.17).

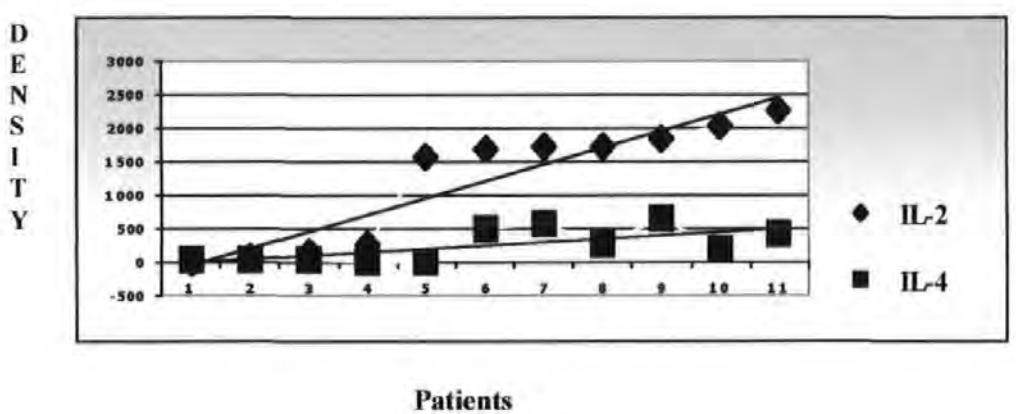
Figure 4.15: Correlation between IL-2 and IL-4 RT-PCR product density in patient B- and T-lymphocytes

A) B-lymphocytes



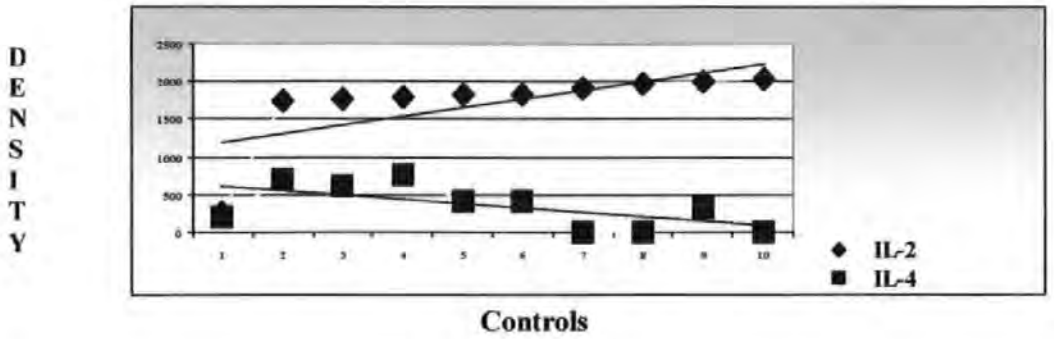
Spearman's Correlation was used to measure the correlation between IL-2 and IL-4 RT-PCR product density in patient B-lymphocytes. A strong positive correlation was found (0.74, p=0.008).

B) T-lymphocytes



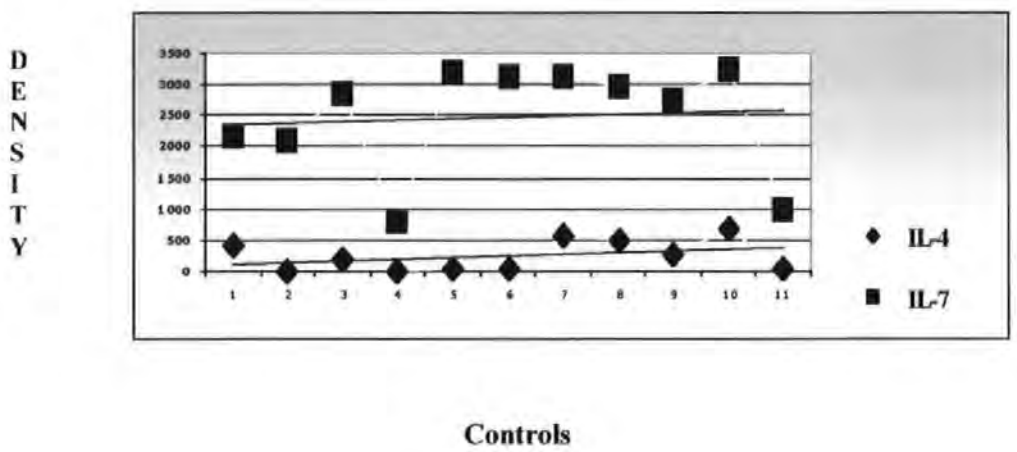
Spearman's Rank Correlation was used to measure the correlation between IL-2 and IL-4 mRNA transcript density in patient T-lymphocytes. A positive correlation was found (r=0.62, p=0.03).

Figure 4.16: Correlation between IL-2 and IL-4 RT-PCR product density in control T-lymphocytes



Spearman's Rank Correlation was used to measure the correlation between IL-2 and IL-4 RT-PCR product density in control T-lymphocytes. A negative correlation was found (r= -0.72, p=0.01).

Figure 4.17: Correlation between IL-4 and IL-7 RT-PCR product density in patient T-lymphocytes



Spearman's Rank Correlation was used to measure the correlation between IL-7 and IL-4 RT-PCR product density in patient T-lymphocytes. A positive correlation was found (r=0.06, p=0.05).

4.7. mRNA analysis of IL-2 wild type, IL-2 δ2, IL-4 wild type and IL-4δ2 RT-PCR products

For the detection of IL-2 RT-PCR products, the following primers were used: for the first round of amplification primers spanning exons 1 to 4; for the second round of amplification primers spanning exons 1 to 3. For the detection of IL-4 RT-PCR products, both sets of primers spanned exons 1 to 3.

There was a significant difference between the IL-2 wild type RT-PCR product density of patient and control B-lymphocytes (mean value: **172.4,** SD+/-73.2 [range: 21.4-249.6] and **53.0**, SD+/-35.3 [range: 0-109.6] respectively, p=0.001). There was no significant difference between the density of patient and normal T-lymphocytes IL-2 wild type RT-PCR products (mean value: **175.3,** SD+/-79.0 [range: 48.3-274.4] and **195.7,** SD+/-67.9 [range: 30.3-268.1] respectively). In addition, the ratio between patient B and T-lymphocyte mean density values of IL-2 wild type RT-PCR products was 1: 1 and in normal healthy controls 1: 3.7 (Appendix 9, Figure 4.18 and 4.19).

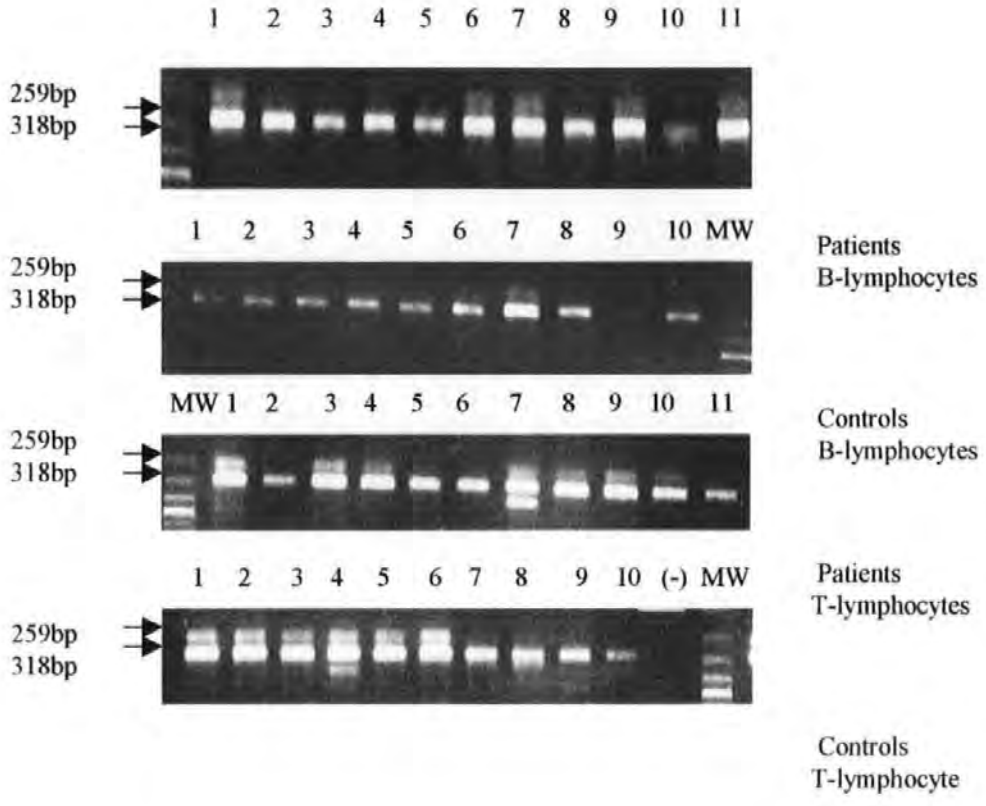
There was a significant difference between the IL-2 splice variant IL-2 delta 2 (IL-2 δ2) RT-PCR product density of patient and control B-lymphocytes (mean value: **13.1,** SD+/-16.7 [range: 0-56.6] and **0.53,** SD+/-1.6 [range: 0-5.3] respectively, p=0.03). There was no significant difference between the density of patient and normal T-lymphocyte IL-2 δ2 RT-PCR products (mean value: **38.8,** SD+/-41.1 [range: 0-138.2] and **80.6,** SD+/-61.7 [range: 0-172.4] respectively). The ratio between patient B and T-lymphocyte mean density values of IL-2 δ2 RT-PCR products was 1: 2 and in normal healthy controls 1: 152 (Appendix 9, Figure 4.18, Table 4.19).

There was a significant difference between the IL-4 wild type RT-PCR product density of patient and control B-lymphocytes (mean value: **27.0**, SD+/-4.1 [range: 18.7-29.9] and **21.2,** SD+/-1.9 [range: 19.0-22.9] respectively, p=0.03). There was no significant difference between the density of patient and normal T-lymphocyte IL-4 wild type RT-PCR products

(mean value: **24.8,** SD+/-10.9 [range: 13.3-35.3] and **21.2,** SD+/-9.6 [range: 11.4-34.4] respectively, p=ns). The ratio between patient B and T-lymphocyte mean density values of IL-4 wild type RT-PCR products was 1: 0.9 and in normal healthy controls 1:1 (Appendix 10, Figure 4.20 and 4.21).

No significant difference between the IL-4 splice variant IL-4 delta 2 (IL-4 δ2) RT-PCR product density of patient and control B-lymphocytes was demonstrated (mean value: **11.4,** SD+/-1.9 [range: 9.0-14.1] and **11.8,** SD+/-2.0 [range: 9.5-14.0] respectively, p=ns). There was a significant difference between the density of patient and normal T-lymphocyte IL-4 δ2 RT-PCR products (mean value: **2.1,** SD+/-0.7 [range: 1.0-3.1] and **15.8,** SD+/-7.6 [range: 7.1-24.3] respectively, p=0.001). The ratio between patient B and T-lymphocyte mean density values of IL-4 δ2 RT-PCR products was 1: 7.6 and in normal healthy controls 1: 1.3 (Appendix 10, Figure 4.20, Table 4.21).

Figure 4.18: IL-2 wild type and IL-2 delta 2 (IL-2δ2) RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify IL-2 wild type and IL-2δ2 splice variant products in B- and T-lymphocytes of patients with B-CLL and normal healthy controls. The figure shows RT-PCR products after a second round of amplification. A 100bp molecular weight ladder was used.
MW-molecular weight

Figure 4.19: Mean density values of IL-2 wild type and IL-2δ2 nested RT-PCR products

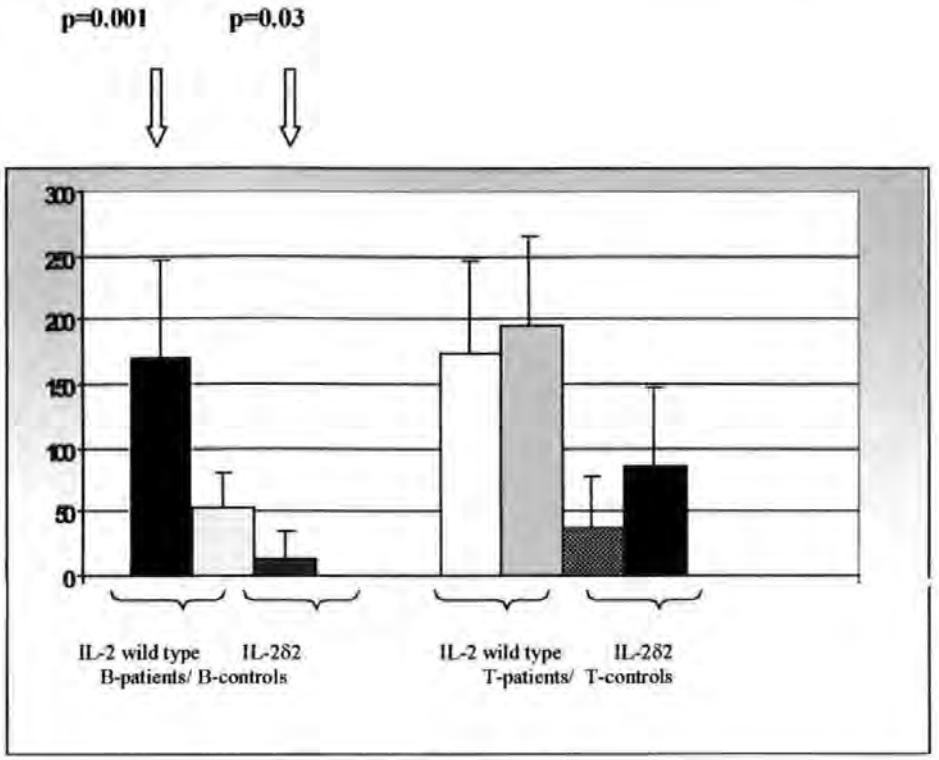
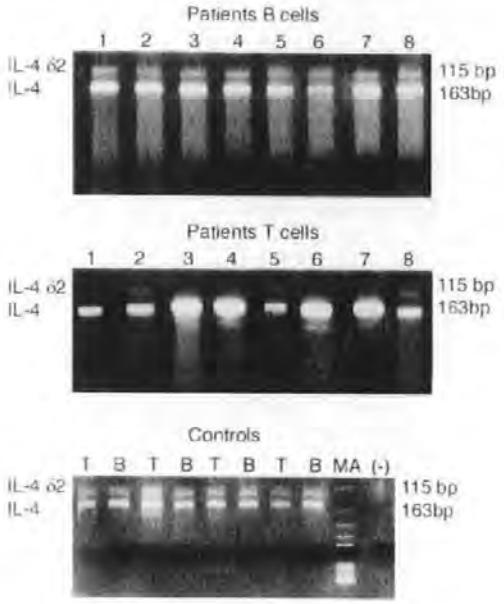


Figure 4.19 shows the densitometry results of the IL-2 and IL-2 δ2 RT-PCR products presented in Appendix 9. The Students t test was used to measure statistical significance. A significant difference between IL-2 wild type RT-PCR product density of patient and control B-lymphocytes was found (mean value: 172.4 [range: 21.4-249.6] and 53.0 [range: 0-109.6] respectively, p=0.001). No significant difference between the density of patient and normal T-lymphocyte IL-2 wild type RT-PCR products was found (mean value: 175.3 [range: 48.3-274.4] and 195.7 [range: 30.3-268.1] respectively, p=ns). In addition, the ratio between patient B and T-lymphocyte mean density values of IL-2 wild type RT-PCR products was 1: 1 and in normal healthy controls 1: 3.7.

There was a significant difference between the IL-2 splice variant IL-2 δ2 RT-PCR product density of patient and control B-lymphocytes (mean value: 13.1 [range: 0-56.6] and 0.53 [range: 0-5.3] respectively, p=0.03). There was no significant difference between the density of patient and normal T-lymphocyte IL-2 δ2 RT-PCR products (mean value: 38.8 [range: 0-138.2] and 80.6 [range: 0-172.4] respectively, p=ns). In addition, the ratio between the mean density values of patient B-lymphocyte IL-2 δ2 RT-PCR products was 1: 2 and in normal healthy controls 1: 152.

SD=Standard deviation, ns=Not significant

Figure 4.20: IL-4 wild type and IL-4 delta 2 (IL-4 δ2) RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify IL-4 and IL-4 δ2 products in B-and T-lymphocytes in patients with B-CLL and in normal controls. The figure shows RT-PCR products after a second round of amplification. A 100 bp molecular weight marker was used.
MW=molecular weight marker
(-)=negative control

Figure 4.21: Mean density values of IL-4 wild type and IL-4δ2 nested RT-PCR products

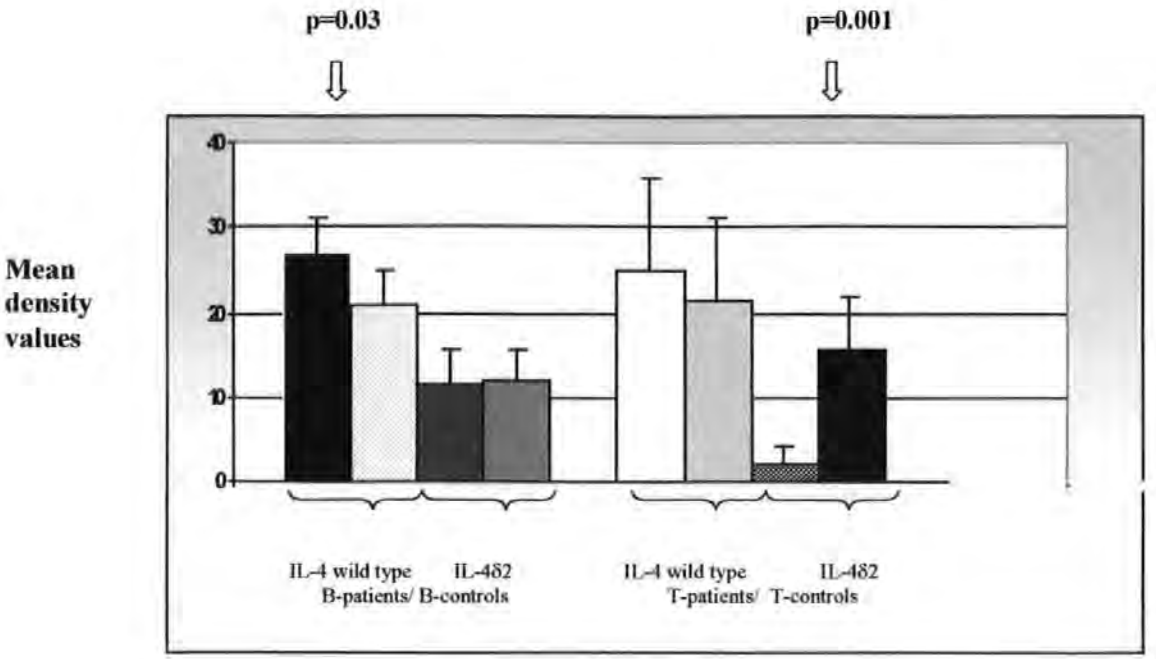


Figure 4.21 shows mean values of the densitometry results of the IL-4 and IL-4 δ2 RT-PCR products presented in Appendix 10. The Students t test was used to measure statistical significance. There was a significant difference between the IL-4 wild type RT-PCR product density of patient and control B-lymphocytes (mean value: 27.0 [range: 18.7-29.9] and 21.2 [range: 19.0-22.9] respectively, p=0.03). No significant difference was found between the density of patient and normal T-lymphocyte IL-4 wild type RT-PCR products (mean value: 24.8 [range: 13.3-35.3] and 21.2 [range: 11.4-34.4] respectively, p=ns). The ratio between patient B and T-lymphocyte mean density values of IL-4 wild type RT-PCR products was 1: 0.9 and in normal healthy controls 1: 1.

No significant difference between IL-4 splice variant IL-4 δ2 RT-PCR product density of patient and control B-lymphocytes was found (mean value: 11.4 [range: 9.0-14.1] and 11.8 [range: 9.5-14.0] respectively, p=ns). However, a significant difference between the density of patient and normal T-lymphocyte IL-4 δ2 RT-PCR products was demonstrated (mean value: 2.1 [range: 1.0-3.1] and 15.8 [range: 7.1-24.3] respectively, p=0.001). The ratio between patient B and T-lymphocyte mean density values of IL-4 δ2 RT-PCR products was 1: 7.6 and in normal healthy controls 1: 1.3.

SD=Standard deviation, ns=Not significant

4.8. Correlation between IL-2 wild type and IL-2 δ2 RT-PCR product densities

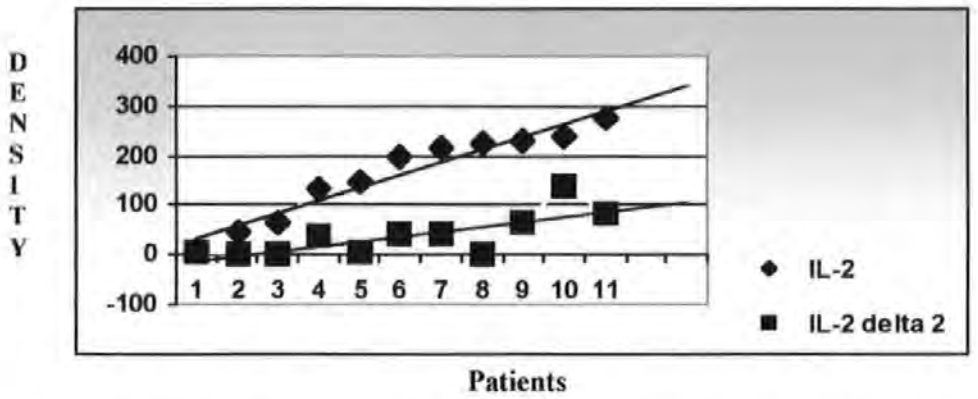
Using Spearman's Correlation, a significant positive correlation was found between IL-2 wild type bands and IL-2 δ2 splice variant bands in patient and control T-lymphocyte samples (r=0.76, p=0.006 and r=0.75, p=0.01 respectively). No significant correlation between IL-2 wild type bands and IL-2 δ2 splice variant bands in patient and control B-lymphocyte samples was found (Figure 4.22 A and B)

4.9. Correlation between IL-4 wild type and IL-4 δ2 RT-PCR product densities

Using Spearman's Rank Correlation, a significant positive correlation between IL-4 wild type bands and IL-4 δ2 spliced variant bands in patient B-lymphocyte samples was found (r=0.87, p=0.005*)* (Figure 4.23). No significant correlation was found between IL-4 wild type bands and IL-4 δ2 splice variant bands in patient and in control T-lymphocyte samples as well as in control B-lymphocyte samples.

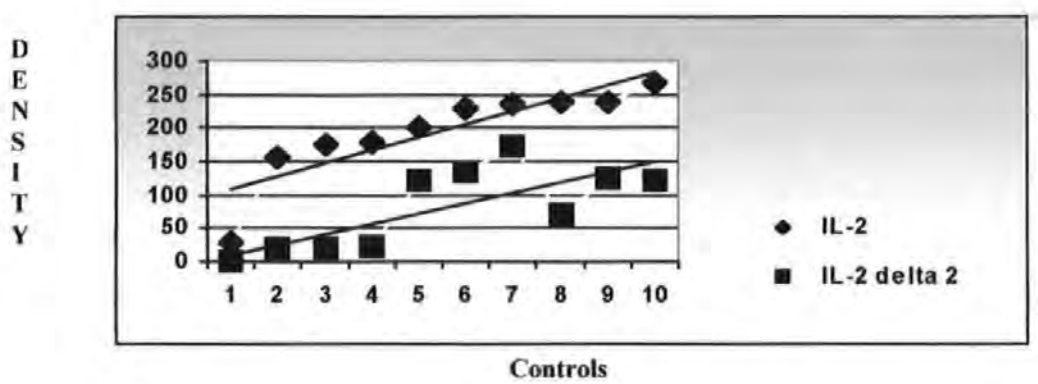
Figure 4.22: IL-2 wild type versus IL-2 δ2 RT-PCR product density in patient and control T-lymphocytes

A) Patient T-lymphocytes



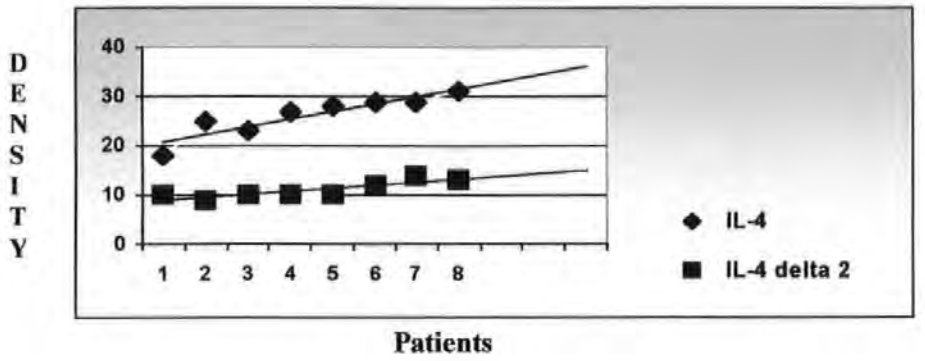
Spearman's Rank Correlation was used to measure the correlation between IL-2 and IL-δ2 RT-PCR product density in patient B-lymphocytes. A significant correlation was found (r=0.76, p=0.006).

B) Control T-lymphocytes



Spearman's Rank Correlation was used to identify the correlation between IL-2 and IL-2δ RT-PCR product density in patient B-lymphocytes. A significant correlation was found (r=0.75, p=0.01).

Figure 4.23: IL-4 wild versus IL-4 δ2 RT-PCR product density in patient B-lymphocytes



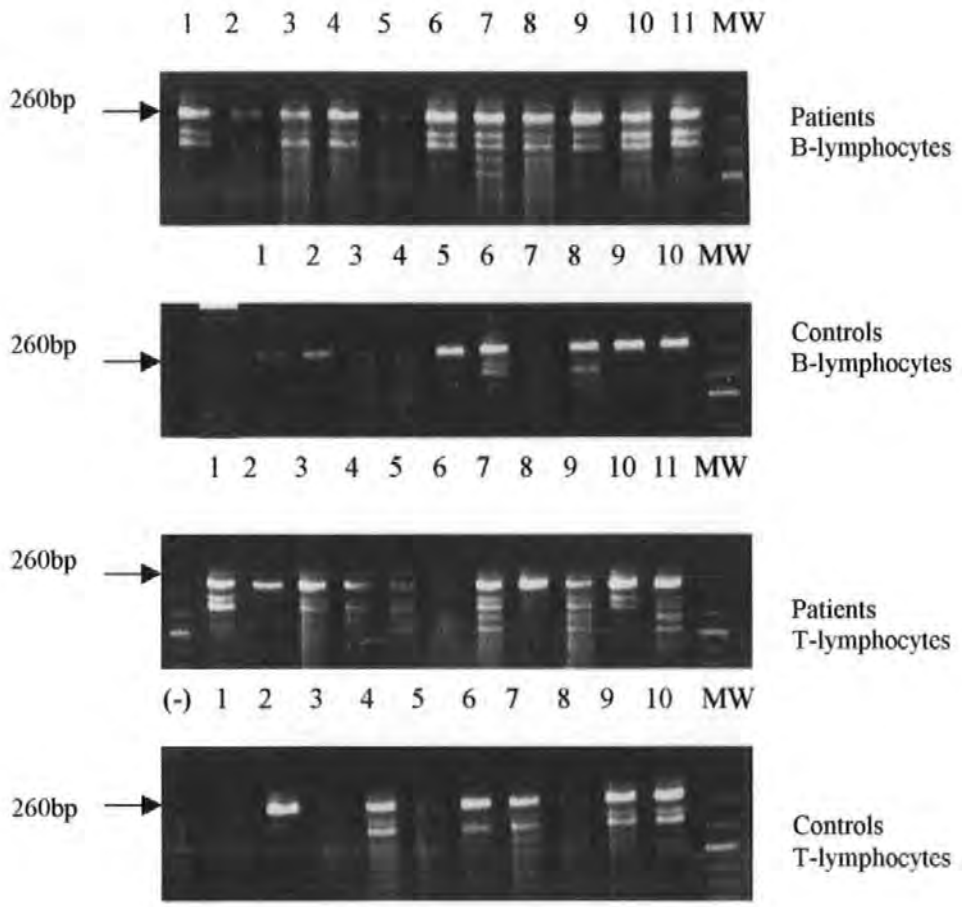
Spearman's Rank Correlation was used to measure the correlation between IL-4 and IL-4 δ2 RT-PCR product density in patient B-lymphocytes. A significant positive correlation was found ($r=0.87$, $p=0.005$).

4.10. mRNA analysis of IL-5 and IL-13 RT-PCR products

A nested RT-PCR technique was also used to identify IL-5 and IL-13 RT-PCR products in B and T-lymphocytes of patients with B-CLL and in normal controls. The reason for investigating IL-5 is that IL-5 is thought to have a strong apoptotic effect on malignant B-CLL cells. IL-13 on the other hand presents many similarities to IL-4. The IL-4R α chain is a common component of IL-4R and the IL-13 R. IL-13 is anti-apoptotic cytokine for malignant B-CLL cells. Throughout the study, the same cDNA samples were used and the primers used for detecting IL-5 and IL-13 were designed to cover the entire mRNA size. No significant difference between the density of patient and control B-lymphocytes IL-5 RT-PCR products was found (mean value: **788.0,** SD+/-373.8 [range: 16.7-1173] and **486.2,** SD+/-353.9 [range: 0-666.3] respectively, p=ns). There was no significant difference between the density of patient and normal T-lymphocyte IL-5 RT-PCR products (mean value: **704.4,** SD+/-374.8 [range: 0-1076.7] and **537.8,** SD+/-465.0 [range: 0-974.5] respectively, p=ns). In addition, the ratio between patient B and T-lymphocyte mean density values of IL-5 RT-PCR products was 1: 0.9. The same ratio in normal healthy controls was 1: 1.1 (Appendix 11, Figure 4.24 and 4.25).

There was no significant difference between the density of patient and control B-lymphocyte IL-13 RT-PCR products (mean value: **328.2,** SD+/-130.6 [range: 39.4-536.2] and **349.5,** SD+/-166.0 [range: 34.8-541.3] respectively, p=ns). There was no significant difference between the density of patient and normal T-lymphocyte IL-13 RT-PCR products (mean value: **308.4,** SD+/-145.8 [range: 48.5-455.7] and **349.0,** SD+/-158.6 [range: 0-510.8] respectively, p=ns). In addition, the ratio between patient B and T-lymphocyte mean density values of IL-13 RT-PCR products was 1: 0.9. The same ratio in normal healthy controls was 1: 1 (Appendix 12, Figure 4.26 and 4.27). Automated sequencing (MWG, Germany) was used to identify the RT-PCR products (Appendices (29-30).

Figure 4.22: IL-5 RT-PCR products after a second round of amplification



A nested RT-PCR technique was used to identify IL-5 products in B- and T-lymphocytes of patients with B-CLL and normal controls. The figure represents RT-PCR products after a second round of amplification. A 100 bp molecular weight ladder was used.
M=-molecular weight marker
(-)=negative control

Figure 4.25: Mean density values of IL-5 nested RT-PCR products

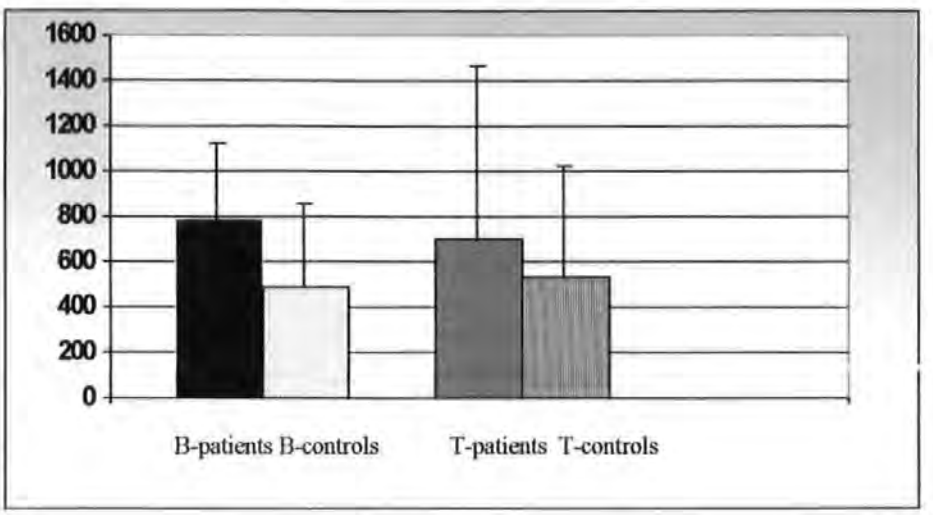
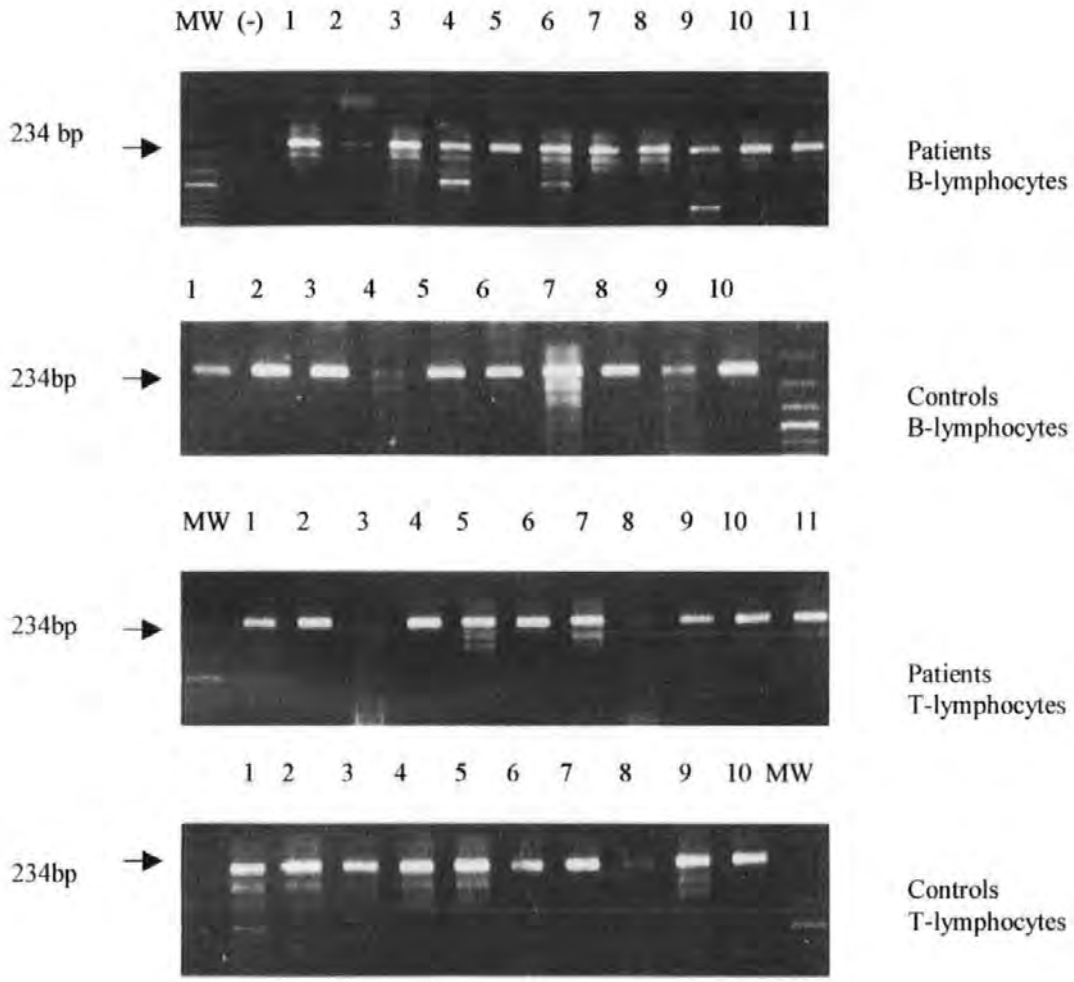


Figure 4.25 shows the densitometry results of the IL-5 RT-PCR products presented in Appendix 11. The Students t test was used to measure statistical significance. There was no significant difference between the density of patient and control B-lymphocyte IL-5 RT-PCR products (mean value: 788.0 [range: 16.7-1173] and 486.2 [range: 0-666.3] respectively, p=ns). There was no significant difference between the density of patient and control T-lymphocyte IL-5 RT-PCR products (mean value: 704.4 [range: 0-1076.7] and 537.8 [range: 0-974.5] respectively, p=ns). The ratio between patient B and T-lymphocyte mean density values of IL-5 RT-PCR products was 1: 0.9. The same ratio in normal healthy controls was 1: 1.1.
SD=Standard deviation, ns=Not significant

Figure 4.24: IL-13 nested RT-PCR products after a second round of amplification



A nested RT-PCT technique was used to identify IL-13 products in B- and T-lymphocytes in patients with B-CLL and in normal controls. The figure represents RT-PCR products after a second round of amplification. A 100 bp molecular weight marker was used.
MW=molecular weight marker
(-)=negative control

Figure 4.27: Mean density values of IL-13 nested RT-PCR products

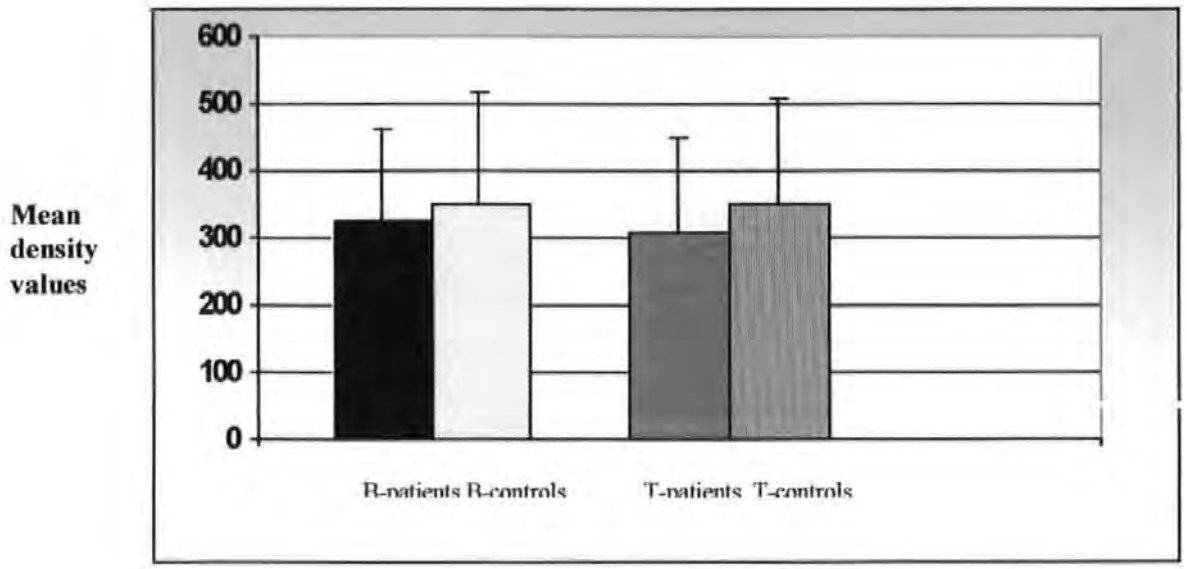


Figure 4.27 shows the densitometry results of the IL-13 RT-PCR products presented in Appendix 12. The Students t test was used to measure statistical significance. There was no significant difference between the density of patient and control B-lymphocyte IL-13 RT-PCR products (mean value: 328.2 [range: 39.4-536.2] and 349.5 [range: 34.8-541.3] respectively, p=ns). There was no significant difference between the density of patient and control T-lymphocyte IL-13 RT-PCR products (mean value: 308.4 [range: 48.5-455.7] and 349.0 [range: 0-510.8] respectively, p=ns). The ratio between patient B and T-lymphocyte mean density values of IL-13 RT-PCR products was 1: 0.9. The same ratio in normal healthy controls was ratio: 1: 1.

4.10. Correlation between IL-4 and IL-13 RT-PCR product densities

Since it is known that IL-4 and 13 use the IL-4Rα chain as part of their individual receptors, inter-regulation of protein expression may occur. In order to determine whether a correlation existed between the densities of RT-PCR products of the above cytokines, the data was analysed using the Spearman's Rank Correlation test.

No significant relationship was found between IL-4 and IL-13 RT-PCR product density in patient B-lymphocytes although there is a strong tendency for IL-13 RT-PCR products to be up-regulated at the same time as IL-4 RT-PCR products. Similarly no significant relationship was found between IL-4 and IL-13 RT-PCR products in control B-lymphocytes.

The correlation between IL-4 and IL-13 RT-PCR product density was not significant in patient and control T-lymphocytes.

4.12. Summary- nested RT-PCR

In this part of the project, RT-PCR products of cytokines belonging to the IL-2 receptor γc chain family as well as γc chain and IL-4R α chain RT-PCR products were investigated in purified unstimulated B- and T-lymphocytes of patients with B-CLL and in normal controls (Table 5.2). In addition, IL-2 and IL-4 spliced variant (IL-2 δ2 and IL-4 δ2 respectively) RT-PCR products were also investigated. All tests were carried out on the same cDNA preparation. A nested RT-PCR technique was applied to all the samples.

- The most striking results were significantly higher IL-2 and IL-4 wild type RT-PCR product densities in malignant B-lymphocytes compared to control B-lymphocytes samples.
- IL-2 δ2 and IL-4 δ2 RT-PCR products were detected. Patient B-lymphocytes had significantly higher IL-2 δ2 product densities than control B-lymphocytes. Control T-lymphocytes had significantly higher IL-4 δ2 RT-PCR product densities compared to patient T lymphocytes.
- IL-7 transcript densities were significantly higher in both B- and T-lymphocytes from patients with B-CLL compared to controls.
- IL-15 RT-PCR products were also identified in all tested samples although B-and T-lymphocytes are not considered to be IL-15 producers. In addition, control T-lymphocyte samples had significantly higher IL-15 RT-PCR product densities when compared to patients T-lymphocytes.
- No differences were found for γc RT-PCR products in patient and control T lymphocytes and in patient and control B-lymphocytes.
- A significant positive correlation was found between IL-2 and IL-4 RT-PCR product mean density values in patient B- and T-lymphocytes and control T-lymphocytes
- In addition, IL-5 and IL-13 RT-PCR products were also detected. However there was no significant difference between the corresponding samples.

- Automated sequencing (MWG, Germany) identified the RT-PCR products.

Table 4.2: Summary of the abundance of cytokines in B-CLL and normal lymphocytes

Characteristics	B-CLL B lymphocyte	Normal B-lymphocyte	B-CLL T-lymphocyte	Normal T-lymphocyte
t-test used to compared B-CLL B cell/normal B-cell and B-CLL T-cell/normal T-cell values				
Intracellular IL-4	++++ *	++	++ *	+
Surface IL-4R α	++++	++++	++ *	+
IL-4 protein(WB)	+	Not done	++++	++++
IL-4Rα mRNA	++++	++++	++++	++++
γc mRNA	++++	++++	++++	++++
IL-2 mRNA	+++ *	+	++++	++++
IL-4 mRNA	+++ *	+	++++	++++
IL-7 mRNA	+++ *	++	++++ *	+++
IL-15 mRNA	+++	+++	++	++++ *
IL-2 δ2 mRNA	++ *	+	+++	++++
IL-4 δ2 mRNA	++	++	+	++++ *
IL-13 mRNA	++++	++++	++++	++++
Spearmans' correlation test performed within each lymphocyte subset				
IL-2 mRNA/ IL-2 δ2 mRNA	ns	ns	Positive correlation	Positive correlation
IL-4 mRNA/ IL-4 δ2 mRNA	Positive correlation	ns	ns	ns
IL-2 mRNA /IL-4 mRNA	Positive correlation	Positive correlation	ns	Negative correlation
IL-4 mRNA /IL-7 mRNA	ns	ns	Positive correlation	ns
IL-4 mRNA /IL-13 mRNA	ns	ns	ns	ns

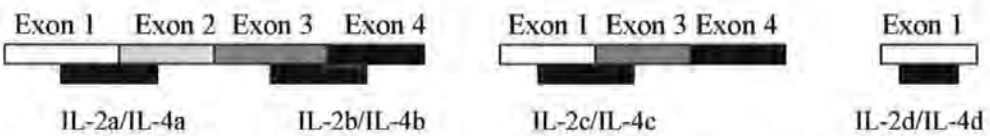
This table indicates the abundance of RT-PCR cytokine products and the percentage of cells expressing IL-4 or IL-4R, ++++= the most aboundant, += the least aboundant, Ns=Not significant, WB=Western blotting, *=significant

CHAPTER 5: RESULTS OF ANTISENSE STUDY

5.1. Introduction

In this part of the study, the influence of antisense oligonucleotides (ONs) on the proliferation and apoptosis of B-CLL and normal healthy control cells was tested. Previous studies have shown that IL-2 and IL-4 have an anti-apoptotic effect on malignant B-lymphocytes (Mainou-Fowler, 1996). This study also provided evidence for increased intracellular expression of IL-4 in malignant B-lymphocytes as well as increased mRNA expression of IL-2 and IL-4 cytokines in these cells. In view of this, MTT{3-(4,5.dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide)} and antisense staining techniques were used to measure the effect of the addition of antisense ONs to B-CLL and normal cell cultures.

The antisense oligonucleotides were designed as follows:



Antisense	Blocking effect
IL-2a or IL-4a	IL-2 or IL-4 wild type and δ3
IL-2b or IL-4b	IL-2δ3 or IL-4δ3
IL-2c or IL-4c	IL-2δ2 or IL-4δ2
IL-2d or IL-4d	IL-2 or IL-4 wild type and δ2 and δ3 spliced variants

Control antisense ON was designed as a reverse reading of IL-4d ON.

5.2. Patients and controls

Details concerning normal healthy controls are listed in table 3.2 and clinical and immunophenotypic features of the patients are listed in table 5.1.

Table 5.1: Clinical and immunophenotypic features of patients used in the antisense study

Patient number	Gender	Age (Years)	% CD5+ve	% CD19 +ve	Absolute lymphocyte count (x 10^9/l)	Stage (Binet/Rai)
22	M	62	95.0	86.0	12.7	A(0)
23	M	68	68.5	75.9	41.1	A(0)
24	M	86	80.3	91.2	62.5	A(0)
25	M	72	99.0	96.0	71.6	A(0)
26	M	82	69.0	79.0	12.7	A(0)
27	M	75	87.5	73.1	38.4	A(0)
28	F	72	50.0	69.9	14.8	A(0)
29	F	80	84.0	29.5	89.6	A(0)
30	M	79	82.0	84.0	84.3	A(0)
31	F	62	43.6	69.8	58.2	A(0)
32	F	78	69.5	89.6	20.4	A(0)
33	M	65	48..0	40.7	43.2	A(0)
34	F	82	66.0	56.0	15.9	A(0)

The percentage of CD5+ve and CD19+ve lymphocytes and the absolute lymphocyte count were obtained from the diagnostic laboratory. The staging system used at the time of the diagnosis is that described by Binet and Rai. The list represents details of all the patients used in the antisense study.
F=Female, M=Male, %=Percentage

5.3. Testing for antisense oligonucleotide (ON) penetrance into PBMCs

Rodamine-red antisense IL-2a ON was tested for its ability to enter lymphocytes. For this purpose, PBMCs from patients with B-CLL resuspended to a concentration of $1x10^5$ and plated into a 96 well plate and rodamine-red conjugated antisense IL-2a ON was added at the following concentrations: 10 μM, 5 μM, 2.5 μM, 1.75μM, 1 μM, 0.5 μM. No carrier was used.

The cells were viewed under UV microscope after 30 min, 1 hour, 2 hours, 3 hours and 4 hours. After 2.5 hours of incubation, a discrete, punctuate staining was visible in the cytoplasm of the cells. The staining did not increase with time.

The experiment was repeated using PBMCs separated from patients with B-CLL and from normal healthy controls. Similarly after 2.5 hours of incubation, a distinctive punctuate staining was visible in the cytoplasm of the cells. Unfortunately, our laboratory does not have the facility to photograph stained cells.

5.4. MTT assay

The TACS™ MTT Assay kit was used to measure cell proliferation and viability of B-CLL cells and normal control PBMCs after incubation with various antisense ONs.

The MTT assay has been developed as an alternative system to conventional ^{3}H-thymidine uptake for measuring cell proliferation.

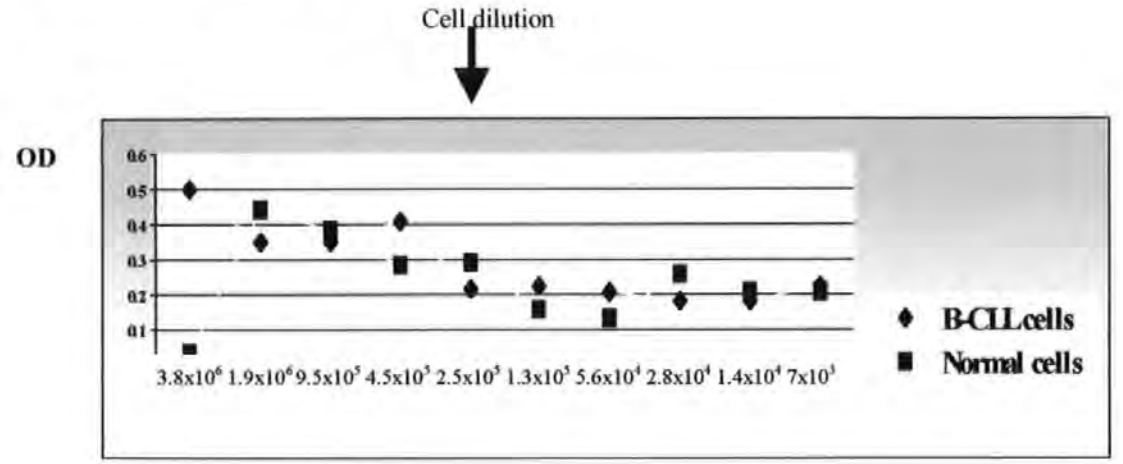
5.4.1. Optimisation of cell concentration

Different concentrations of PBMCs separated from B-CLL patients and normal controls were used to establish the optimal concentration of cells to be used in the MTT assay. Double diluted PBMCs (from $3.8x10^6$ to $7x10^3$) were used. All experiments were performed in triplicate. Results are given as the mean optical density (OD) value of each experiment. The optimal concentration of PBMCs for use in the MTT assay was calculated on the basis of OD

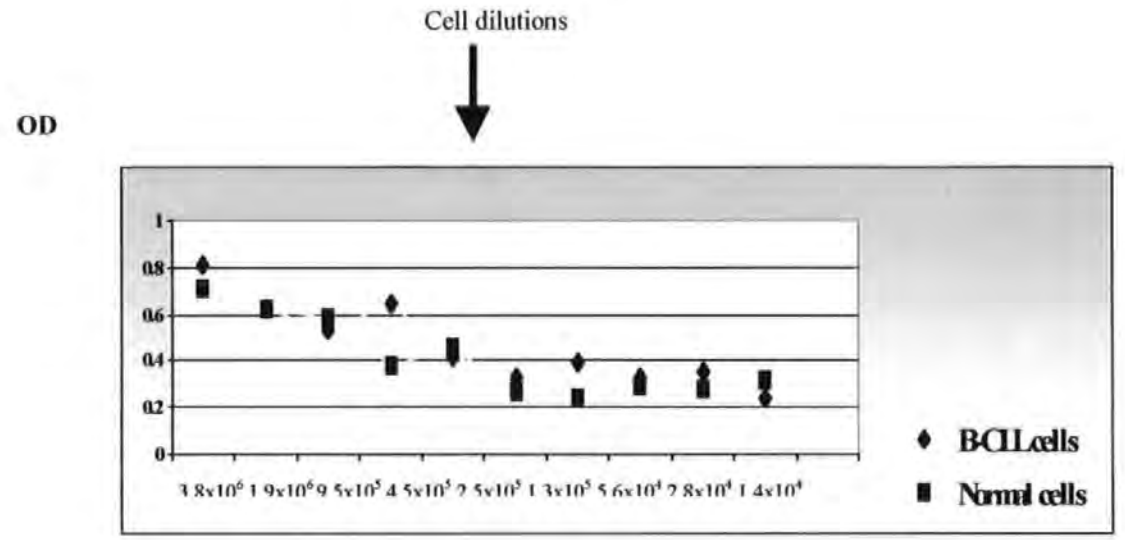
distribution. The OD decreased between the cell concentrations $3.8x10^6$ and $2.5x\ 10^5$. At lower cell concentrations (between $1.25x10^5$ to $7x10^3$) very similar readings were obtained. Thus, the concentration of PBMCs at $2.5x10^5$ was chosen as the optimal concentration to be used in further MTT studies (Figure 5.1 A and 5.1 B).

Figure 5.1: Optimisation of the concentration of PBMCs separated from patients with B-CLL and from normal controls for use in the MTT assay

A) Unstimulated PBMCs



B) PHA-stimulated PBMCs



The graph shows OD results of the MTT assay. PHA-stimulated PBMCs from one normal control and from one patient with B-CLL were double diluted from $3.8x10^6$/ml to $7x10^3$/ml and incubated for 24 hours in a 96 well plate. Optical density measurements show that $2.5x10^5$/ml is the optimal concentration of PHA-stimulated cells to be used for further studies.

5.4.2. Optimisation of antisense ONs concentrations for the MTT assay

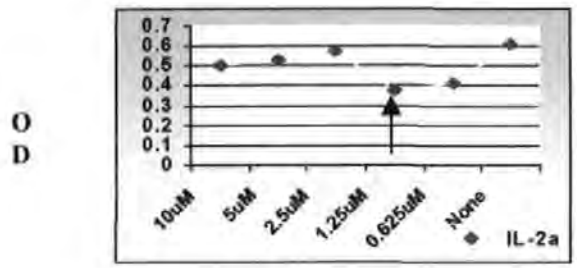
The effect of different concentrations of antisense oligonucleotides (ONs) on the viability and proliferation of PBMCs from patients 22 and 23 with B-CLL and two normal controls was examined. IL-2a, IL-2b, IL-2c IL-2d as well as IL-4a, IL-4b, IL-4c, IL-4d and control antisense were used in this study. The control antisense ON was designed as a reverse reading of IL-4d antisense ON. The following concentrations of antisense oligonucleotides were used: 10μM, 5μM, 2.5μM, 1.25μM, 0.625μM. All experiments were performed in triplicate. The ONs were added to the cells twice (one hour before the addition of PHA and 1 hour after). The results are given as the mean OD value of each experiment performed in triplicate.

MTT assay mean OD values were lowest when antisense ONs IL-2a, IL-2b, IL-2c were added at a concentration of 1.25μM. The strongest suppression of proliferation in normal PHA-stimulated cells after the addition of IL-2d occurred at a concentration of 5μM. PTO-modified ON at a concentration of 1.25 μM were therefore considered the strongest suppressors of proliferation of normal PHA-stimulated PBMCs (Figure 6.2 A, B, C, D and E). Similarly IL-4a, IL-4b, IL-4c, IL-4d ONs at a concentration of 1.25 μM were the strongest suppressors of cell viability of normal PHA-stimulated PBMCs (Figure 5.3 A, B, C, D and E).

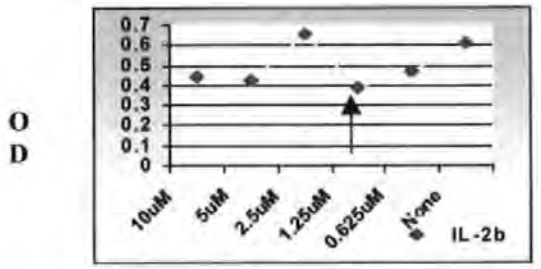
PTO-modified IL-2a, IL-2b, IL-2c, IL-2d ONs at a concentration of 1.25 μM were the strongest suppressors of proliferation of PHA-stimulated B-CLL PBMCs (Figure 5.4 A, B, C, D and E). Finally, PTO-modified IL-4a and IL-4b at a concentration of 1.25 μM were the strongest suppressors of proliferation of PHA-stimulated B-CLL PBMCs (Figure 5.5 A, B, C, D and E). The optimal concentration of antisense ON chosen for further studies was 1.25μM.

Figure 5.2: Optimisation of PTO-modified IL-2 antisense ONs concentrations for the MTT assay, using PHA-stimulated PBMCs from normal controls

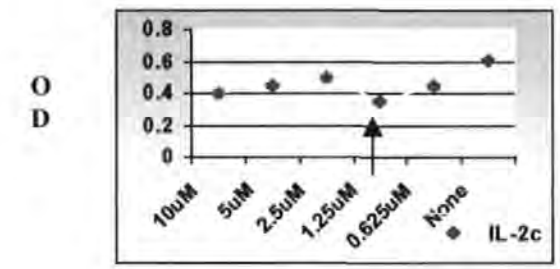
A) Incubation with IL-2a



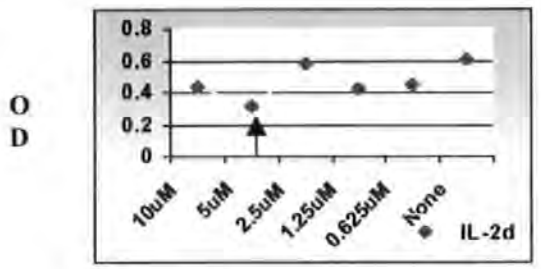
B) Incubation with IL-2b



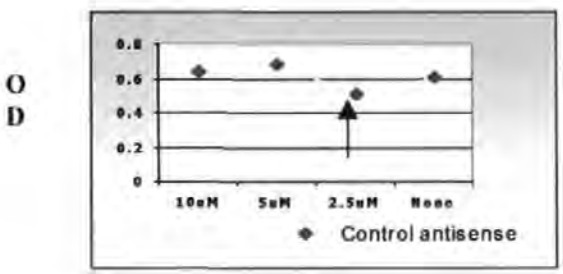
C) Incubation with IL-2c



D) Incubation with IL-2d



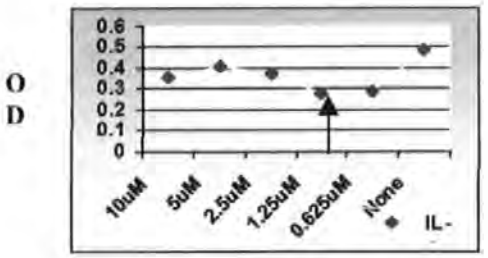
E) Incubation with control antisense ON



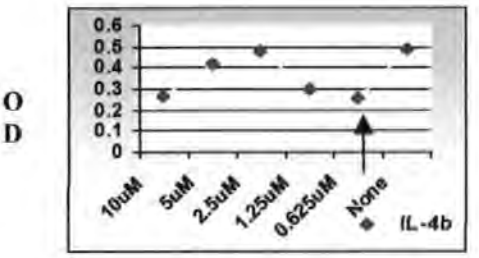
This figure represents MTT assay mean optical density (OD) values of each experiment performed in triplicate. PBMCs from two normal controls were incubated with various PTO-modified IL-2 antisense oligonucleotides added at different concentrations to the cell culture (Range: 10 μM-0.625 μM). PBMCs were stimulated with PHA at a concentration of 1μg/ml and incubated in 96 well plates for 24 hours at 37°C. ONs were added at the beginning of the incubation, 1 hour before PHA-stimulation and 1 hour after PHA addition. IL-2a, IL-2b, IL-2c ONs at a concentration of 1.25 μM were the strongest suppressors of proliferation of normal PHA-stimulated PBMCs (Figure A-C. The strongest suppression of proliferation was after addition of IL-2d antisense ON at a concentration of 5μM. The optimal concentration of antisense ONs chosen for further studies was 1.25μM. Arrows indicate the lowest OD values after addition of antisense ON. OD-Optical density

Figure 5.3: Optimisation of PTO-modified IL-4 antisense ON concentrations for the MTT assay, using PHA-stimulated PBMCs from normal controls

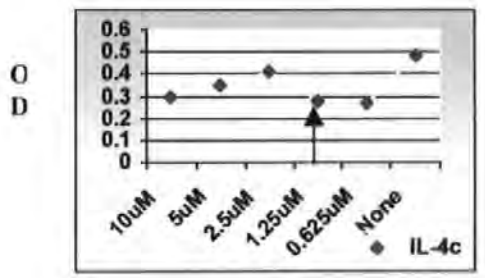
A) Incubation with IL-4a



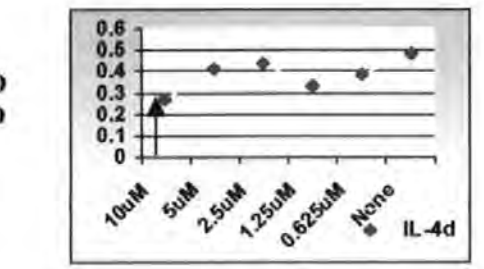
B) Incubation with IL-4b



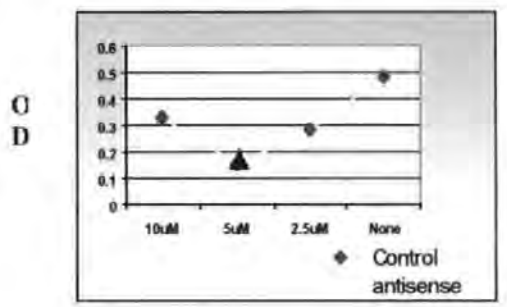
C) Incubation with IL-4c



D) Incubation with IL-4d



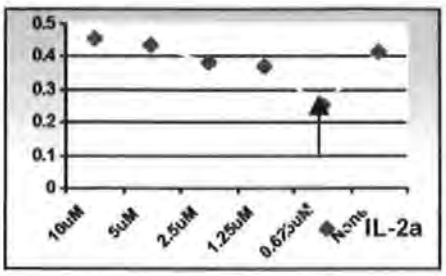
E) Incubation with control antisense ON



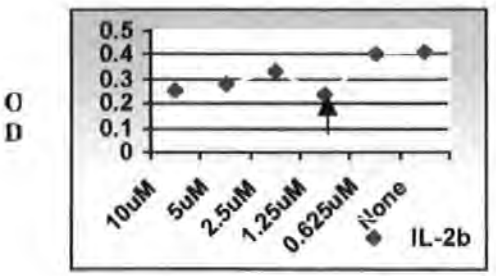
This figure represents MTT assay mean optical density (OD) values. All experiments were performed in triplicate. PBMCs from two normal controls were incubated with various PTO-modified IL-4 antisense oligonucleotides added at different concentrations to the culture (Range: 10μM-0.625μM). Cells were stimulated with PHA. ONs were added at the start of the incubation and 1 hour after PHA addition. IL-4a ON at a concentration of 1.25 μM was the strongest suppressor of proliferation of normal PHA-stimulated PBMCs (Figure A). IL-4b and IL-4c ONs at a concentration of 0.625 μM were the strongest suppressors of proliferation of normal PHA-stimulated PBMCs (Figures B and C). The strongest suppression of proliferation was after addition of IL-4d antisense ON at a concentration of 10μM. Arrows indicate the lowest OD values after addition of antisense ON. OD=Optical density

Figure 5.4: Optimisation of PTO-modified IL-2 antisense ON concentrations for the MTT assay, using PHA-stimulated PBMCs from patients with B-CLL

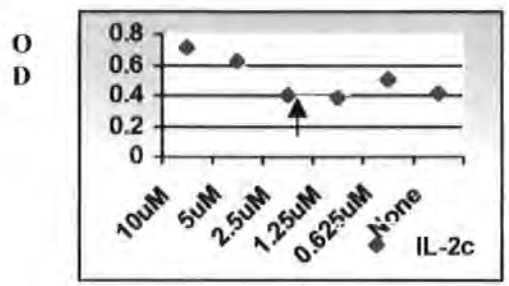
A) Incubation with IL-2a



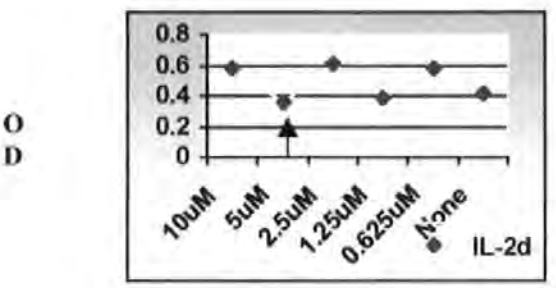
B) Incubation with IL-2b



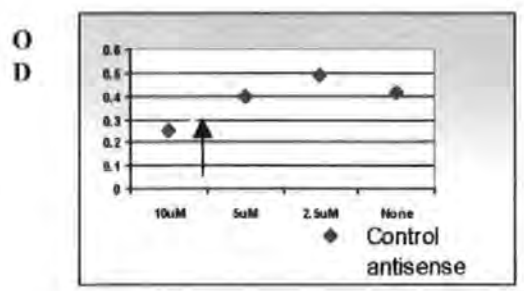
C) Incubation with IL-2c



D) Incubation with IL-2d



E) Incubation with control antisense ON

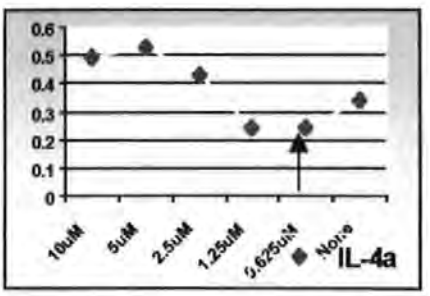


This figure represents MTT assay mean optical density (OD) values. All experiments were performed in triplicate. PBMCs from two patients with B-CLL were incubated with various PTO-modified IL-4 antisense ONs added at different concentrations to the culture (Range: 10μM-0.625 μM). Cells were stimulated with PHA, which was added one hour after the start on the incubation. Antisense ONs were added at the start of the incubation and 1 hour after the addition of PHA.

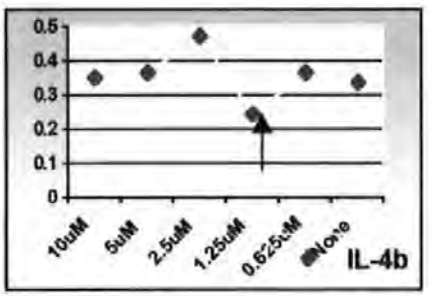
IL-2b and IL-2c antisense ONs at a concentration of 1.25 μM were the strongest suppressors of proliferation of PHA-stimulated PBMCs from B-CLL patients (Figure B and C). IL-2a antisense ON at a concentration of 0.625 μM was the strongest suppressor of proliferation of PHA-stimulated PBMCs (Figures A) and IL-2d at the concentration of 5μM was the strongest suppressor of proliferation (Figure D). Arrows indicate the lowest OD values after addition of antisense ON.

Figure 5.5: Optimisation of PTO-modified IL-4 antisense ON concentrations for the MTT assay, using PHA-stimulated PBMCs from patients with B-CLL

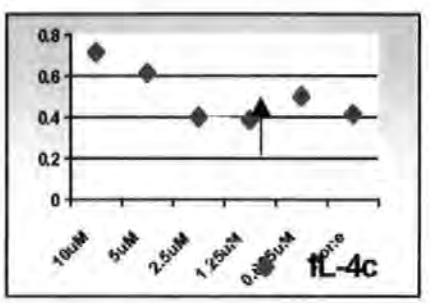
A) Incubation with IL-4a



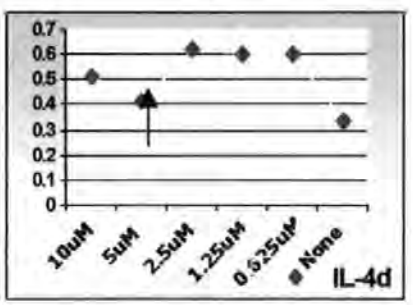
B) Incubation with IL-4b



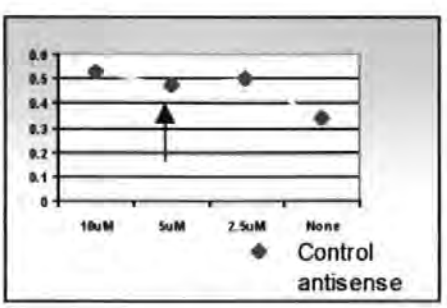
C) Incubation with IL-4c



D) Incubation with IL-4d



E) Incubation with control antisense ON



This figure represents MTT assay mean optical density (OD) values. PBMCs from two patients with B-CLL were incubated with various PTO-modified IL-4 antisense ONs at different concentrations (Range: 10 μM-0.625 μM). PHA was added one hour after the start of the incubation. IL-4 antisense ONs were added at the start of the incubation time and an hour after PHA stimulation.

IL-4a, IL-4b and IL-4c ONs at a concentration of 1.25 μM were the strongest suppressors of proliferation of PHA-stimulated PBMCs from B-CLL patients (Figures A, B and C). IL-4d ON at a concentration of 5 μM was the strongest suppressor of proliferation of PHA-stimulated PBMCs (Figure D). Arrows indicate the lowest OD values after addition of antisense ON. Arrows indicate the lowest OD values after addition of antisense ON. OD=Optical density

5.4.3. Effect of PTO-modified IL-2 and IL-4 antisense ONs on the viability of PBMCs from patients with B-CLL

In this experiment, PBMCs from patient Patient 22, Table 6.1) with B-CLL were isolated by density gradient centrifugation. All antisense ONs were added three times at a concentration of 1.25μM. The first dose of antisense ONs was added at the start of the incubation time, the second dose one hour after the addition of PHA and the third dose was added 24 hours later, just prior to the cells being used for the MTT assay.

Spectrophotometrical analysis revealed that the viability of PBMCs decreased after incubation for 48 hours with IL-2a, IL-2b, and IL-2c as well as in negative controls and increased at 72 hours of (Figure 5.6 A, B, C, D and E).

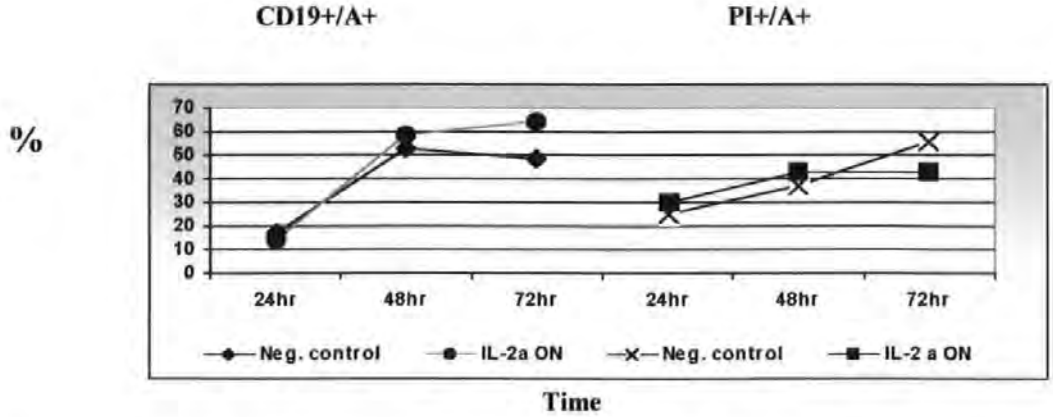
OD readings after 24 hours of incubation were higher in PHA-stimulated cells compared to unstimulated PBMCs. OD readings after 48, 72 and 96 hours of incubation did not differ very much between PHA-stimulated and unstimulated PBMCs incubated with IL-2 antisense ONs. In conclusion, addition of IL-2 antisense ONs to unstimulated and PHA-stimulated PBMCs from B-CLL patients partially decreased the proliferation of the cells when compared to negative controls as shown by a decrease in OD readings (Figure 5.6 A, B, C, D and E).

Similarly, incubation of unstimulated and PHA-stimulated PBMCs from B-CLL patient with IL-4a, IL-4b, IL-4c and IL-4d antisense ONs dramatically decreased the OD after incubation for 48 hours and increased the OD at 72 hours of incubation (Figure 5.7 A, B, C, D and E). In addition, OD readings after 24 and 48 hours of incubation were higher in PHA-stimulated cells compared to unstimulated cells. At 72 hours of incubation, OD readings of the non-stimulated cells incubated with IL-4 antisense ONs were slightly higher than those of PHA-stimulated cells.

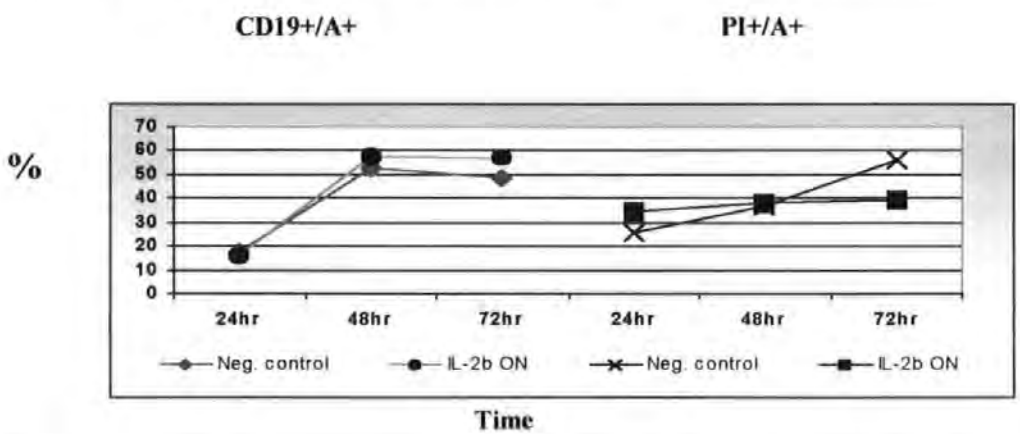
In summary, addition of PTO-modified IL-4a, IL-4b, IL-4c and IL-4d PTO-modified antisense ONs decreased proliferative responses in PBMCs from B-CLL patients compared to negative controls (Figure 5.7).

Figure 5.11: Flow cytometric analysis of the effect of different incubation times with PTO-modified IL-2 antisense ONs on apoptosis of CD19+ve lymphocytes from B-CLL patients

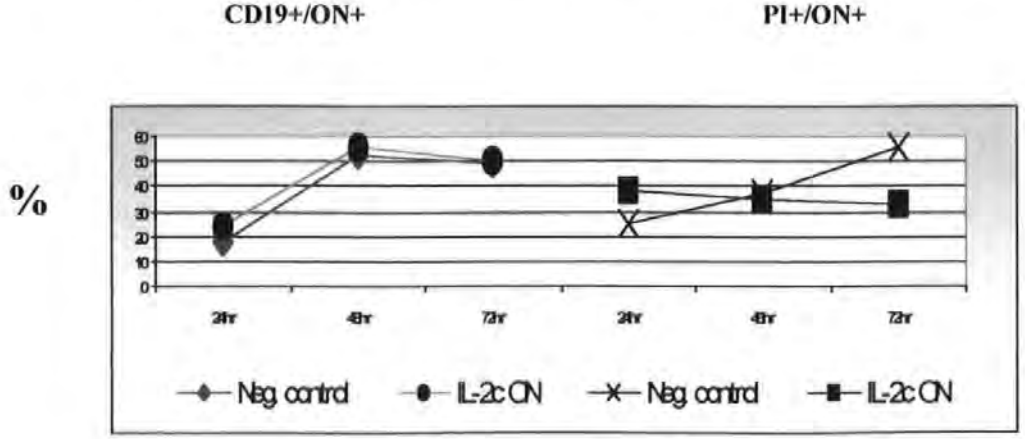
A) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-2a ON



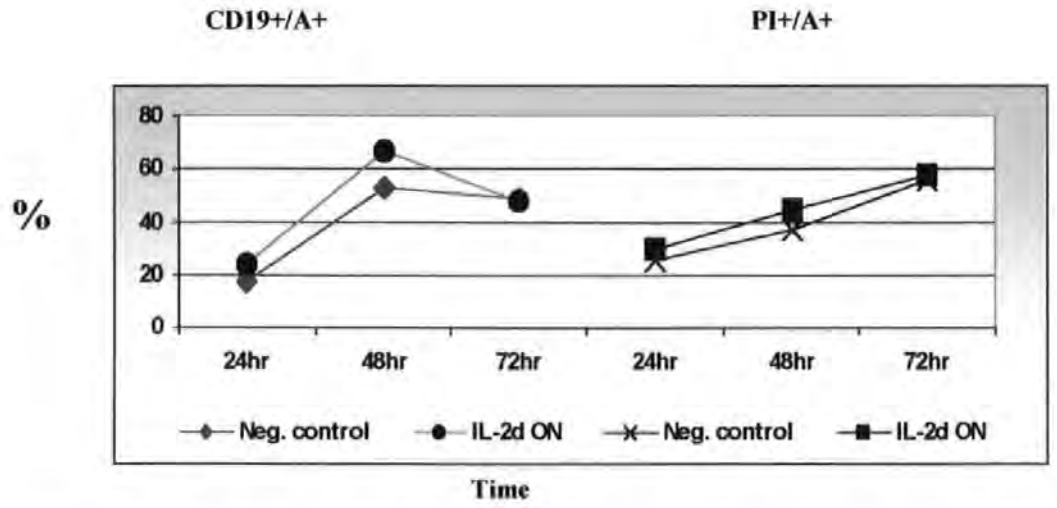
B) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-2b ON



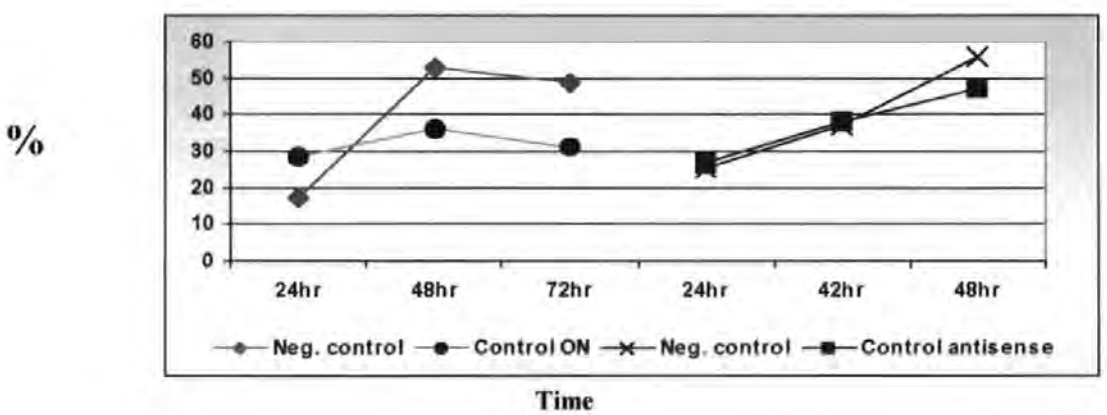
C) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-2c ON



D) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-2d ON



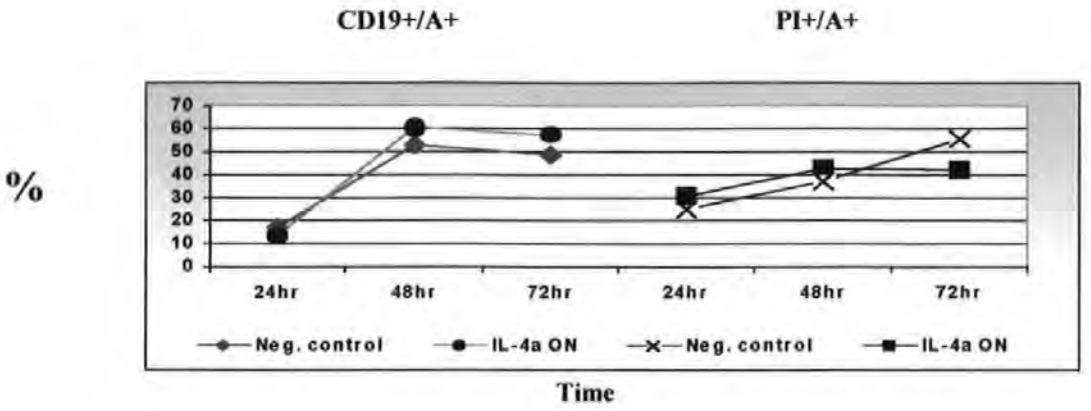
E) Percentage of CD19+/A+ and PI+/A+ cells after incubation with control antisense ON



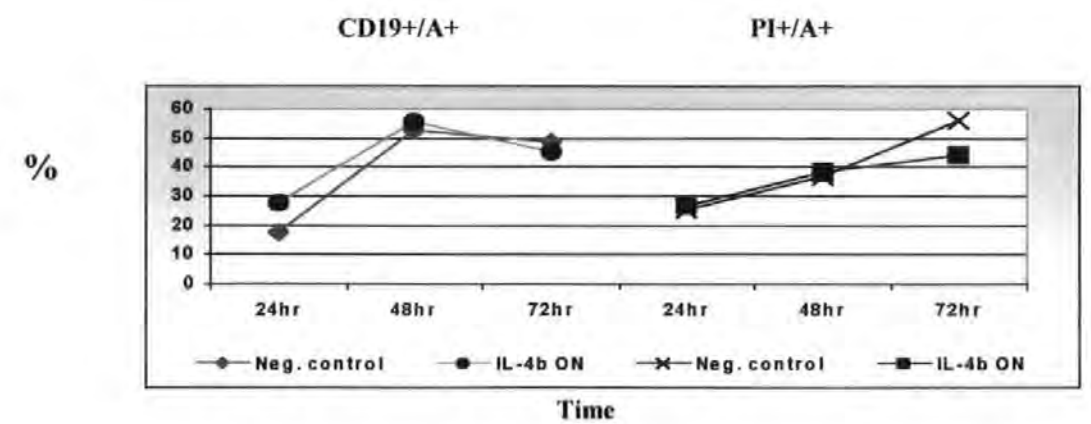
The figure shows the effect of different incubation times on the percentage of CD19+/A+ and PI+/A+ cells after addition of IL-2 antisense ONs. PBMCs were isolated by density gradient centrifugation from patients with B-CLL. The graph shows the percentage of double stained CD19+/A+ B-CLL lymphocytes incubated with IL-2 and IL-4 antisense ONs for 0, 24, 48, and 72 hours. The percentage of double positive CD19 PE/ Annexin V FITC increased with the incubation time and was highest after 48 hours. The percentage of PI+/A+ positive cells in the negative control also increased with time. However, after addition of IL-4a, b and c, the percentage of PI+/A+ cells decreased after 72 hours of incubation.
%=Percentage, A=Annexin V, ON=Oligonucleotide, PI=Propidium iodide

Figure 5.12: Flow cytometric analysis of the effect of different incubation times with PTO-modified IL-4 antisense ONs on apoptosis of CD19+ve lymphocytes from a B-CLL patient

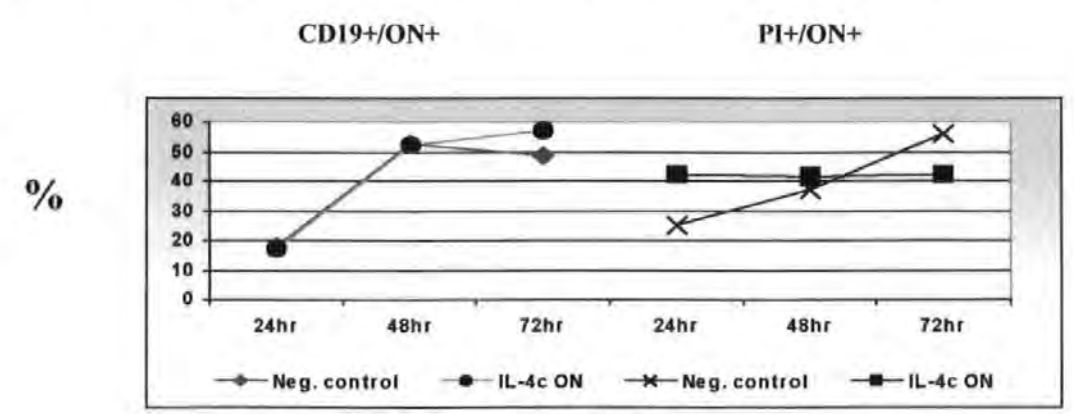
A) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-4a ON



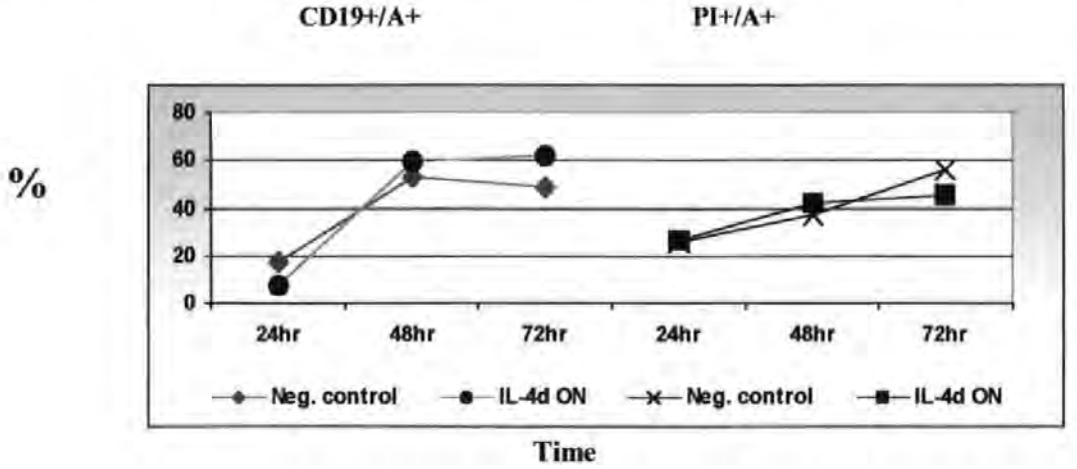
B) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-4b ON



C) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-4c ON



D) Percentage of CD19+/A+ and PI+/A+ cells after incubation with IL-4d ON



The figure shows the effect of different incubation times on the percentage of CD19 PE/ Annexin V FITC (CD19+/A+) and Propidium Iodine/Annexin V FITC (PI+/A+) cells after addition of IL-4 antisense ONs. PBMCs were isolated by density gradient centrifugation from one patient with B-CLL This graph shows the percentage of double stained CD19+/A+ lymphocytes incubated with IL-4 antisense oligonucleotides for 0, 24, 48, and 72 hours. The percentage of double positive CD19+/A+ lymphocytes increased with incubation time after incubation with IL-4c and IL-4d ONs (Figures C and D) The highest percentage of the CD19+/A+ lymphocytes was seen after 48 hours of incubation with the addition of IL-4a and b antisense ONs (Figures A and B). Thus, 48 hours was chosen to be the optimal incubation time to be used for further studies.
%=Percentage, A=Annexin V, ON=Oligonucleotide, PI=Propidium iodide, Neg. control=Negative control

5.6.2. Effect of the addition of PTO-modified antisense ONs on apoptosis of B-CLL cells

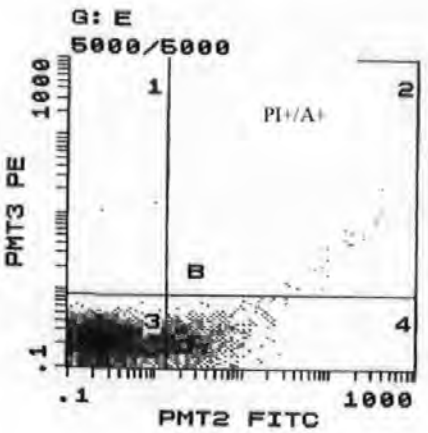
After determining the optimal concentrations of antisense oligonucleotides and optimal incubation times, the following series of experiments was performed. PBMCs isolated from five patients with B-CLL and from five normal controls were used. Cells were stained with anti-CD19 PE, double stained with anti-CD19 PE/Annexin V FITC (CD19+/A+) and double stained with Propidium Iodide/Annexin V FITC (PI+/A+) (Appendix 13 and 14). Staining with Annexin V is an indicator of cells undergoing early apoptosis. Staining with Annexin V and PI shows the percentage of the cells in the late apoptotic stage and staining with PI shows the percentage of dead cells (Figure 5.13).

The percentage of CD19+ve lymphocytes undergoing apoptosis was significantly higher when the PBMCs from B-CLL patients were incubated with the following antisense ONs: IL-4b ($p=0.03$), 2b ($p=0.04$), 2c ($p=0.02$) and 2d ($p=0.002$) (Table 5.2, Figure 5.14 and 5.15).

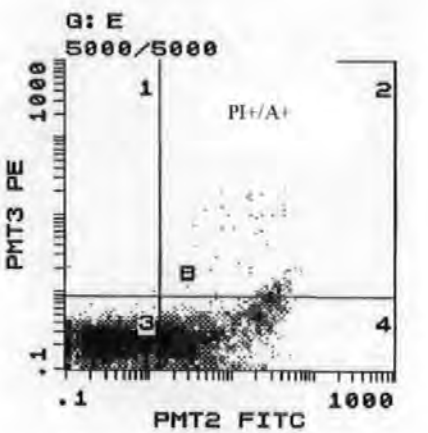
No significant difference was present between the percentage of double positive PI+/A+ cells from B-CLL patients incubated with IL-2 and IL-4 antisense ON and the negative control (Figure 5.16). However, it is clear that addition of different antisense ONs and control antisense increased the percentage of PI+/A+ cells.

Figure 5.13: Flow cytometric analysis of B-CLL cells stained with Annexin V FITC and anti-CD19 PE or Propidium iodide (PI)

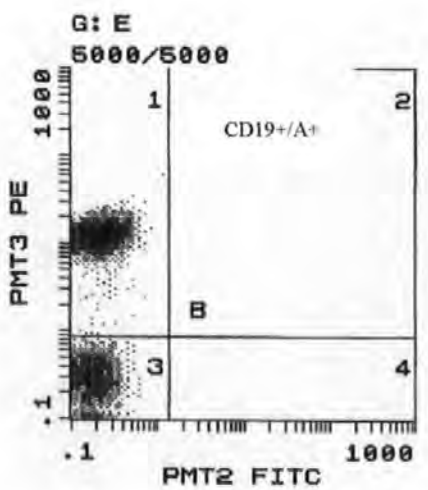
A) Propidium Iodide+/Annexin V+



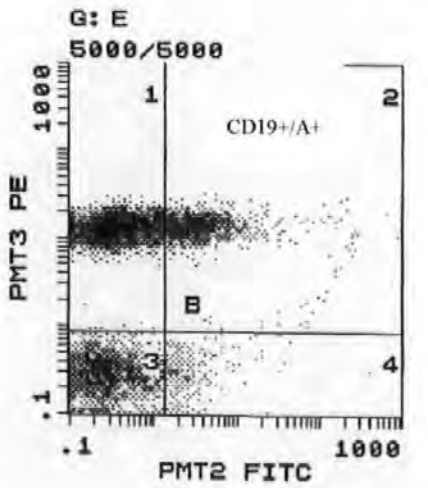
B) Propidium Iodide+/+Annexin V + IL-2a ON



C) CD19+/Annexin V+



D) CD19+/+Annexin V + IL-2a ON



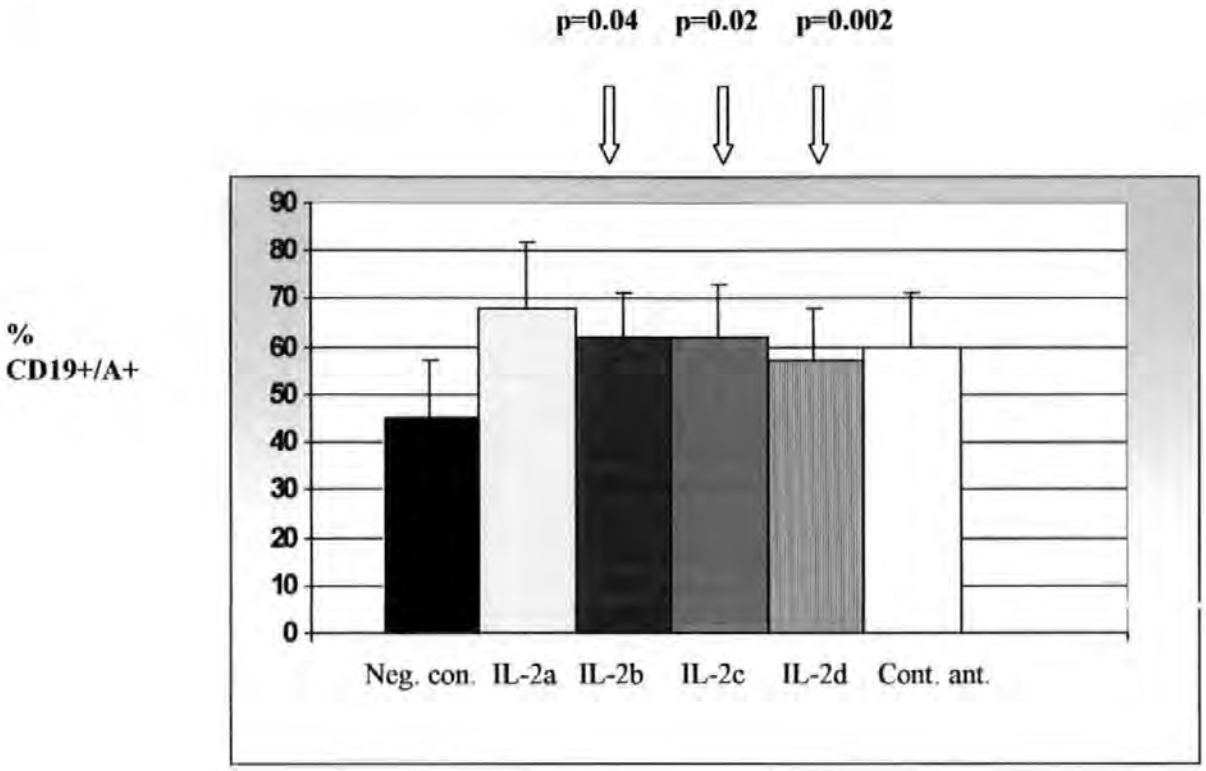
PBMCs from patients with B-CLL were stained with PI and Annexin V (A) FITC or anti-CD19 PE conjugated monoclonal antibody and Annexin V FITC. Figure B and C shows flow cytometric analysis of lymphocytes from patients with B-CLL after incubation with IL-2a antisense oligonucleotide.

Table 5.2: Statistical analysis of the data presented in Appendix 13 and 14

T test						
Variable: CD19+/A+ neg. cont. versus CD19+/A+ plus ON				**Variable: PI+/A+ neg. cont. versus PI+/A+ plus ON**		
Mean:	45.7	57.5	**IL-4a**	Mean:	29.3	31.9	**IL-4a**
SD+/-	11.5	13.6		SD+/-	13.2	15.3	
p=ns				p=ns			
Mean:	45.7	55.8	**IL-4b**	Mean:	29.3	36.5	**IL-4b**
SD+/-	11.6	7.2		SD+/-	13.2	8.7	
p=0.032				p=ns			
Mean:	45.7	56.1	**IL-4c**	Mean:	29.3	39.6	**IL-4c**
SD+/-	11.6	13.0		SD+/-	13.2	8.5	
p=ns				p=ns			
Mean: :	45.7	52.4	**IL-4d**	Mean:	29.3	36.2	**IL-4d**
SD+/-	11.6	4.4		SD+/-	13.2	11.7	
p=ns				p=ns			
Mean: :	45.7	68.3	**IL-2a**	Mean:	29.3	40.6	**IL-2a**
SD+/-	11.7	14.1		SD+/-	13.2	10.2	
p=ns				p=ns			
Mean: :	45.7	61.9	**IL-2b**	Mean:	29.3	41.9	**IL-2b**
SD+/-	11.7	10.9		SD+/-	13.2		
p= 0.036				p=ns			
Mean: :	45.7	61.2	**IL-2c**	Mean:	29.3	39.8	**IL-2c**
SD+/-	11.6	10.5		SD+/-	13.2	10.5	
p=0.021				p=ns			
Mean: :	45.7	56.8	**IL-2d**	Mean:	29.3	31.8	**IL-2d**
SD+/-	11.6	12.0		SD+/-	13.2	12.5	
p=0.002				p=ns			
Mean: :	45.6	60.0	**Cont.**	Mean:	29.3	42.2	**Cont.**
SD+/-	11.6			SD+/-	13.2	7.7	
p=ns				p=ns			

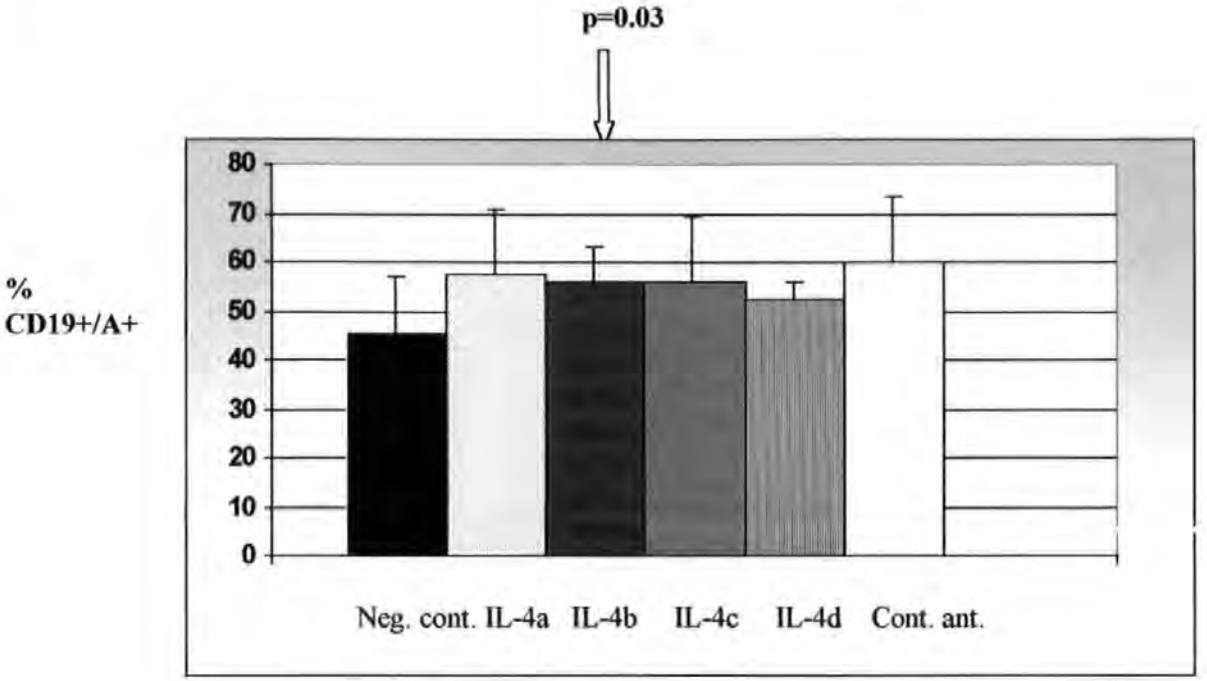
This table shows statistical analysis of the data presented in Appendix 13 and 14. The Student t test was used. Values equal or lower than p=0.05 were considered statistically significant. The percentage of CD19+/A+ lymphocytes was significantly higher when PBMCs from B-CLL patients were incubated with the following PTO-modified antisense ONs: IL-4b (p=0.04), 2b (p=0.04), 2c (p=0.02) and 2d (p=0.002).
No significant difference was present between the percentage of double positive PI+/A+ cells from B-CLL patients incubated with different PTO-modified IL-2 and IL-4 antisense ONs and the negative control.
ns= Not-significant, SD=Standard deviation

Figure 5.14: Flow cytometric analysis of the effect of PTO-modified IL-2 antisense ONs on unstimulated CD19+ve lymphocytes from patients with B-CLL



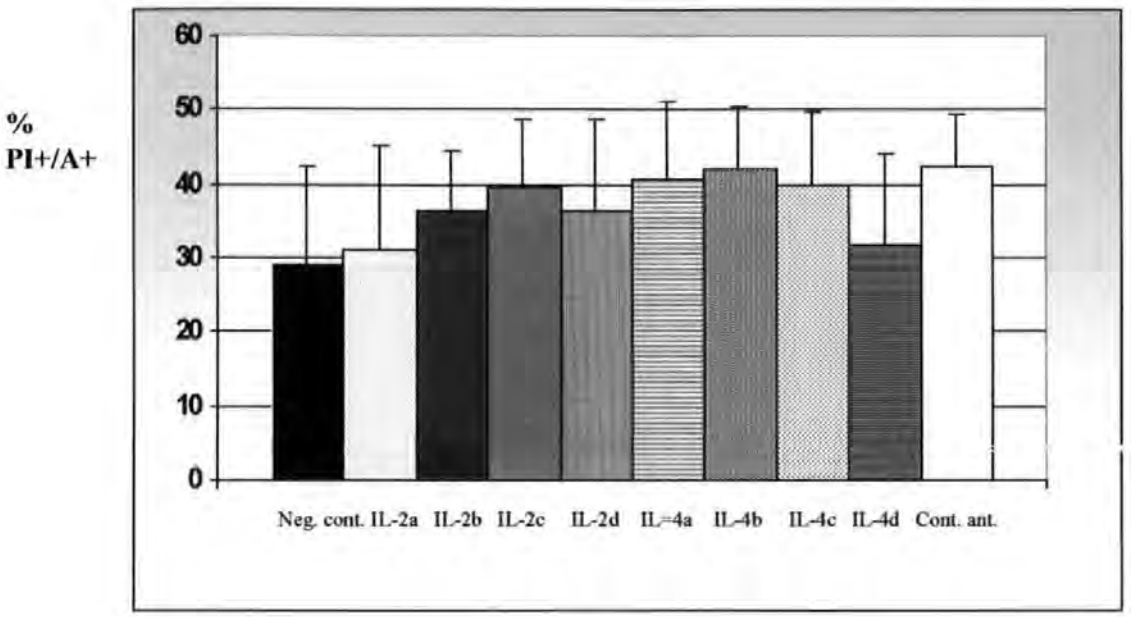
This graph shows the results from Appendix 13. PBMCs from five patients with B-CLL were double stained with anti-CD19 PE antibody and Annexin V FITC. Cells were incubated for 48 hours with PTO-modified ONs at a total concentration of 7.5 μM (3 x 2.5 μM). Stained cells were analysed by flow cytometry and the results are shown as the mean percentage of positive cells. The percentage of CD19+/A+ lymphocytes was significantly higher when PBMCs from B-CLL patients were incubated with the following antisense ONs: 2b (p=0.04), 2c (p=0.02) and 2d (p=0.002) compared to negative controls. This graph shows that addition of control antisense also increased the percentage of CD19+/A+ lymphocytes.
A = Annexin V, neg. cont. =Negative control, cont. ant. =Control antisense

Figure 5.15: Flow cytometric analysis of the effect of PTO-modified IL-4 antisense ONs on unstimulated CD19+ve lymphocytes from patients with B-CLL



This graph shows the results from Appendix 14. PBMCs from five patients with B-CLL were double stained with anti-CD19 PE antibody and Annexin V FITC. Cells were incubated for 48 hours with PTO-modified ONs at a total concentration of 7.5μM (3 x 2.5 μM). Stained cells were analysed by flow cytometry and the results shown as the mean percentage of positive cells. The percentage of CD19+/A+ lymphocytes was higher significantly higher when PBMCs from B-CLL patients were incubated with IL-4b antisense ONs (p=0.03) compared to negative controls. The percentage of CD19+/A+ lymphocytes was also higher after incubation with IL-4a, c and d as well as control antisense ONs.
A = Annexin V, neg. cont. =Negative control, cont. ant. =Control antisense.

Figure 5.16: Flow cytometric analysis of the effect of PTO-modified IL-2 and IL-4 antisense ONs on unstimulated PBMC from patients with B-CLL



This graph shows the data from Appendix 13 and 14. PBMCs from B-CLL patients were double stained with PI and Annexin V FITC. Cells were incubated for 48 hours with PTO-modified ONs at a total concentration of 7.5μM (3 x 2.5 μM). Stained cells were analysed by flow cytometry and the results presented as the mean percentage of positive cells. The cells double stained for PI and Annexin V FITC (PI+/A+) represent the percentage of cells in the late apoptotic stage. No significant difference was found between the percentage of double positive PI+/A+ cells from patients with B-CLL incubated with different PTO-modified IL-2 and IL-4 antisense ONs and the negative control. However, it is clear that addition of different antisense ONs and control antisense increased the percentage of PI+/A+ cells.
PI=Propidium iodide, A=Annexin V, cont. ant.= Control antisense, neg. cont.= Negative control

5.6.3. Effect of the addition of PTO-modified antisense ONs on apoptosis of normal control cells

PBMCs isolated from five normal control samples were stained with anti-CD19 PE, double stained with anti-CD19 PE and Annexin V FITC and double stained with Annexin V FITC and Propidium iodine (PI) (Appendix 15 and 16).

No significant difference was found between the percentage of double stained CD19 +/Annexin V+ (CD19+/A+) positive lymphocytes incubated with antisense ONs and the negative control. Only incubation of the cells with PTO-modified IL-2d antisense ONs significantly increased the percentage of PI+/Annexin V (PI+/A+) positive cells compared to a negative control ($p=0.013$) (Table 5.3, Figure 5.17, 5.18 and 5.19).

Normal PBMCs were double stained for CD3 PE and Annexin V FITC (Appendix 16, Table 6.4). No significant difference was present between the percentage of double stained CD3+/A+ lymphocytes incubated with various PTO-modified IL-2 and IL-4 antisense ONs and the negative control (Figure 5.20 A and B).

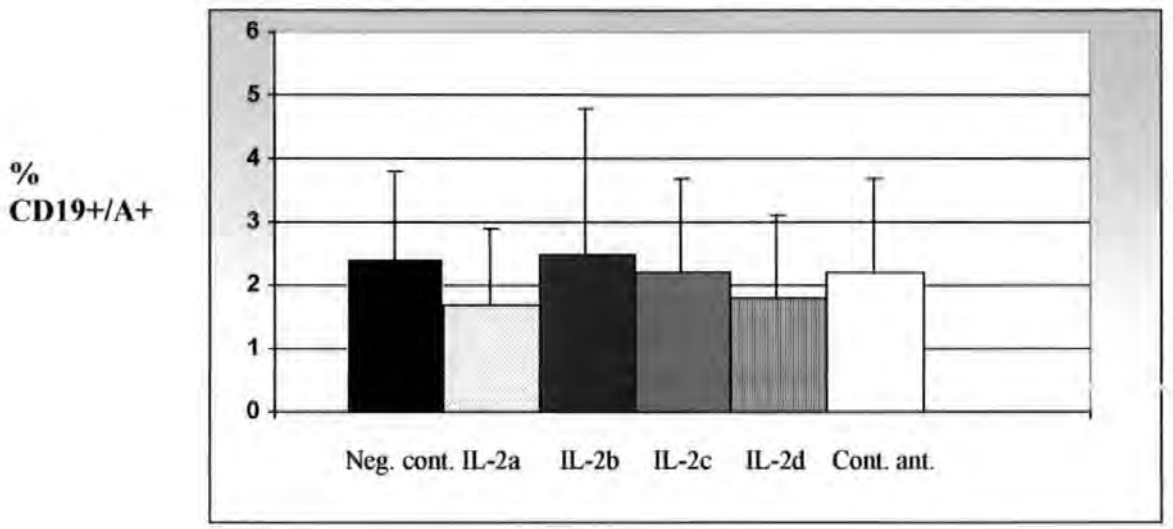
When PBMCs from patients with B-CLL were stimulated with PHA and incubated with PTO-modified IL-2 and IL-4 antisense ONs the results showed that, contrary to previous experiments, addition of ONs did not increase the percentage of CD19+/A+ cells compared to the negative control (Figure 5.21 A and B).

Table 5.3: Statistical analysis of data from Appendix 15 and 16

T test						
Variable: CD19+/A+ neg. cont. versus CD19+/A+ plus antisense ON				**Variable: PI+/A +neg. cont. vesus PI+/A+ Plus antisense ON**		
Mean:	2.5	1.8	**IL-4a**	Mean:	4.8	4.1	**IL-4a**
SD+/-	1.5	1.4		SD+/-	3.0	2.7	
p=ns				p=ns			
Mean:	2.5	1.8	**IL-4b**	Mean:	4.8	4.6	**IL-4b**
SD+/-	1.5	1.4		SD+/-	3.0	2.6	
p=ns				p=ns			
Mean:	2.5	1.6	**IL-4c**	Mean:	4.8	4.7	**IL-4c**
SD+/-	1.5	1.0		SD+/-	3.0	2.2	
p=ns				p=ns			
Mean :	2.5	2.2	**IL-4d**	Mean:	4.8	6.9	**IL-4d**
SD+/-	1.5	1.3		SD+/-	3.0	4.2	
p=ns				p=ns			
Mean:	2.5	1.7	**IL-2a**	Mean:	4.8	4.0	**IL-2a**
SD+/-	1.5	1.3		SD+/-	3.0	2.1	
p=ns				p=ns			
Mean:	2.5	2.5	**IL-2b**	Mean:	4.8	5.0	**IL-2d**
SD+/-	1.5	2.3		SD+/-	3.0	3.9	
p=ns				p=ns			
Mean:	2.5	2.2	**IL-2c**	Mean:	4.8	7.5	**IL-2c**
SD+/-	1.5	1.5		SD+/-	3.0	5.0	
p=ns				p= ns			
Mean:	2.5	1.8	**IL-2d**	Mean:	4.8	6.1	**IL-2d**
SD+/-	1.5	1.3		SD+/-	3.0	2.5	
p=ns				p=0.01			
Mean:	2.5	2.2	**Cont.**	Mean:	4.8	5.0	**Cont.**
SD+/-	1.5	1.5		SD+/-	3.0	3.1	
p= ns				p=ns			

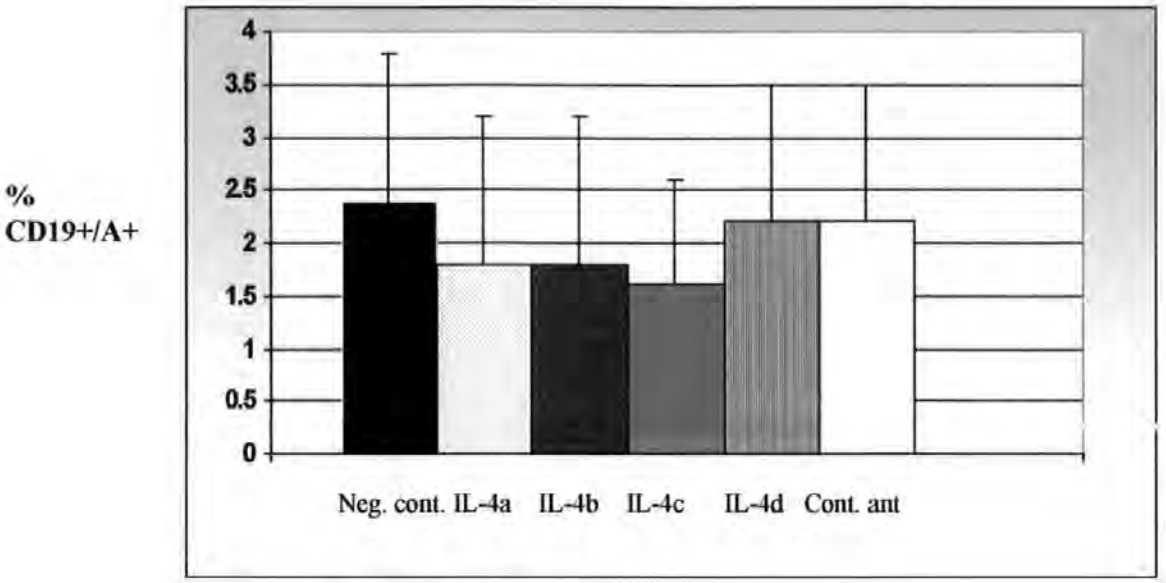
The Student t test was used to measure the statistical significance of the data presented in Appendix 15 and 16. Values equal or lower than p=0.05 were considered statistically significant.
No significant difference was found between the percentage of double stained CD19 PE/Annexin V FITC (CD19+/A+) lymphocytes incubated with PTO-modified antisense ONs and the negative control. Only incubation of the cells with PTO-modified IL-2d antisense ON significantly increased the percentage of Propidium iodine/Annexin V FITC (PI+/A+) positive cells compared to the negative control (p=0.01).
Ns=not-significant, A=Annexin, PI=Propidium iodine

Figure 5.17: Flow cytometric analysis of the effect of PTO-modified IL-2 antisense ONs on nstimulated CD19+ve lymphocytes from normal controls



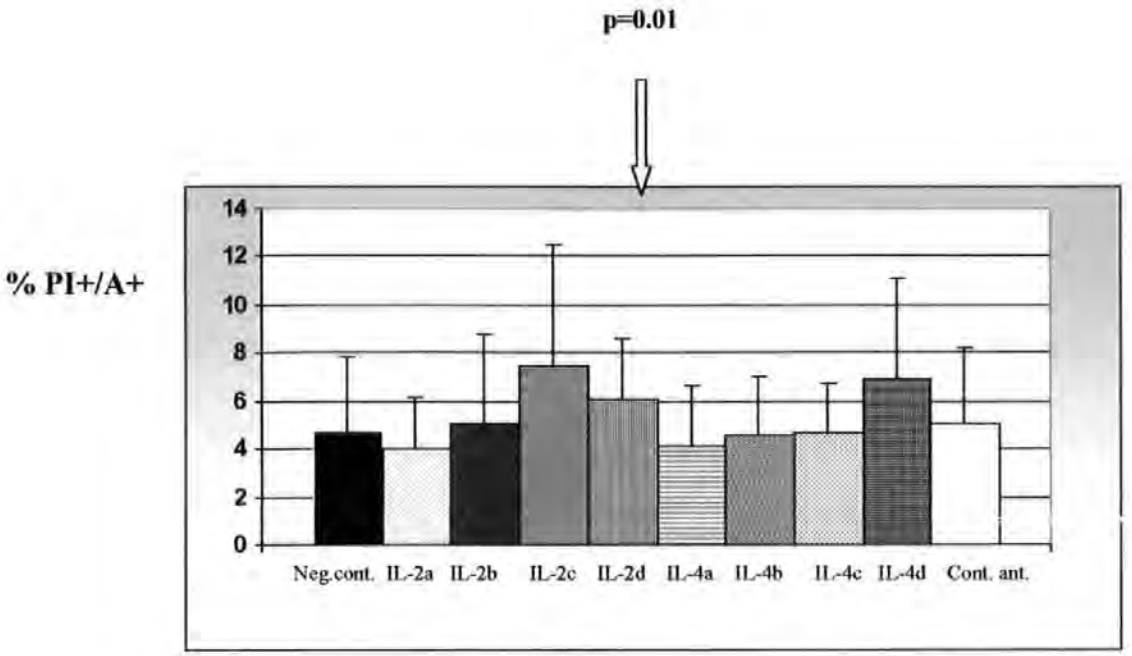
This graph shows the result from Table 5.3. PBMCs from five normal controls were used. PBMCs were incubated for 48 hours with IL-2 PTO-modified ONs at a total concentration of 7.5 μM (3 x 2.5 μM). Collected PBMCs were double stained with anti-CD19 PE antibody and Annexin V FITC (CD19+/A+) and were analysed by flow cytometry. The results are shown as the mean percentage of positive cells. No significant difference was present between the percentage of double stained CD19+/A+ positive lymphocytes incubated with IL-2 PTO-modified antisense ONs and the negative control. In fact the percentage of CD19+/A+ lymphocytes was lower after addition of IL-2a, c, d and control antisense ONs.
A = Annexin V, neg. cont. =Negative control, cont. anat. =Control antisense

Figure 5.18: Flow cytometric analysis of the effect of PTO-modified IL-4 antisense ONs on unstimulated CD19+ve lymphocytes separated from normal controls



This graph shows the result from Table 5.3. PBMCs from five patients with B-CLL were used. PBMCs were diluted in 5% FCS culture medium to a concentration of $0.5x10^5$/ml. Cells were incubated for 48 hours with IL-4 PTO-modified ONs at a total concentration of 7.5 μM (3 x 2.5 μM). Collected PBMCs were double stained with anti-CD19 PE monoclonal antibody and Annexin V FITC (CD19+/A+) and were analysed by flow cytometry. The results are shown as the mean percentage of positive cells. No significant difference was present between the percentage of double stained CD19+/+A positive cells incubated with IL-2 PTO-modified antisense ONs and the negative control. Overall, the percentage of CD19+/A+ lymphocytes was lower after the addition of antisense ON compared to a negative control.
A = Annexin V, neg. cont. =Negative control, cont. ant.= Control antisense

Figure 5.19: Flow cytometric analysis of the effect of PTO-modified IL-4 antisense ONs on unstimulated PBMCs from normal controls



This graph shows the results from Table 5.3. PBMCs from five patients with B-CLL were used. PBMCs were diluted in 5% FCS culture medium to a concentration of $0.5x10^5$/ml. Cells were incubated for 48 hours with IL-4 PTO-modified ONs at a total concentration of 7.5μM (3 x 2.5 μM). Collected PBMCs were double stained with anti-CD19 PE monoclonal antibody and Annexin V FITC (CD19+/A+) and were analysed by flow cytometry. The results are shown as the mean percentage of positive cells. No significant difference was present between the percentage of double stained PI+/A+ cells incubated with IL-2 PTO-modified antisense ONs and the negative control. Overall, the percentage of PI+/A+ was higher after addition of IL-2b, c, d, and IL-4d antisense ONs compared to negative control.
A = Annexin V, neg. cont. =Negative control, cont. ant. = Control antisense

Table 5.4: Statistical analysis of the data from Appendix 17

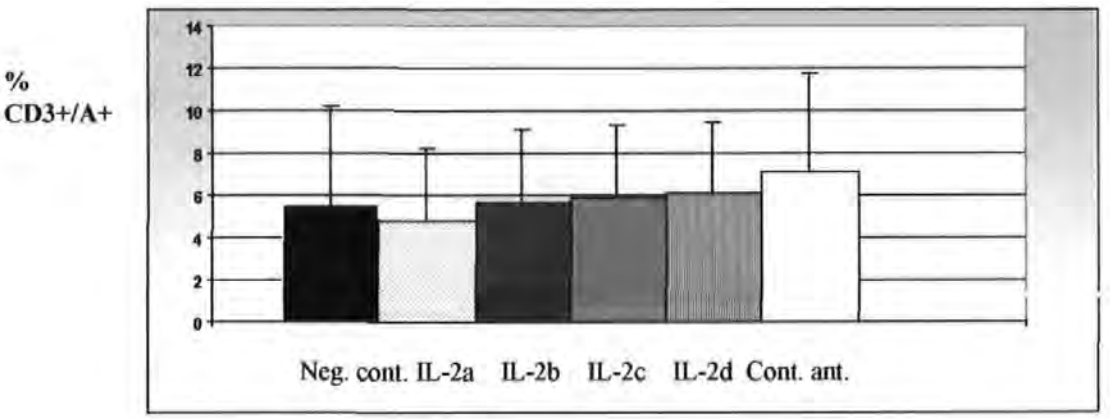
T test						
Antisense	**Variable:** **CD19+/A+ neg. cont. vs CD19+/A + plus ON**			**Antisense**	**Variable:** **CD19+/A+ neg. cont. vs CD19+/A+ plus ON**	
IL-2a	Mean:	5.6	4.8	**IL-4a**	Mean:	5.6	4.4
	SD+/-	4.4	3.3		SD+/-	4.4	2.5
	p=ns				p=ns		
IL-2b	Mean:	5.6	5.6	**IL-4b**	Mean:	5.6	5.6
	SD+/-	4.4	3.2		SD+/-	4.4	4.4
	p=ns				p=ns		
IL-2c	Mean:	5.6	5.8	**IL-4c**	Mean:	5.6	4.7
	SD+/-	4.4	3.4		SD+/-	4.4	3.4
	p=ns				p=ns		
IL-2d	Mean:	5.6	6.1	**IL-4d**	Mean:	5.6	5.9
	SD+/-	4.4	3.3		SD+/-	4.4	3.9
	p=ns				p=ns		
Control antisense	Mean:	5.6	7.1				
	SD+/-	4.4	4.8				
	p=ns						

PBMCs from five normal healthy controls were double stained with CD3 PE monoclonal antibody and Annexin V FITC (CD3+/A+). The Student t test was used. Values equal or lower than p=0.05 were considered statistically significant. No significant difference was present between the percentage of double stained CD3+/A+ positive lymphocytes incubated with PTO-modified IL-2a, b, c, d and IL-4a, b, c, d antisense ONs and the negative control.

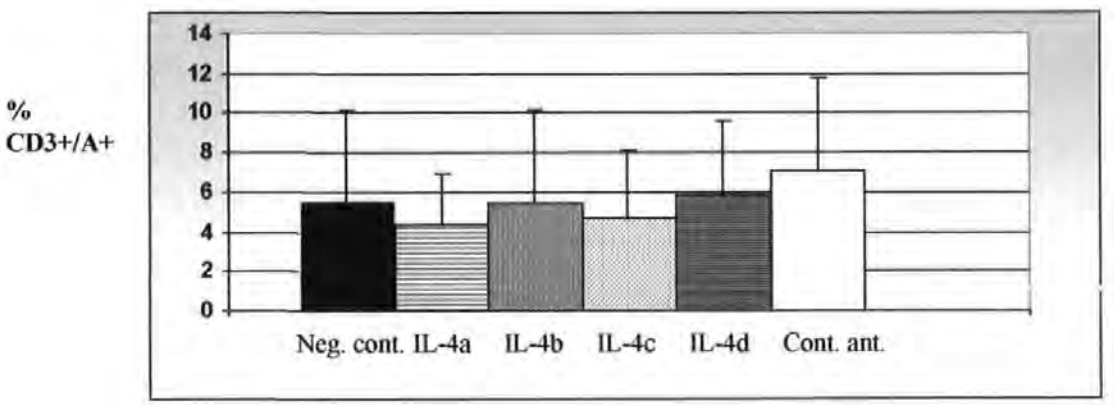
ns= not significant, A=antisense, SD=standard deviation, ON=oligonucleotide

Figure 5.20: Flow cytometric analysis of the effect of PTO-modified IL-2 and IL-4 antisense ONs on unstimulated normal CD3+ve lymphocytes

A) Percentage of CD3+/A+ lymphocytes in PBMCs from normal control after incubation with PTO-modified IL-2a, b, c, and d and control antisense ONs



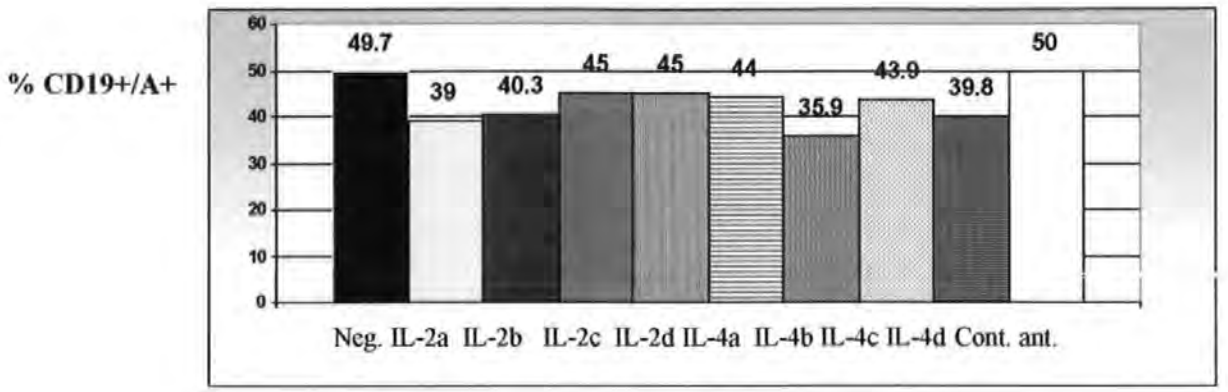
B) Percentage of CD3+/A+ lymphocytes in PBMCs from normal control after incubation with different PTO-modified IL-4a, b, c, and d and control antisense ONs



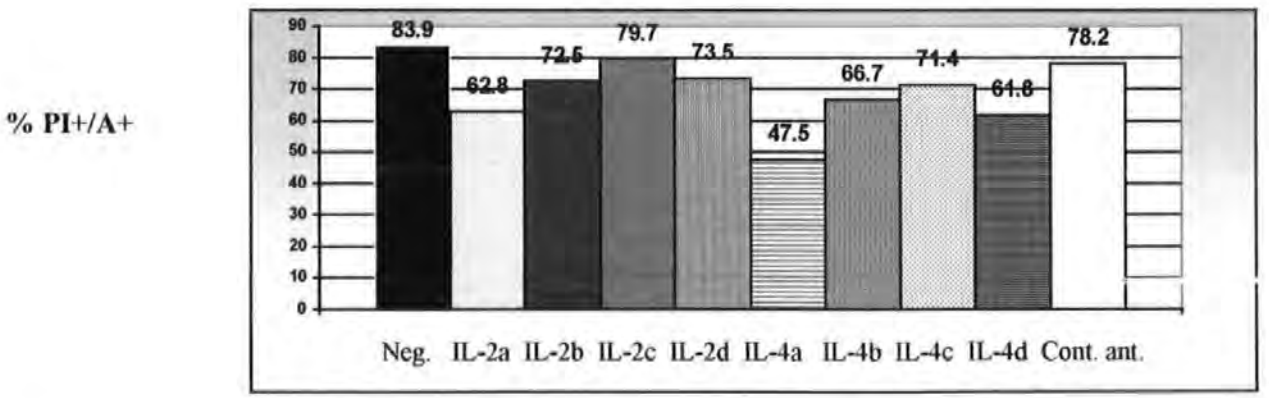
This graph shows data from Table 5.4. Normal PBMCs were incubated for 48 hours with PTO-modified IL-2a, b, c, d and IL-4a, b, c d and control antisense ONs at a total concentration of 3 x 2.5 μM. The cells were then double stained with anti-CD3 PE monoclonal antibody and Annexin V FITC (CD3+/A+). The stained cells were immediately analysed by flow cytometry and the results presented as the mean percentage of positive cells. The Student t test was used. Values equal or lower than p=0.05 were considered statistically significant. No significant difference was present between the percentage of double stained CD3+/A+ positive lymphocytes incubated with various IL-2 and IL-4 antisense ONs compared to the negative control.
A= Annexin V, cont. ant. = Control antisense, neg. cont.= Negative control

Figure 5.21: Flow cytometric analysis of the effect of PTO-modified IL-2 and IL-4 antisense ONs on PHA-stimulated CD19+ve lymphocytes from patients with B-CLL

A) Percentage of CD19+/A+ lymphocytes in PBMCs from patients with B-CLL after incubation with IL-2 and IL-4 antisense ONs



B) Percentage of PI+/A+ lymphocytes in PBMCs from patients with B-CLL after incubation with IL-2 and IL-4 antisense ONs



PHA-stimulated PBMCs from two patients with B-CLL were were double stained with anti-CD19 PE and Annexin V FITC (CD19+/A+) or Annexin V FITC and Propidium iodine (PI+/A+). The cells were incubated for 48 hours with various PTO-modified IL-2 and IL-4 ONs at a total concentration of 3 x 2.5 μM. Stained cells were analysed by flow cytometry and the results are presented as the mean percentage of positive cells. The CD19+ve lymphocytes stained with Annexin V FITC represent the percentage of CD19+ve B-lymphocytes cells undergoing apoptosis. The graph shows that the percentage of PHA-stimulated CD19+/A+ and PI+/A+ cells from patients with B-CLL decreases after addition of IL-2a, b, c, d and IL-4a, b, c, d antisense ONs compared to a negative control.
PI=Propidium iodine, A=Annexin V, cont. ant.= Control antisense, neg. cont.= Negative control

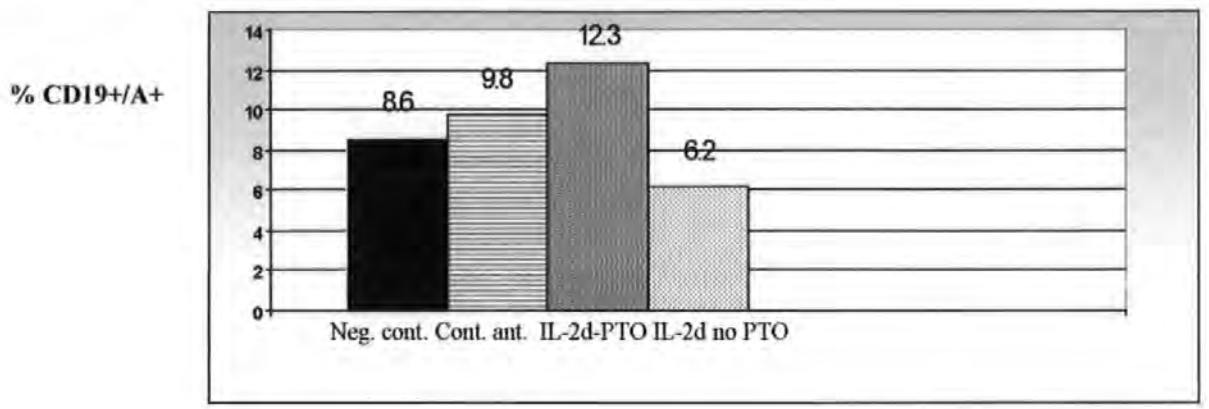
5.6.4. Comparison between PTO- modified and unmodified IL-2d antisense ONs

Previous experiments have shown that the addition of PTO-modified IL-2 and IL-4 antisense ONs to B-CLL cell cultures increased the percentage of CD19+/Annexin V+ and PI+/Annexin V+ positive cells when compared to a negative control. In this experiment, unmodified antisense ONs as well as Phosphorothioate (PTO)-modified antisense ONs were used. A different antisense control (Table 2.3, antisense 2) was also used in this experiment.

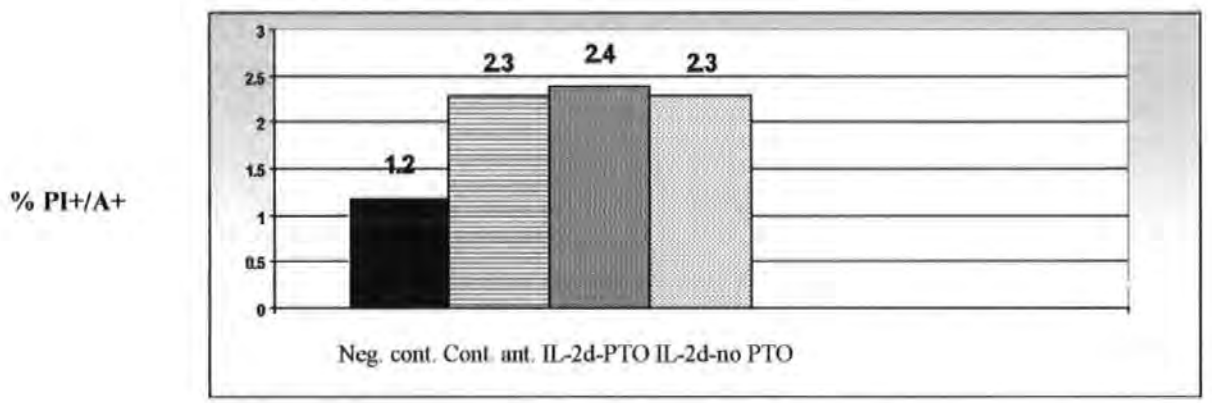
The addition of PTO-modified IL-2d antisense ON to the PBMC cell culture from three patients with B-CLL increased the percentage of CD19+/A+ positive lymphocytes (12.3%) when compared to incubation with unmodified antisense ONs (6.2%) and a negative control (8.6%). Control antisense also increased the percentage of CD19+/A+ positive lymphocytes but not as much as PTO-modified antisense ON (9.6%) (Appendix 18, Figure 5.22).

Figure 5.22: Flow cytometric analysis of B-CLL cells after the addition of control, IL-2d PTO-modified and unmodified IL-2d antisense ONs

A) Percentage of CD19+/A+ lymphocytes after incubation with PTO-modified and unmodified ONs



B) Percentage of PI+/A+ lymphocytes after incubation with PTO-modified and unmodified ONs



PBMCs from three patients with B-CLL were incubated with unmodified IL-2d and IL-2 PTO-modified antisense ONs as well as control antisense ON for 48 hours. Cells were stained with CD19 PE monoclonal antibody and Annexin V FITC (CD19+/A+) or PI and Annexin V FITC PI+/A+). This graph shows that only PTO-modified IL-2d and PTO-modified control antisense ONs increased the percentage of double positive CD19+/A+ lymphocytes compared to a negative control. The percentage of PI+/A+ lymphocytes that are thought to be in a late state of apoptosis was overall very small and increased slightly after incubation with antisense ONs.

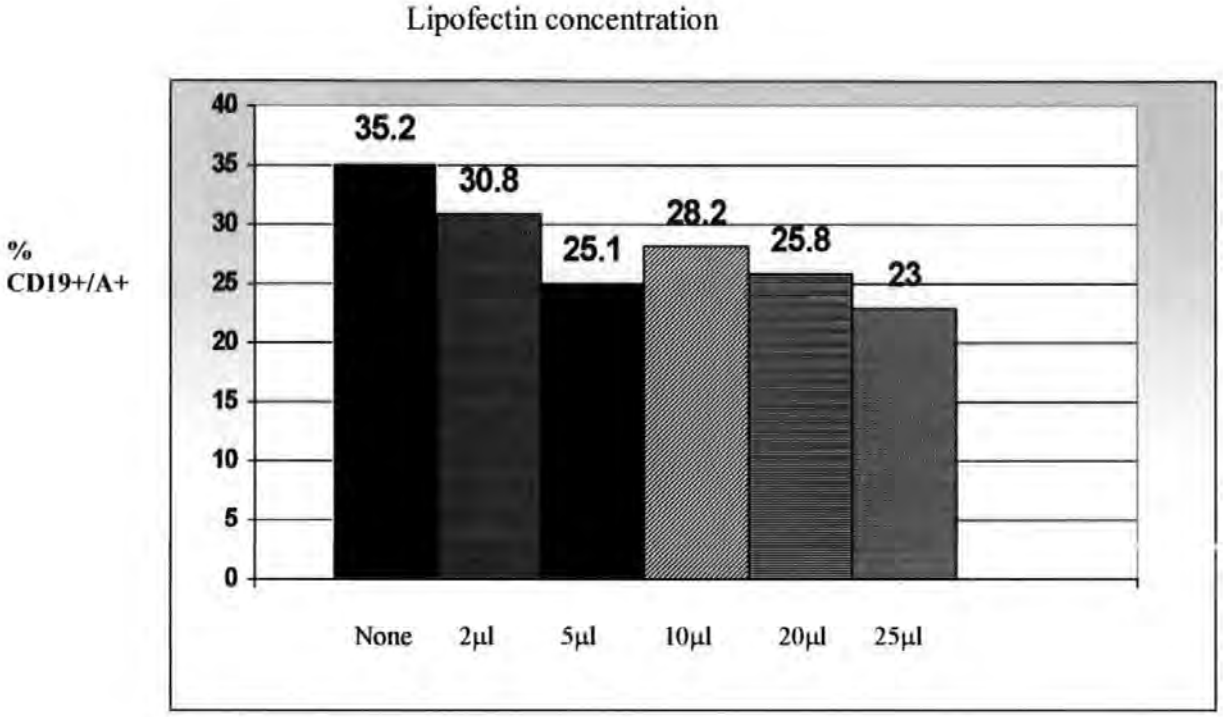
Neg. cont.= negative control, cont. ant. =Control antisense, A=Antisense, PI=Propidium iodine, PTO= Phosphorothioate modified

5.6.5. Measurements of apoptotic cell death after incubation with PTO-modified antisense ON plus Lipofectin

In order to enhance cellular uptake and to promote nuclear accumulation of PTO-modified IL-2d antisense ONs, cationic lipids were used as a delivery system. *Lipofectin*, which has been described as one of the most efficient carriers was used. This preliminary experiment was carried out only once. PBMCs from one patient with B-CLL were incubated with IL-2d antisense ON and was supplemented with Lipofectin at various concentrations (Range: 2-25μl).

As shown in the Figure 5.23, the addition of Lipofectin to the PBMCs incubated with IL-2d antisense ON decreased the percentage of double positive CD19+/A+ lymphocytes in a dose dependent manner (30.8%, 25.1%, 28.2%, 25.8% and 23.0%). Addition of a higher concentration of Lipofectin decreased the percentage of CD19+ve B-CLL cells compared to lower concentrations of Lipofectin and to a negative control. As mentioned before, this experiment was performed only once using a much lower concentration of antisense (0.1μM) than in all previous experiments (3 x 2.5μM).

Figure 5.23: Flow cytometric analysis of unstimulated B-CLL cells after incubation with PTO-modified IL-2d antisense ON plus Lipofectin



PBMCs isolated from one patient with B-CLL cells were incubated with PTO-modified IL-2d antisense ON for 48 hours. To increase delivery of the antisense ON, Lipofectin was used at various concentrations (range: 2-25µl). Cells were stained anti-CD19 PE and Annexin V FITC (CD19+/A+). Antisense ON was added at a concentration of 0.1µM together with different concentration of Lipofectin.
Addition of Lipofectin as a cationic carrier to the PBMCs decreased the percentage of double positive CD19+/A+ lymphocytes in a dose dependent manner. In other words, addition of the higher concentration of Lipofectin decreased the percentage of CD19+ve B-CLL cells compared to lower concentrations of Lipofectin and to a negative control.
A=Antisense, PTO = Phosphorothioate modified

5.7. Summary-Annexin V staining

Flow cytometric analysis was used to determine the percentage of CD19+ve and CD3+ve lymphocytes from patients with B-CLL and from normal healthy controls undergoing apoptosis after incubation with different PTO-modified IL-2 and IL-4 antisense ONs.

- Flow cytometric measurements of the percentage of CD19+/A+ as well as PI+/A+ positive lymphocytes had shown that the addition of various PTO-modified IL-2a, b, c, d and IL-4a, b, c, d antisense ONs to unstimulated PBMCs increased the percentage of CD19+/A+ and PI+/A+ cells when compare to a negative control. CD19+ve lymphocytes stained with Annexin V are considered to be in an apoptotic state. Therefore IL-2 and IL-4 ONs might be considered pro-apoptotic. This effect was most prominent in B-CLL cells, reaching statistical significance after the addition of PTO-modified IL-2b, IL-2c and IL-2d antisense ONs.
- PTO-modified control antisense ONs also increased the percentage of CD19+/A+ lymphocytes in unstimulated PBMCs from patients with B-CLL compared to a negative control. Therefore PTO-modified ONs are cytotoxic.
- The addition of PTO-modified IL-2 and IL-4 antisense ONs had no significant effect on unstimulated normal healthy control CD3+ve lymphocytes. Although a small percentage of CD3+/A+ positive cells were often present, in some cases the percentage of double positive cells actually decreased.
- Only PTO-modified antisense ON was able to increase the percentage of CD19+/A+ lymphocytes compared to unmodified antisense ON. Therefore, unmodified ONs are not biollogically effective.
- In PHA-stimulated PBMCs from patients with B-CLL, the percentage of CD19+/A+, lymphocytes after incubation with IL-2 and IL-4 antisense ONs decreased compared to a negative control.

- Addition of Lipofectin as a cationic carrier to PBMCs from B-CLL patients decreased the percentage of CD19+/A+ lymphocytes compared to a negative control.

5.8. Measurement of intracellular IL-2 and IL-4 in unstimulated and PMA-stimulated B-CLL cells after incubation with various PTO-modified antisense ONs

The effect of PTO-modified IL-4c antisense ON on intracellular expression of IL-2 and IL-4 was studied in PBMCs from four patients with B-CLL (patients 23-28, table 5.1).

The optimal incubation time was chosen using normal unstimulated and PMA-stimulated PBMCs. Flow cytometric analysis of intracellular stainning was performed after 2, 4 and 6 hours of incubation with IL-4c antisense ON. The percentage of unstimulated normal CD19+ve cells expressing intracellular IL-2 and IL-4 was higher after incubation with IL-4c antisense ON for 2 hours (1.5% and 14.0% respectively) when compared to a negative control (0.3% and 6.8% respectively). After 4 and 6 hours of incubation, the percentage of CD19+ve lymphocytes expressing intracellular IL-2 and IL-4 was much lower and not very different from a negative control (Appendix 19, Figure 5.24 A).

The percentage of unstimulated CD3+ve lymphocytes expressing intracellular IL-2 was low. After incubation with antisense ON for 2 hours, the percentage of CD3+/IL-2+ positive lymphocytes decreased from 2.6% to 1.1%. After incubation with PTO-modified antisense ON for 6 hours, the percentage of CD3+/IL-2+ positive lymphocytes decreased from 1.7% to 1.0% (Figure 6.24). The percentage of PMA-stimulated CD19+/IL-2+ cells decreased after 2 hours of incubation with IL-4c antisense ON compared to a negative control (from 4.7% to 0.7%) (Appendix 19, Figure 5.24 A).

After incubation with PTO-modified antisense ON for 2 hours, the percentage of CD3+/IL-4+ positive lymphocytes decreased from 26.1% to 19.6%, after 4 hours from 23.3% to 12.7 % and after 6 hours from 27.6% to 9.4% (Appendix 19, Figure 5.24).

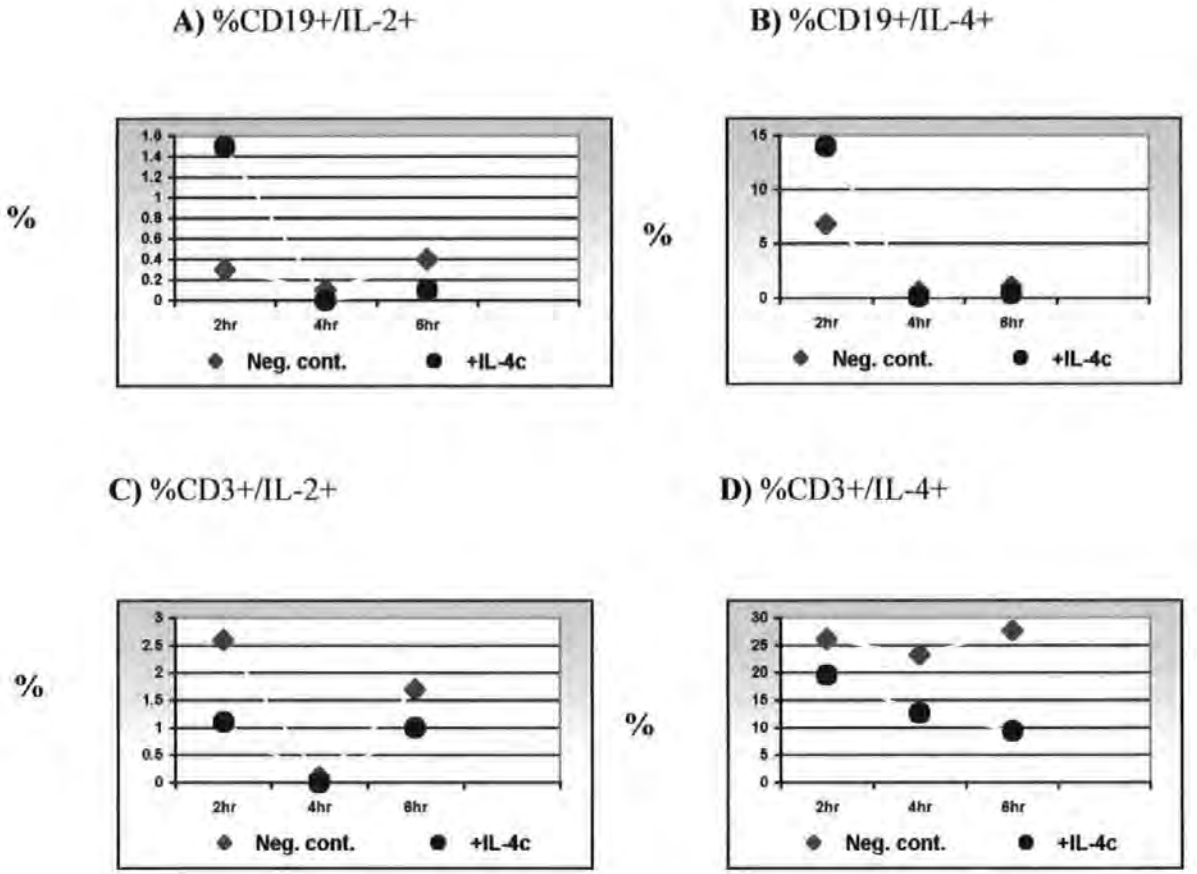
Incubation of PMA-stimulated PBMCs from normal controls with IL-4c antisense had a varied effect on the percentage of CD3+ve and CD19+ve lymphocytes expressing intracellular IL-2 and IL-4. The percentage of CD19+/IL-2+ lymphocytes decreased after 2 hours of

incubation with IL-4c antisense ON compared to a negative control (from 4.7% to 0.7%) (Appendix 19, Figure 5.25).

PBMCs from four patients with B-CLL were incubated with IL-4c antisense ON for 6 hours. The percentage of unstimulated patients CD19+ve and CD3+ve lymphocytes expressing intracellular IL-2 and IL-4 decreased after addition of IL-4c antisense ON compared to a negative control [(CD19+/IL-2+ from 4.6%, SD+/-5.5 to 1.6%, SD+/-1.1), (CD19+/IL-4+ from 10.3%, SD+/-12.3 to 3.3%, SD+/-2.8), (CD3+/IL-2+ from 1.4%, SD+/-1.3 to 1.1%, SD+/-1.8), (CD3+/IL-4+ from 3%, SD+/-3.3 to 1.1%, SD+/-1.0)] (Appendix 20, Table 5.5, Figure 5.26B).

In contrast, the percentage of PMA-stimulated CD19+ve lymphocytes expressing intracellular IL-2 and the percentage of CD3+ve cells expressing intracellular IL-2 and IL-4 increased after addition of IL-4c antisense ON when compared to a negative control [(CD19+/IL-2+ from 6.5%, SD+/-2.4 to 9.0%, SD+/-2.7), (CD3+/IL-2+ from 2.1%, SD+/-2.6 to 4.6%, SD+/-4.5), (CD3+/IL-4+ from 1.8%, SD+/-2.4 to 6.7%, SD+/-8.8)] (Appendix 20, Table 5.5, Figure 5.26B). None of these differences was significant.

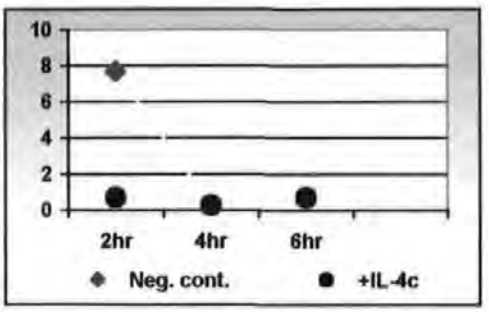
Figure 5.24: Flow cytometric analysis of unstimulated CD19+ve and CD3+ve normal cells stained for intracellular IL-2 and IL-4



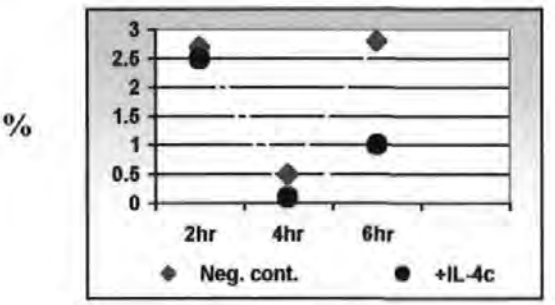
This graph shows the data from Appendix 19. Unstimulated PBMCs from one normal healthy control were incubated for 2, 4, and 6 hours with PTO-modified IL-4c antisense ON at the concentration of 2.5μM. The percentage of CD19+ve cells expressing intracellular IL-2 and IL-4 was higher after incubation with antisense ON for 2 hours (1.5% and 14.0% respectively) when compared to a negative control (0.3% and 6.8% respectively). The percentage of CD3+ve cells expressing intracellular IL-2 and IL-4 was lower after incubation with PTO-modified antisense ON for 2, 4 and 6 hours when compared to a negative control.
Neg. cont.= Negative control, hr=Hours

Figure 5.25: Flow cytometric analysis of PMA–stimulated CD19+ve and CD3+ve normal cells stained for intracellular IL-2 and IL-4

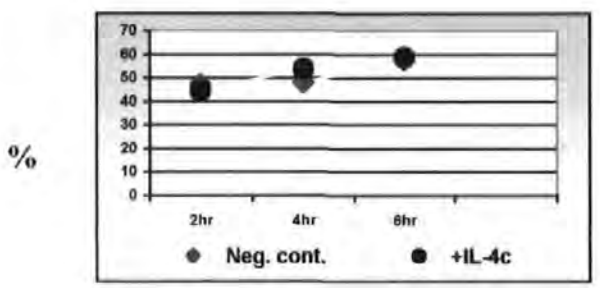
A) %CD19+/IL-2+



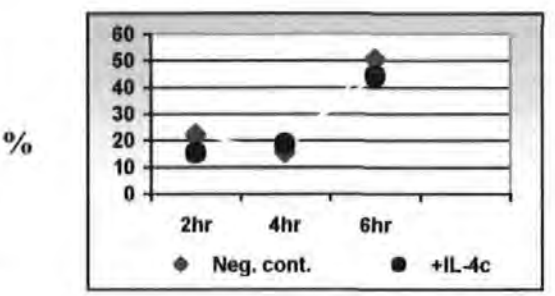
B) %CD19+/IL-4+



C) %CD3+/IL-2+



D) %CD3+/IL-4+



PMA-stimulated PBMCs from one normal healthy control were incubated for 2, 4, and 6 hours with PTO-modified IL-4c antisense ON at a concentration of 2.5μM. This graph shows that incubation of PMA-stimulated PBMCs with IL-4c antisense had a varied effect on the percentage of CD3 and CD19+ve cells expressing intracellular IL-2 and IL-4.
Neg. cont.= Negative control, hr=Hours

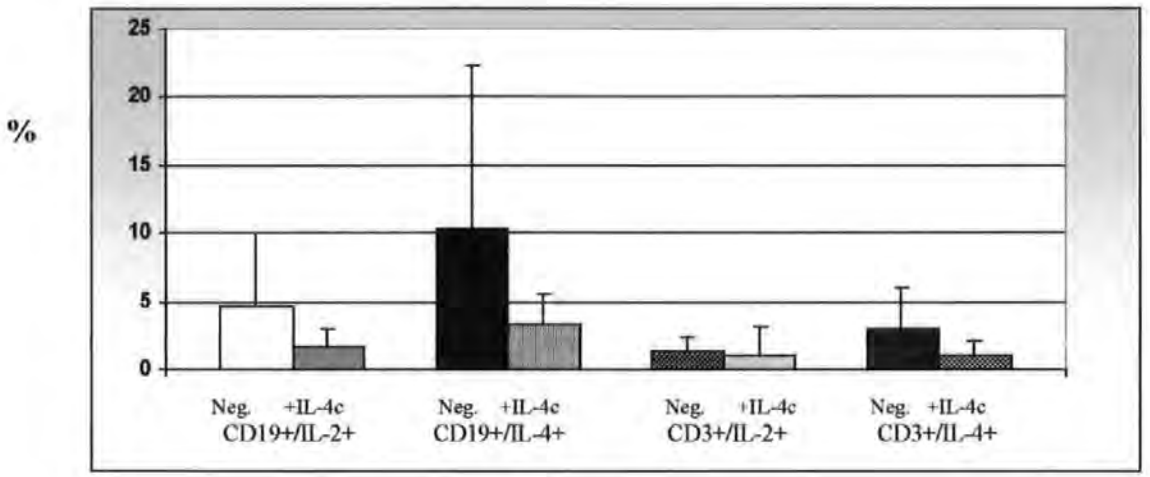
Table 5.5: Significance of the addition of antisense ONs to stimulated and unstimulated PBMCs from patients with B-CLL on intracellular IL-2 and IL-4 protein expression

T test			
%CD19+/IL-4+neg. control /%CD19+/IL-4+ +IL-4c antisense ON	**%CD19+/IL-2+neg. control /%CD19+/IL-2+ + IL-4c antisense ON**	**%CD3+/IL-4+neg. control /%CD3+/IL-4 + + IL-4c antisense ON**	**%CD3+/IL-2 +neg. control /%CD3+/IL-2+ + IL-4c antisense ON**
Unstimulated cells			
Mean: 10.3 3.3 SD+/- 12.3 2.8 p= ns	Mean: 4.6 1.6 SD+/- 5.5 1.1 p= ns	Mean: 3.0 1.1 SD+/- 3.3 1.0 p= ns	Mean: 1.4 1.1 SD+/- 1.3 1.8 p= ns
Stimulated cells			
Mean: 6.6 5.9 SD+/- 2.8 2.5 p= ns	Mean: 6.5 9.0 SD+/- 2.4 2.7 p=ns	Mean: 1.8 6.7 SD+/- 2.4 8.8 p=ns	Mean: 2.1 4.6 SD+/- 2.6 4.5 p= ns

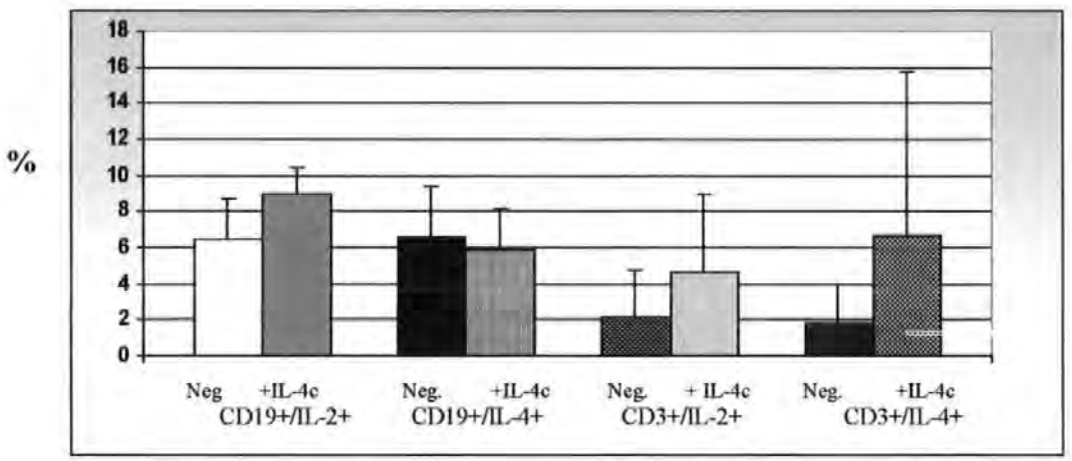
This table shows the statistical significance of the data presented in Appendix 20. PBMCs from four patients with B-CLL were double stained with either of the following; CD3 PE, CD19 PE and IL-2 FITC or IL-4 FITC monoclonal antibodies. The Students t-test was used to analyse the results. Values equal or lover than p=0.05 were considered statistically significant. No statistically significant difference was present in the percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-2 and IL-4 after incubation with PTO-modified IL-4c antisense ONs compared to a negative control.
Neg.=negative control, cont.=Control, ant.=Antisense, SD=Standard deviation, ns=Not significant, ON=oligonucleotide

Figure 5.26: Flow cytometric analysis of unstimulated and PMA-stimulated CD19+ve and CD3+ve B-CLL cells expressing intracellular IL-2 and IL-4

A) Intracellular staining of unstimulated CD19+ve and CD3+ve lymphocytes



B) Intracellular staining of PMA-stimulated CD19+ve and CD3+ve lymphocytes



This graph shows the data presented in Appendix 20 A and B. The percentage of unstimulated patient CD19+ve and CD3+ve lymphocytes expressing intracellular IL-2 and IL-4 decreased after the addition of IL-4c antisense ON when compared to negative control (Figure A).
In contrast, the percentage of PMA-stimulated CD19+ve lymphocytes expressing intracellular IL-2 and the percentage of CD3+ve cells expressing intracellular IL-2 and IL-4 increased after the addition of IL-4c antisense ON when compared to a negative control (Figure B).
Neg. cont.= Negative control, hr=Hours

5.8.1. Summary –intracellular staining

The percentage of unstimulated CD19+ve and CD3+ve normal cells expressing intracellular IL-4 and IL-2 decreased after 4-6 hours incubation with PTO-modified IL-4c ON, compared to a negative control. The percentage of PMA-stimulated CD19+ve normal cells expressing intracellular IL-4 and IL-2 decreased after incubation with IL-4c ON for 4 and 6 hours, compared to a negative control. However the percentage of CD3+ve cells expressing intracellular IL-4 and IL-2 increased after incubation for 4 and 6 hours with antisense ON.

- Measurement of intracellular IL-2 and IL-4 expression in normal and CD19+ve and CD3+ve B-CLL cells has shown that the addition of PTO-modified IL-4c antisense decreased the percentage of unstimulated CD19+ve and CD3+ve normal and B-CLL cells expressing intracellular IL-4 and IL-2. The addition of IL-4c to PMA stimulated B-CLL cells increased the percentage of CD19+ve and CD3+ve cells expressing intracellular IL-2 and IL-4.

5.9. Measurements of IL-2 and IL-4 protein secretion by ELISA after incubation with PTO-modified antisense ONs

The capacity of five five unstimulated and PHA-stimulated BPMCs from patients with B-CLL (patients 29-34, Table 5.1) to secrete IL-2 and IL-4 protein was further evaluated. Protein levels were measured in cell-culture supernatant following 48 hours incubation with PTO-modified antisense ONs. Antisense ONs were added at a concentration of 3 x 2.5μM.

The addition of PTO-modified IL-2 antisense ONs reduced IL-2 protein level in PHA-stimulated cells compared to negative controls (Table 5.12). The addition of PTO-modified IL-4 antisense ON slightly increased the level of IL-4 protein in the supernatant when compared to a negative control (Appendix 21, Table 5.6 and Figure 5.27)

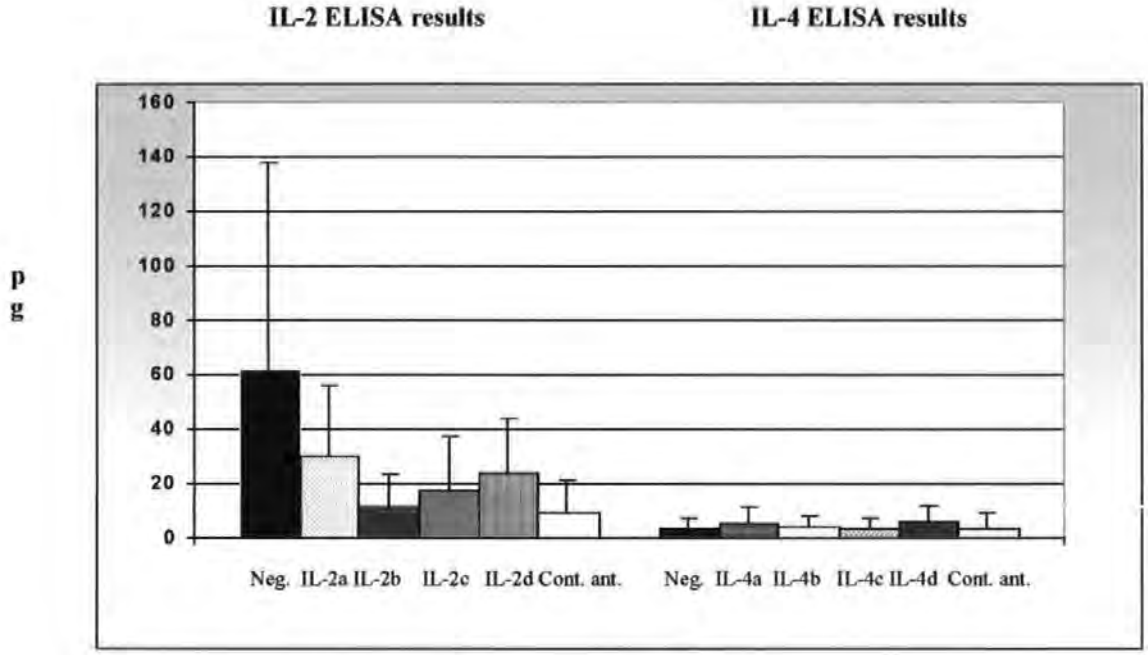
No measurable amounts of IL-2 were detected in the supernatants of unstimulated PBMCs from patients with B-CLL incubated with PTO-modified IL-2 antisense ONs. The level of detectable IL-4 protein was very small and increased after incubation with PTO-modified control antisense, IL-4b and IL-4c antisense ONs (Table 5.7).

Table 5.6: Statistical analysis (t-test) of IL-2 and IL-4 protein secretion of PHA stimulated PBMCs isolated from patients with B-CLL and incubated with IL-2 and IL-4 antisense ONs

T test						
IL-2 ELISA readings (pg)				**IL-4 ELISA readings (pg)**		
Variable: Neg. cont. / cells + antisense ON				Variable: Neg. cont. / cells + antisense ON		
Mean:	62.09	9.25	**Cont.**	Mean:	3.26	3.21	**Cont.**
SD+/-	75.8	12.5	**antisense**	SD+/-	3.8	6.0	**antisense**
p= ns				p= ns			
Mean:	62.09	30.24	**IL-2a**	Mean:	3.26	5.20	**IL-4a**
SD+/-	75.8	26.1	**ON+**	SD+/-	3.8	5.8	**ON+**
p=ns				p= ns			
Mean:	62.09	11.32	**IL-2b**	Mean:	3.26	3.98	**IL-4b**
SD+/-	75.8	10.9	**ON+**	SD+/-	3.8	4.2	**ON+**
p=ns				p=ns			
Mean:	62.09	17.47	**IL-2c**	Mean:	3.26	3.62	**IL-4c**
SD+/-	75.8	20.6	**ON+**	SD+/-	3.8	4.3	**ON+**
p=ns				p= ns			
Mean :	62.09	24.08	**IL-2d**	Mean:	3.26	5.69	**IL-4d**
SD+/-	75.7	19.6	**ON+**	SD+/-	3.8	5.9	**ON+**
p= ns				p= ns			

PHA-stimulated PBMCs from only four patients with B-CLL were incubated with various PTO-modified IL-2 and IL-4 antisense ONs for 48 hours. Collected supernatants were tested for the concentration of IL-2 and IL-4 protein by ELISA. The Students t-test was performed. Values equal or lover than p=0.05 were considered statistically significant. Addition of different IL-2 and IL-4 antisense oligonucleotides reduced the IL-2 protein secretion but the results were not statistically significant.
ns= not significant, Cont. = control antisense

Figure 5.27: Measurement of IL-2 and IL-4 protein secretion by ELISA



This graph shows data from Table 5.6. Levels of IL-2 and IL-4 protein were measured in supernatant of PBMCs from B-CLL patients. Cells were stimulated for 48 hours with PHA added at a concentration of 1μg/ml. The addition of PTO-modified IL-2a, IL-2b, IL-2c and IL-2d antisense ONs reduced IL-2 protein level in PHA-stimulated PBMCs when compared to negative controls. The addition of PTO-modified IL-4a, IL-4b, IL-4c and IL-4d antisense ON slightly increased the level of IL-4 protein in the supernatant when compared to a negative control.
Pg=picogram, IL=interleukin, Cont. ant.= Control antisense, neg. cont. = Negative control

Table 5.7: Analysis of IL-2 secretion by unstimulated normal cells incubated for 48 hours with IL-2 and IL-4 antisense ONs

IL-2 ELISA readings (in pg): No IL-2 ELISA reading was recorded with unstimulated normal cells

Concentration of IL-4 (in pg)

Control	Negative control	Control antisense	IL-4a	IL-4b	IL-4c	IL-4d
1	0.064	0.281	0	0.292	0.028	0.015
2	0	0.040	0.040	0.132	0.143	0
3	0.040	0.087	0.028	0	0.110	0.064
4	0.121	0.121	0.040	0.040	0.076	0.028
Mean	0.056	0.132	0.027	0.206	0.089	0.026

Unstimulated PBMCs from normal healthy controls were incubated for 48 hours with various PTO-modified IL-2 and IL-4 antisense ONs.
No measurable amounts of IL-2 were demonstrated in unstimulated supernatants incubated with different PTO-modified IL-2 antisense ONs. The level of detectable IL-4 protein was very small and increased after incubation with PTO-modified control antisense, IL-4b and IL-4c antisense ONs.

5.10.1. Summary-ELISA

- Addition of IL-2 antisense ONs reduced the level of IL-2 protein by PHA-stimulated B-CLL cells. However in unstimulated cells, IL-2 protein was not detectable by ELISA.
- The addition of PTO-modified IL-4 antisense ONs to PHA stimulated B-CLL cells increased the level of IL-4 protein in supernatants compared to negative controls. However in unstimulated cells, IL-2 protein secretion was undetectable in all tested samples.
- The addition of PTO-modified IL-4 antisense minimally increased the secretion of IL-4 protein.

CHAPTER 6: DISCUSSION

6.1. FLOW CYTOMETRIC ANALYSIS OF IL-4 AND IL-4R

6.1.1. Intracellular expression of IL-4 in B-CLL and normal B-lymphocytes

Previous studies have shown that IL-4 has an anti-apoptotic effect on the malignant B-lymphocytes in B-CLL (Mainou-Fowler et al. 1995 and 1996). In view of this, the intracellular expression of IL-4 in B-CLL and normal cells was studied by flow cytometry. The results demonstrated an increased expression of intracellular IL-4 in B and T lymphocytes from patients with B-CLL patients compared to normal controls.

Many cytokines have been shown to be involved in the induction or regulation of B-CLL proliferation (Nerl et al. 1988, Touw et al. 1987 and Cordingley et al. 1988). Exactly which cytokines or cell-cell contacts are important to the survival of B-CLL cells *in vivo,* and whether the cytokines are produced by the tumour cells themselves or by accessory cells has proven to be a difficult question to answer. IL-4 was shown to prevent B-CLL cell clones from entering spontaneous apoptosis by up-regulating bcl-2 expression (Dancescu et al. 1992). Production of IL-4 appears to be mainly but not exclusively, found in T cells, designated Th2 cells (Mosmann 1997). It has also been shown that B-lymphocytes from normal mice are able to produce cytokines previously considered as T-lymphocyte cytokines, depending on the cytokine environment in which the cells were stimulated during their primary encounter with antigen and T-lymphocytes (Harris et al. 2000). These B-lymphocytes have been divided into two populations called Be1 and Be2 and produce polarised cytokine profiles (Harris et al. 2000). Be1 cells mainly produce cytokines such as IL-2, INF-γ, while Be2 cells produce IL-4, IL-6 and IL-10.

There are contradictory reports about the production of IL-4 by B-CLL B-lymphocytes. In some studies, spontaneous expression of IL-4 mRNA or the presence of IL-4 protein in germinal centre and normal mantle zone B-lymphocytes or by B-CLL B-lymphocytes cannot be detected (Schena et al. 1992). Others have clearly demonstrated that malignant B-CLL B-lymphocytes are able to express IL-4 mRNA (Mertz et al. 1990 and di Celle et al. 1994). In

addition, IL-4 and IL-10 were found to be produced by neoplastic B-cell lines that were considered a murine homologue of human B-CLL since they express the CD5 surface marker (O'Garra et al. 1990). Plate et al. (1993), have shown that the majority of individuals studied constitutively express IL-4 m-RNA, but this is most probably unrelated to leukaemic transformation as it was expressed as frequently in normal as in leukaemic CD5+ve cells. More recently the presence of a greater percentage of CD19+ve malignant expressing intracellular IL-4 when compared to normal controls was also confirmed (Kay et al. 2001). It was also shown that CD8+ve T lymphocytes had an increased expression of intracellular IL-4 compared to their normal counterparts (Kay et al. 2001).

Thus the presence of cytokines detected *in vitro* varies from study to study. This may be due to many variable factors, such as incubation time, activation, type of culture media, presence of other cells or cytokines which may influence the results. However, a picture has emerged about the range of cytokines involved in autocrine or paracrine growth loops, with TNF, IL-1, IL-6, IL-7, IL-8 and IFN-γ being found to be produced by B-lymphocytes from patients with B-CLL (Cordingley et al. 1988, Aqualines et al. 1991 and Rigley et al. 1991). It seems likely that, apart from IL-4, other cytokines are involved in cell-cell or cell-matrix interactions and may be important in preventing apoptosis of B-CLL cells in vivo (Dancescu et al. 1992 and Panayiotidis et al. 1993). Although it has been known for some time that IL-4 protects cells from death via apoptosis, little is known about the mechanism by which IL-4 exerts its effect (Mainou-Fowler et al. 1995). One way in which IL-4 protects malignant B-lymphocytes from apoptosis is by upregulating the expression of the anti-apoptotic gene bcl-2 (Dancescu et al. 1992). It has been speculated that both Stat6 and PI 3-kinase pathways may not be needed for bcl-2/bcl-x induction by IL-4, suggesting involvement of an additional signal transduction pathway (Aronica et al. 2000). However, IL-4 deprivation induces apoptotic cell death but does not modulate bcl-2 or bcl-x expression (Rebollo et al. 2000). In this case, the role of the bcl-2 as a survival factor in the absence of IL-4 growth factor is taken over by bcl-3 (Rebollo

et al. 2000). IL-4 may protect against apoptosis via the insulin receptor substrate pathway and a second independent signalling pathway (Zamorano et al. 1996).

The main action of IL-4 on normal B-lymphocytes is to promote proliferation, increased MHC class II, CD23, CD40 antigen expression and the secretion of Ig by anti-IgM stimulated cells (Defrance et al. 1987, Rigley et al. 1991). It also enhances the activation of B-CLL cells via a selective up-regulation of CD54 (ICAM1) (Carlsson et al.1993). IL-4 abrogates B-CLL proliferation in response to TNF, IL-2 and low molecular weight B-lymphocyte growth factor and inhibits autocrine and paracrine TNF induced proliferation (Luo et al. 1991). It plays a particularly important role in the up-regulation of expression of one of the isoforms of the CD23 antigen, CD23b, which is mainly found on B-CLL cells (Bonnefoy et al. 1995). In B-CLL patients, soluble CD23 levels provide additional prognostic information (Sarfati et al. 1996). IL-4 can also protect B-CLL cells against anti-APO1-induced apoptosis (Mainou-Fowler et al. 1995).

The increased intracellular IL-4 expression in B-CLL B- and T-lymphocytes found in this study can be explained in two ways. Firstly, B-CLL B-lymphocytes can secrete IL-4 in an autocrine fashion. Secondly, B-CLL T-lymphocytes can over-secrete IL-4 in a paracrine fashion, which is then internalised by the malignant B-lymphocytes, either through IL-4 receptors or in a non-receptor mediated fashion. Pre-incubation of B-CLL cells with anti-IL-4 R antibody prior to intracellular staining did not influence the percentage of the cells expressing intracellular IL-4. This suggests that any paracrine IL-4 pathway may not require significant IL-4R expression.

6.1.2. Intracellular expression of IL-4 in B-CLL and normal T lymphocytes

The data from this study has shown a significantly increased expression of intracellular IL-4 in B-CLL T-lymphocytes. Previous studies have reported a variety of abnormalities in the T-lymphocyte compartment in B-CLL (Kay et al. 1979, Gale et al. 1987). Untreated patients

have been shown to have a significant increase in T and NK cells, decreased T helper cells, increased T cytotoxic/suppresser cells and an inversion of the CD4 / CD8 ratio (Kay et al. 1979, Vuillier et al. 1988 and Gale et al. 1987). The pattern of cytokine secretion by T-lymphocytes is influenced not only by antigen but also by antigen presenting cells (Pharam, 2000) and since accessory cells in B-CLL are predominantly malignant B-lymphocytes, they greatly alter the immune function of T helper cells *in vitro*. In contrast, in normal individuals, the addition of antigen presenting cells, which are predominantly monocytes, resulted in a significant reduction in IL-4 (Decker et al. 1995). Multiple expansions of unique T cell clones may be derived in vivo from B-CLL tumour associated antigen stimulation (Farace et al. 1994). Interestingly Mu and his co-workers reported that freshly isolated CD4+ve and CD8+ ve T-lymphocytes from B-CLL patients had significantly elevated levels of intracellular IL-4 (Mu et al. 1997). However, a recent study has shown that CD4+ve cells in B-CLL have a reduced expression of intracellular IL-4 and INF-γ (Hill et al. 1999). An altered pattern of IL-4 production was also found in PHA stimulated CD2+ve cells from patients with B-CLL (Reyes et al. 1998).

Since studies of T-lymphocyte populations in B-CLL are sometimes conflicting and inconsistent, they may reflect the heterogeneity of the disease (Mu et al. 1997, Reys et al. 1998).

6.1.3. IL-4 receptor (IL-4R) expression in B-CLL and normal B and T lymphocytes

IL-4 acts via high affinity receptors expressed on a wide range of hematopoietic and non-hematopoietic cell lineages (Russell et al. 1993). Activated T-lymphocytes express high levels of IL-4R in response to IL-4 (Chromat et al. 1997).

This study has demonstrated that B-CLL patients have a greater percentage of CD3+ve T-lymphocytes expressing surface IL-4 receptors than T-lymphocytes from normal individuals. However CD19+ve B-lymphocytes from patients with B-CLL did not show this difference.

This may be further evidence that a paracrine IL-4 pathway may not be involved in IL-4-induced B-CLL B-lymphocyte survival.

IL-4 responses are induced through the high affinity receptor that is composed of an IL-4Rα chain and a γc chain. The γc chain is a functional subunit of the receptors for interleukins, 2, 4, 7, 9, and 15 and plays an important role in lymphoid development (Kondo et al. 1993, Russel et al. 1993, Noguchi et al. 1993 and Giri et al. 1994). Resting normal B-lymphocytes express on average 360 high affinity IL-4 receptors, and IL-4 may display different biological activities on resting and activated B-lymphocytes depending on the numbers of high affinity IL-4 receptors expressed (Zuber et al. 1990). Although the effect of IL-4 has been shown to be related to the expression of its receptor in solid tumours and resting T- and B- lymphocytes (Ohara et al. 1988), the data from this thesis shows that pre-blocking with anti-IL-4R antibody does not effect intracellular expression of IL-4.

In vitro experiments have shown that receptor expression is not necessarily an essential requirement for responsiveness to cytokines (Taga et al. 1997). Many cytokines stimulate cells through a complex receptor structure, which may involve a chain or chains primarily involved in cytokine binding and another chain that transduces the signal (Taga et al. 1997). One study analysed cytokine receptor expression in various types of leukaemic cells, but failed to demonstrate a direct association between the leukaemia type and cytokine receptor expression (Zola et al. 1994). However this study did not include a comparison with normal control counterparts. In another study, the same group showed that when cells were stimulated with IL-4 they failed to proliferate (Zola et al. 1997).

The significantly increased expression of IL-4 receptors on B-CLL T-lymphocytes could indicate an increased state of activation or a skew towards a Th2 response.

6.1.4. IL-4 protein secretion by B-CLL and normal cells

Intracellular IL-4 expression studies demonstrated that there was a significant difference in IL-4 production between PBMCs from patients with B-CLL and normal controls. In view of these results it was necessary to establish if PBMCs from patients with B-CLL were able to produce and release IL-4 and compared these results to normal controls. Several techniques can be used to measure the amount of cytokine secretion by stimulated cells. Enzyme-linked immunoabsorbent assay (ELISA) uses enzyme-labelled antibodies for detection of particular protein. Is a highly sensitive and specific assay and allows accurate measurements of very low protein levels (Bienvenue et al. 2000). Similarly enzyme-linked ImmunoSPOT (ELISPOT) or CellELISA can be used can be used to enumerate cytokine producing cells allowing detection of the protein of interest in the environment immediately surrounding the cell secreting it. In general this technique is more sensitive than sandwich ELISA and allows quantification of cytokines per cell basis (Bienvenue et al. 2000, Zhang et al. 2001). Recently very novel technique was developed by Zhang. This technique termed IDAT (immuno-detection amplified by T7 RNA polymerase), combines the peotein binding abilities of an antibody with the sensitivity of nucleic acid amplification technique (Zhang et al. 2001).

Hovewer, for the present study sandwich ELISA technique was used. Although expensive it allows very rapid quantification of PBMCs capacity to release IL-4 after mitogenic stimulation.

When the PBMCs from B-CLL patients were stimulated for 48 hours with PHA or PWM, no measurable amount of IL-4 protein was detected by ELISA after PWM stimulation and also in the majority of PHA-stimulated cells. In contrast all exept one normal control sample tested had measurable amouts of IL-4 after stimulation with PHA and PWM. It has been reported previously that B-CLL B-lymphocytes have an impaired proliferative response to mitogenic signals such as PWM (Catovsky et al. 1972, Karray et al. 1987). In addition, T-lymphocytes from B-CLL patients exhibit defective proliferative responses to PHA, however this response

was close to normal in patients with "smouldering B-CLL" (Gale et al. 1987, Prieto et al. 1993).

IL-4 on its own, displays anti-proliferative activity towards B-CLL cells. It inhibits autocrine and paracrine tumour necrosis factor (TNF) induced proliferation of B-CLL cells as well as spontaneous and TNF induced proliferation (van Kooten et al. 1992 and Reittie). Recombinant IL-4 profoundly inhibits the proliferative response of B-CLL cells to recombinant IL-2 (Karray et al. 1988). A recent observation that apoptosis-inducing toxins stimulated intracellular IL-4 expression in normal PBMC with decreased level of anti-apoptotic bcl-2 protein add additional complexity to the anti-apoptotic effect of IL-4 (Stein et al. 2000).

The inability to detect a measurable amount of IL-4 protein in stimulated PBMCs from B-CLL, despite the significant expression of intracellular IL-4 in both B and T lymphocytes is intriguing. It is possible that the IL-4 is secreted in an autocrine fashion by the B-CLL B-lympocytes or in a paracrine fashion by the B-CLL T-lymphocytes and immediately taken up by the B-CLL B lymphocytes to be used for their own survival. This hypothesis was tested further by Western Blotting studies. Using a Western blotting technique incorporating enhanced chemiluminescence, the presence of a strongly positive IL-4 band in B-CLL T-lymphocytes and very weakly positive bands in some B-CLL B-lymphocytes was found (data not shown). However, as with Northern blotting, Schena et al. were also unable to detect IL-4 protein by Western blotting (1992). One problem with Western Blotting is that it require large amount of protein. The very weak presence of IL-4 protein in some but not all purified B-CLL B-lymphocyte samples may be yet another indication of B-CLL heterogeneity These results may also indicate the presence of IL-4 protein internalised or bound to its receptor on malignant and normal B-lymphocytes.

6.2. ANALYSIS OF COMMON GAMMA CHAIN RELATED CYTOKINES BY NESTED RT-PCR

To understand better the biology of cytokines and the ability of the malignant and normal cells to produce cytokines, other techniques had to be applied. Several methods can be used to quantitate or semi-quantitated mRNA of the cytokines in question. Unfortunately Northern Blotting proved to be unsuccessful. The lack of positive results could be explained by a limited number of IL-4 producing cells. However, the percentage of CD19+ve cells expressing intracellular IL-4 was high in many patient samples. There is also a possibility that only a small number of mRNA copies were present in unstimulated cells or the prepared RNA was degraded ornot enough mRNA was present in the samples. RNA used in those experiments was a total RNA of each mRNA is only 1-2%. These results are in agreement with the study by Schena et al. in which they were unable to detect IL-4 mRNA using Northern blotting (1992). In no other studies known to the author was this technique used to detect mRNA in malignant B-CLL cells.

Other techniques such as Real-Time PCR, RNase Protection Assay, competetive PCR, RT-PCR or nested RT-PCR are all designed to quantitate or semi-quantitate RNA in question (Giulietti et al. 2001, Vet et al. 2002). Hovewer many of them are relatively new and require specialized equipment not evalible at the time in our laboratory. cDNA arrays method is still limited in its use by cost consideration (Butcher, 1999). Therefore, a nested RT-PCR technique was used to further study the presence of IL-4 mRNA in malignant B-lymphocytes, since initial question to be ansered was only the confirmation or absense of specific mRNA transcripts. RT-PCR has become an important technique for the detection of mRNA. It is the most sensitive and the most flexible of the quantification methods and can be used to compare the levels of mRNAs in differentsamles population, to descriminate between closely related RNAs. However the sensitivity of this technique can be improved by using a nested RT-PCR (Haff et al. 1994). This is because fewer non-specific amplification products are produced,

which could otherwise interfere with interpretation of the data. In addition, nested PCR lowers the threshold of target detection (Haff et al. 1994) as it is 10 000 times more sensitive than a single round of RT-PCR alone. Using a nested RT-PCR technique, the presence of mRNA transcripts of common gamma chain related cytokines were studied (section 2.12). To ensure the quality and the presense of equal amount of mRNA in all tested samples beta-actin was aplied. Beta actin is expressed at moderately aboundant levels in most cell types. It was the first RNAs to be used as a quantitive reference for RT-PCR assays (Bustin et al. 2000), despite the evidence that its levels vary widely. In addition , the presence of pseudogenes interferes with the interpretation of the results (Dirnhofer et al. 1995). In this study the presence of beta-actin mRNA was confirmed only after first round of amplification not permiting using beta-actin RT-PCR products to be used as a quantitive marker.

6.2.1. Measurement of IL-2 wild type RT-PCR products

The first of the common γc cytokines studied was IL-2. The expression of IL-2 wild type mRNA was analysed in unstimulated B- and T-lymphocytes from B-CLL patients and normal controls. Wild type IL-2 RT-PCR products (amplification between exon 1 and 4) were significantly more abundant in patient compared to normal B-lymphocytes. In addition, when amplification was performed between exons 1 and 3, the density of wild type IL-2 RT-PCR product density was also significantly higher in patient B-lymphocyte samples than in controls In contrast however, there was no significant difference in the density of IL-2 RT-PCR products in T-lymphocytes between patients and controls.

A previous study of the molecular expression of cytokines by normal and malignant B-lymphocytes showed that IL-2 production was limited to the actively proliferating germinal centre B cell blasts (Schena et al. 1992). Another study identified IL-8 mRNA transcripts but failed to identify IL-2, IL-3, IL-4, IL-5 and IL-7 (Di Celle et al. 1994). However leukaemic B-

lymphocytes stimulated with PMA, ionomycin and lipopolysaccharide can produce high amounts of IL-2 protein for non-T cells (Mouzaki et al. 1995).

The effect of IL-2 on apoptosis has also been well documented. Co-culture of normal and leukaemic B-lymphocytes with IL-2 results in proliferation and protection from apoptosis (Mainou-Fowler et al. 1995, Malkovska et al. 1987). This enhancement of B-lymphocyte viability and suppression of apoptosis was associated with a delay in the down regulation of the bcl-2 molecule (Tangye et al. 1997). IL-2 also enhances the production of tumour necrosis factor (TNF) and its receptor in activated B-CLL cells as well as expression of the CD23 type B antigen on malignant B-lymphocytes (Bonefoy et al. 1995). The CD23 molecule has been reported to play a role in normal B-lymphocyte proliferation and survival and TNF-α is thought to be an autocrine growth factor for B-CLL. In B-CLL, soluble CD23 is significantly increased (Larsson et al. 1993, Fournier et al. 1992, and Fournier et al. 1995). The idea that in B-CLL a paracrine or autocrine system of IL-2 production and utilisation may occur has been supported by the findings of an IL-2 like factor (IL-2lf) by PHA stimulated leukaemic B cells and that IL-2 and IL-2lf were able to use the same receptor (Giovarelli et al. 1988).

Thus the presence of anti-apoptotic IL-2 mRNA in malignant B-lymphocytes but not in their normal counterparts may indicate another strong candidate the production of which can contribute to the survival of malignant B-lymphocytes.

6.2.2. Measurement of IL-2 delta 2 RT-PCR products

The next cytokine studied was IL-2 δ2, which is a splice variant of the wild type IL-2. The density of IL-2 δ2 RT-PCR products was also found to be significantly higher in patient than in normal B-lymphocytes. Atamas et al. have shown that the IL-2δ2 splice variant inhibited IL-2 co-stimulation of T-lymphocyte proliferation and cellular binding of recombinant IL-2 to high affinity IL-2 receptors. (1997). This could be due to engagement of β and γc chains but not the α chain of the IL-2R complex (Atamas et al. 1997). Thus the altered expression of a

wild type and alternatively splice variant protein may lead to disease (Glare et al. 1990 and Sakkos et al. 1999). Since very little work has been performed to support results obtained in this thesis it can be speculated that the increased expression of the IL-2 splice variant mRNA transcripts in malignant B-lymphocytes may have regulatory effect on IL-2 wild type production. It was also shown that a positive significant correlation exists between IL-2 wild type mRNA expression and its splice variant in patient and control T-lymphocytes samples but not in malignant and normal B-lymphocytes. In another words, increased expression of wild type of IL-2 leads to the increased expression of splice variant type. Thus malignant B-lymphocyte may not be able to increase its IL-2 splice variant expression in response to IL-2 wild type expression.

6.2.3. Measurement of IL-4 wild type RT-PCR products

The next cytokine belonging to the γc chain family is IL-4. When primers spanning exons 1 and 4 were used, the density of wild type IL-4 RT-PCR product was greater in patient B-lymphocytes than in controls but this did not reach statistical significance. When primers spanning exons 1 and 3 were used, the density of wild type IL-4 RT-PCR products was significantly higher in patient B-lymphocytes than in controls. Therefore this difference in IL-4 wild type RT-PCR products when using different primers may be due to the fact that smaller size transcripts are much easier to identify or it may reflect the quality of the primers.

There are conflicting reports about the expression of IL-4 mRNA and protein by malignant B-lymphocytes. In some studies, IL-4 mRNA or protein could not be demonstrated in the germinal centre and normal mantle zone B-lymphocytes or in B-CLL cell samples (Mertz et al. 1990, Di Celle et al. 1994). In another, 11 of 16 B-CLL patient malignant B-lymphocytes were shown to express IL-4 mRNA and this expression has been confirmed in normal B-lymphocytes (Schena et al. 1992, Klein et al. 1996).

6.2.4. Measurement of IL-4 δ2 mRNA transcripts

IL-4 δ2 is a splice variant of the wild type IL-4. The expression of IL-4 δ2 RT-PCR products was significantly lower in patient T-lymphocytes than in controls but not significant when IL-4δ2 RT-PCR products from control and patient B-lymphocytes were compared. In addition a positive significant correlation was found between IL-4 wild type and IL-4 δ2 RT-PCR products in patients B lymphocytes. This means that the expression of one type increases the expression of the other type. It is then difficult to explain why in patients T-lymphocytes the expression of the splice variant is significantly lover than in controls but the IL-4 intracellular level is significantly higher than in their normal counterparts (Kaminski et al. 1998 and Kay et al. 2001).

A natural spliced variant of IL-4, IL-4 δ2 could represent yet another potential mechanism for the regulation of human IL-4 activity (Alm et al. 1996). In his study, the entire coding region of IL-4δ2 was detected in all PBMC tested as well as purified CD2+ve lymphocytes and was shown to increase after stimulation. However IL-4δ2 RNA was expressed in PBMC at lower levels than IL-4 RNA. Additionally, IL-4 δ2 was found to be expressed at much higher levels in thymocytes and bronchoalveolar lavage cells (Atamas, et al. 1996).

Recombinant IL-4δ2 does not act as a co-stimulator of T-lymphocyte proliferation but independently inhibits the action of recombinant IL-4 (Atamas et al. 1996). IL-4δ2 is a widely expressed isoform in many cell types and its expression seems to be tissue dependent (Atamas at al 1996). Previous studies have shown that IL-4 δ2 protein can inhibit the action of IL-4. This is thought to occur by competitive binding to the IL-4 receptor (Atamas et al 1996). It is known that protein variants formed by alternative splicing can inhibit the function of native protein (Kitamura et al. 1993). This is because alternative splicing generates structural and functional diversity of protein as well as differential expression in certain tissues (Boyd et al. 1993 and Huang et al. 1993).

Promoters, enhancers and regulatory elements within the IL-4 gene have been studied successfully (Lee et al. 1994, Todd et al. 1993 and LiWeber et al. 1993). It was shown that a positive significant correlation exists between wild type and splice variant RT-PCR products in malignant B-lymphocytes only. Presence of alternative splicing of the IL-4 gene and the inter-relationship between IL-4 wild type and IL-4 δ2 RT-PCR product densities adds to the complexity of IL-4 regulation but this study does not provide any evidence to explain how this phenomenon can affect the proliferation or apoptosis of the cells.

6.2.5. Measurement of IL-7 RT-PCR products

IL-7 is another important cytokine playing a role in B- and T-lymphocytes development and function and belongs to the γc chain receptor family. It is a stromal cell-derived growth factor (Welch et al. 1989). Normal B-lymphocytes do not make detectable quantities of IL-7 mRNA (Hickman et al. 1990). In this study, the existence of IL-7 mRNA transcripts in B- and T-lymphocytes of B-CLL patients and controls was detected. Patient B and T-lymphocyte samples had significantly increased mRNA transcripts when compared to their normal counterparts. This study also revealed several additional bands suggesting a number of additional spliced variants of IL-7 but attempts to sequence them proved to be inconclusive.

The entire coding sequence for IL-7, as well as an alternatively spliced IL-7 mRNA lacking exon 4 (IL-7δ4), was found to be transcribed in leukaemic B-lymphocytes but not in normal cells. Unexpected IL-7 amplification products were also found. Three additional spliced variants resulting from skipping of exon 3 and 5 or both in combination with exon 4, were demonstrated. They were named IL-7δ3/4, IL-7δ4/5 and IL-7δ3/4/5. In addition three out-of-frame spliced variants were identified: IL-7 (-56bp exon2), IL-7δ4 (-56bp exon 2), and IL-7 δ3/4/5 (-56bp exon 2), in which, in addition to exon skipping, 56bp of the 3' end of exon 2 were omitted (Korte et al. 1999). The significance of the spliced transcripts remains unknown. Malignant B-lymphocytes were also shown to express IL-7 receptors, suggesting that IL-7

could be relevant to the growth of leukaemic cells (Frishman et al. 1993). Some studies suggested that IL-7 could trigger proliferation in a variety of leukaemic cells, including CLL cells (Digel et al. 1991). In summary, B-CLL and control B-lymphocytes had a greater number of additional bands than T-lymphocytes. The existence of such a wide variety of spliced variants may be important for the regulation of IL-7 function and further studies are needed to investigate their involvement in the pathogenesis of B-CLL.

6.2.6. Measurement of IL-9 RT-PCR products

In this study, IL-9 nested RT-PCR products were not detected in B and T-lymphocytes separated from patients with B-CLL and normal controls. IL-9 is produced preferentially by Th2 lymphocytes and, although unstimulated freshly isolated PBMCs do not express IL-9 mRNA, stimulation with PHA or anti-CD3 antibody has been shown to induce substantial expression of IL-9 in CD4 T-lymphocytes (Renauld et al. 1990). In addition, expression of IL-9 has been shown to be restricted to the CD45RO+ve T-lymphocyte subset (memory cells) following stimulation with CD3 and CD28 (Houssiau et al. 1995). IL-9 expression is associated with HTLV-I, a retrovirus involved in adult T cell leukaemia and the existence of an IL-9-mediated autocrine loop has been suggested for Hodgkin's disease (Kelleher et al. 1991 and Renauld et al. 1995).

It is therefore surprising that it was not possible to detect IL-9 products in normal T-lymphocytes in this study. This could be due to number of reasons. Firstly, some faint bands were visible in patient B and T-lymphocytes and control T-lymphocytes after the first round of amplification. The results were negative in normal control B-lymphocytes. Secondly in unstimulated cells, IL-9 mRNA transcripts may be not very abundant. Lastly, despite many attempts to optimise IL-9 RT-PCR in this study, the optimal conditions for visualising IL-9 products may not have been achieved.

6.2.7. Measurement of IL-15 RT-PCR products

IL-15 is another member of the γc chain receptor family. IL-15 RT-PCR products were found in all tested samples, but they were significantly higher in control than in patient T-lymphocytes. Even though IL-15 mRNA is constitutively expressed in monocytes/macrophages but not on human T- and B-lymphocytes (also on cell lines; Mt4, M9, C5966, Jurkat, Daudi, Raji and Ebstein-Barr *virus-immortalised* B-cell clones) it can be upregulated by a variety of stimuli (Musso et al. 1999). In lymphocytes, IL-15 secretion is only found in pathological conditions such as chronic inflammation. Autocrine production of IL-15 was detected in myeloma cell lines as well as in cutaneous T-cell lymphoma (Tinhofer et al. 2000 and Asadullah et al. 2000). Constitutive gene expression of IL-15 was also found in bone marrow blasts from children with relapsed B-lymphocyte precursor acute *lymphoblastic* leukaemia (Kebelmann-Betzing et al. 2001). IL-15 has been found to be a potent survival factor in the prevention of spontaneous apoptosis but not CD95 induced apoptosis in CD8+ve and CD4+ve lymphocytes (Naora et al. 1999). IL-15 is not constitutively secreted and is possibly regulated at the transcriptional level (Onu et al. 1997). IL-15 has similar activities to IL-2. B-CLL cells stimulated with IL-15 plus thiorexin could be induced to proliferate. The stimulatory activity achieved by IL-15 was triggered through the IL-2 R system subunit since blocking the beta and gamma chains of the IL-2R resulted in decreased stimulatory activity of IL-15 (Trentin et al. 1996). IL-15 is able to trigger both proliferation and antibody production by normal B-lymphocytes, however this is acquired only after the B-lymphocytes have been pre-activated *in vitro*. In contrast, B-CLL cells are able to proliferate in response to IL-15 regardless of *in vitro* preactivation. This activity appears to be related to the presence of the α and γc chain of the IL-2R system on malignant cells (Trentin et al. 1997). Both IL-15 and its receptor selectively regulate differentiation of sIgM+, IgA- and IgM-, IgA+ B-1 cells (CD5 expressing B cells) expressing IL-15R, into IgA-producing cells in mucosal tissues (Hiroi et al. 2000). IL-15 can enhance antibody-dependent cellular cytotoxicity and natural killer (NK)

cell activity (Nguyen et al. 1998). It is also known that NK activity is impaired in B-CLL patients (Katrinakis et al. 1996). Thus decreased IL-15 mRNA transcripts in B-CLL T-lymphocytes may have a negative effect on patients NK cell activity as well as antibody-dependent cellular cytotoxicity which is already impaired by the lack of proper antibody producing B-lymphocytes.

The presence of such strong IL-15 mRNA transcripts in B- and T-lymphocytes of patients and controls in this study is of interest, since mRNA transcripts have not been detected in these cells before. However using a nested RT-PCR technique, amplification of very small amounts of mRNA is possible. The results obtained in this study were negative after the first round of amplification (data not shown). The intensive, and in many cases similar, density of the IL-15 mRNA transcripts after a second round of amplification may be also explained by the plateaux effect and needs to be studied further.

6.2.8. Measurement of γc chain and IL-4R αRT-PCR products

All the cytokines described above use the γc chain as part of their individual receptor. Thus studying the mRNA expression of this chain may shed some light on its role in cytokine regulation. Since IL-4 was the first cytokine looked at in this study, it was decided to also look for the presence of IL-4R α chain mRNA transcripts.

The analysis of full-length γc chain mRNA transcripts showed that there was no significant difference between patient and control B- and T-lymphocytes. The γc chain is constitutively expressed on essentially all cells of hematopoietic origin. Some studies have shown that the γc chain is present in purified normal tonsil B-lymphocytes and in all B-CLL patients but at lower levels of expression compared with that found in HCL (Totero et al. 1995). The presence of γc transcripts only in immature leukaemic B-lymphocytes adds to the complexity of receptor systems in regulation of lymphoid cell development and function (Totero et al. 1995). Two types of γc transcripts are expressed differing in their carboxyl terminal coding

regions. This newly identified transcript consists of a deletion of 72 nucleotides close to the 3'-end of the open reading frame. The result is a loss of 24 amino acids, which includes a conserved tyrosine residue shared by several members of the cytokine receptor family (Shi et al. 1997). The importance of this new variant is as yet unknown.

IL-4Rα mRNA transcripts were detected in all patient and control samples. However, no significant difference in RT-PCR product densities was found between patient and control B and T-lymphocytes. This study has also demonstrated that B-CLL patients have a greater percentage of CD3+ve T-lymphocytes expressing surface IL-4 receptors than T-lymphocytes from normal individuals. This discrepancy between the flow cytometric and RT-PCR results may come from the fact that flow cytometry only shows the percentage of lymphocytes expressing IL-4R but not the density of the receptor on their surface. However, this may be further evidence that a paracrine IL-4 pathway may not be involved in IL-4-induced B-CLL B-lymphocyte survival.

6.3. MEASUREMENT OF IL-5 AND IL-13 RT-PCR PRODUCTS

Finally, IL-5 and IL-13 were studied. The reason for this was that IL-5 was shown to act as a pro-apoptotic factor that acts on malignant B-CLL cells (Mainou-Fowler et al 1996). IL-13 uses the IL-4R α chain as part of its own receptor and is also anti-apoptotic for malignant B cells (Tangye et al, 1997).

A nested RT-PCR technique detected the presence of IL-5 and IL-13 mRNA transcripts in B- and T-lymphocytes of B-CLL patients and normal controls but no significant difference was found between the corresponding samples. However, patient B-lymphocytes had more abundant IL-5 mRNA transcripts when compared to normal B cells. Similarly to other cytokines, the secretion of IL-5 and IL-13 is associated with the Th2 cell subset and, in conjunction with IL-4, IL-5 and IL-13, are organised within 140 kb of DNA in humans (Kelly et al. 2000). Only one study looked for the presence of IL-5 mRNA in B-CLL cells but was

unable to find any (di Celle et al. 1994). However the role of IL-5 in controlling B-CLL proliferation has been studied. In one study, B-CLL cells were found to have an impaired response to IL-5 (Hayes et al. 1993). This was evident by the lack of IgM production when IL-5 was added to B-CLL cells. However, other studies have shown that IL-5 increased spontaneous apoptosis of B-CLL cells in a probably bcl-2 independent manner (Mainou-Fowler et al. 1994, 1995). The detection of IL-5 transcripts in patient and control B-lymphocytes in our study may be the result of using nested RT-PCR since the results obtained after the first round of amplification showed negative results with normal B-lymphocytes but positive bands with patient B lymphocytes. The question arises, if IL-5 plays a role as a pro-apoptotic factor, why do the malignant B-lymphocytes have high density IL-5 mRNA transcripts? This is difficult to explain unless the malignant cells have indeed impaired IL-5 responses (Hayes et al. 1993).

The role of IL-13 in B-CLL has also been tested, but the presence of mRNA transcripts in B-CLL cells is not documented. Similarly to IL-2 and IL-4, IL-13 suppresses apoptosis of leukaemic CD5+ve B-lymphocytes and preserves the expression of bcl-2 (Tangye et al. 1997). IL-13 also inhibits IL-2 induced proliferation although this may dependent on the experimental conditions (Fluckinger et al. 1994). Similarly to IL-4, IL-13 enhances CD23 expression as well as soluble CD23 secretion by B-CLL cells (Chaouchi et al. 1996). Thus the presence of IL-13 mRNA transcripts at similar densities in both malignant and normal B-lymphocytes, as well as patient and normal T-lymphocytes, is difficult to explain since IL-13 transcripts were faintly positive after a first round of amplification in the majority of B-CLL B-cell samples and completely negative in all normal B-lymphocyte samples (data not shown). One could speculate that similarly to IL-4, IL-13 secretion is tightly regulated and a small amount of protein is needed for the execution of its action.

6.3.1 Correlation between cytokine RT-PCR product density

When all the molecular cytokine data were colated and analysed, a significant positive correlation was demonstrated between IL-2 and IL-4 RT-PCR product densities in B-CLL B- and T-lymphocytes populations. In contrast, a significant negative correlation was found between IL-2 and IL-4 RT-PCR product densityies in normal T-lymphocyte samples. In addition, a significant correlation was found between IL-4 and IL-7 mRNA transcript densities in patient T-lymphocyte samples.

The T-lymphocyte encounter with antigen leads to the development of a Th1 or Th2 polarised subset (Mossmann, 1996). Th1 cells produce IL-2 and INF-γ, while Th2 cells produce IL-4, IL-5 and IL-13. It is possible that the negative correlation between IL-2 and IL-4RT-PCR product densities seen in normal T-lymphocytes is an indication of T-lymphocyte polarisation. Contrary to normal T-lymphocytes, B and T-lymphocytes from patients with B-CLL displayed a positive correlation between the above cytokines. When mitogen (PHA and OKT3) stimulated T-lymphocytes were measured for their capacity to produce IL-2 and IL-4 protein, no correlation was found between the IL-2 and IL-4 protein levels, in particular the expected inverse correlation was not found (Cartwright et al. 2000). Thus the positive correlation shown between IL-2 and IL-4 RT-PCR product densities in patients with B-CLL may indicate that in malignant cells regulation of IL-4 and IL-2 mRNA expression is deregulated when compared to normal cells.

Although, no significant correlation was seen between IL-4 and IL-13 product densities, a noticeable positive trend was demonstrated in patient B and control T-lymphocytes. IL-4, IL-13 and IL-5 are markers for the Th2 subset of effector T cells and are often expressed together (Kelly et al. 2000; Takemoto et al. 2000). It is therefore not surprising that activation of one cytokine induces the others. What is however surprising is that this pattern is seen in malignant B-lymphocytes. In this respect malignant B-lymphocytes are more similar in their behaviour and cytokine production to T than to B-lymphocytes. These positive and T-

lymphocyte-like correlations of cytokines production may be important for malignant B-lymphocyte survival.

6.4. THE EFFECT OF ANTISENSE OLIGONUCLEOTIDES ON THE SURVIVAL OF B-CLL CELLS

6.4.1 Measurement of proliferation and viability of B-CLL and normal cells by MTT assay

In view of the fact that higher densities of IL-4 and IL-2 RT-PCR were demonstrated in malignant B lymphocytes it was decided to attempt to block these cytokines and their splice variants in an attempt to intefere with their anti-apoptototic effects.

The MTT assay was used to investigate the proliferative abilities of B-CLL and normal control cells after the addition to the cultures of antisense oligonucleotide (ONs). Spectrophotometrically quantified changes in cell proliferation showed that the addition of IL-2 and IL-4 antisense ONs decreased B-CLL cell viability compared to controls. However the experiments aiming to establish the optimal concentration of antisense ONs were not very consistent as far as the different ONs were concerned. Common methods for determining cell viability depend upon membrane integrity (e.g. trypan blue exclusion) or incorporation of nucleotides during cell proliferation (3H-thymidine). These methods are limited by their impracticality in processing large numbers of cell samples. The MTT assay provides a rapid and versatile method for assessing cell viability. Studies using the MTT assay showed excellent linearity between absorbance and leukaemic or other cell numbers viability (Alley et al. 1988 and Van de Loosdrecht et al. 1994). These were the reasons for using MTT assay in this study. However the MTT assay has drawbacks. Unless the experiment is performed using a pure cell population it is impossible to establish exactly which cell is undergoing proliferation or apoptosis. A more specific test was thus needed to answer these questions.

6.4.2 Measurement of apoptosis by Annexin V and Propidium iodide staining

In this part of the study, flow cytometry was used to analyse the percentage of CD19+ve and CD3+ve B-CLL and normal control cells undergoing apoptosis after incubation with antisense ONs. A series of antisense ONs were specially designed to attempt to suppress expression of IL-2 and IL-4 wild type and IL-2 and IL-4 spliced variant mRNA transcripts. The results show that all cytokine specific antisense ONs and control antisense ONs are able to induce apoptosis in patient CD19+ve lymphocytes. Using IL-2b, IL-2c and IL-2d antisense ONs, the percentage of apoptotic double positive CD19/Annexin V cells was significantly increased. This increase in apoptosis coincided with the suppression of PHA-stimulated IL-2 protein production after the addition of IL-2 and IL-4 ON, as measured by ELISA. However, IL-4 protein was increased after the addition of IL-4 antisense. Apoptosis was also unexpectedly induced by control antisense ONs. Since all the antisense were checked against GenBank Sequence Data to avoid similarity with any other human mRNA, this evidence suggests that increased apoptosis is at least partially due to a non specific effect of phosphorothioate oligonucleotides. However, it is also conceivable that IL-2 antisense ON has supressed an IL-2 autocrine ant-apoptotic pathway.

Since IL-2 has been shown to act as an anti-apoptotic cytokine for malignant B-lymphocytes, this "non-specific" effect of antisense ONs can be partially related to the decrease in IL-2 expression. Most B-lymphocytes immortalised with EBV virus, as well as normal stimulated B-lymphocytes, can be induced to produce IL-2 protein (Mouzaki et al. 1995). Recently, human carcinoma cell lines have also been shown to constitutively express protein and mRNA for IL-2 *in vivo* and *in vitro,* attributed to the regulation of cell cycle progression and protection from apoptosis (Reichert et al. 2000).

The concept of an increased or decreased balance between wild type mRNA and IL-4 spliced variant IL-4 δ2 comes from studies in type I diabetes as well as systemic sclerosis and stable asthma (Sakkos et al. 1999 and Glare et al. 1999). It has been suggested that these changes in

alternative splicing may lead to disease (Boyd et al. 1993). However, although successful usage of antisense to block IL-2 and IL-4 mRNA and in consequence IL-2 and IL-4 protein have been published, this is the first study to use antisense to block cytokine spliced variants at the exon-exon junction level. In view of the fact that IL-2 and IL-4 cytokines are able to prevent programmed cell death (Huang et al 1993, Panayiotidis et al. 1993), it seems logical to develop methods to modulate the secretion of IL-2 or IL-4 protein by malignant cells, which may in turn induce apoptosis.

The concept behind the use of antisense is simple in that the ON is provided to inhibit the expression of a single gene product at the mRNA level. However evaluating the effect of antisense proved to be a very complicated procedure. Phosphorothioate antisense ONs are internalised and distributed in a manner, which is dependent on time, temperature, concentration, sequence and cell line (Crooke et al. 1995). Lymphoid sub-populations also differ in their ability to uptake ONs. B-lymphocytes take up ONs one third more readily than T-lymphocytes and this uptake is increased with mitogenic stimulation (Krieg et al. 1991).

One of the major concerns is that the body might recognise antisense ONs as foreign and attack them. Yamamoto and his colleagues identified palindromic single-stranded immunostimulatory DNA sequences including CpG (Cytosine-phosphate-Guanine) containing hexamers, 5'-GACGTC-3', 5'-AGCGCT-3' and 5'-AACGTT-3' and concluded that bacterial DNA containing these sequences can activate the immune system (Yamamoto et al. 1992 and Yamamoto et al. 1994). Experiments have shown that ON fragments containing a two-base sequence, CpG, activated mammalian B and natural killer cells in culture (Krieg et al. 1995). However this immune response was only triggered when the CpG sequence was un-methylated. It is hypothesised that because the un-methylation is very common in bacterial DNA but not in mammalian DNA, un-methylated ON sequences may be seen as bacterial and stimulate an immune response (Wagner et al 1999, Stein 1996 and Engelhard et al. 1998). However, the antisense ONs used in this study did not contain CpG sequences.

The other common side effect of antisense ON treatment in animal models is a drop in the white blood cell count. This side effect can be attributed to the inability of some antisense ONs to correctly bind to the target cell or tissue. They can bind to various proteins within the vicinity of the targeted region thus inhibiting production of certain crucial proteins important within the body (Ma et al. 1996, Tidd, 1992, Wagner 1994, Engelhard et al. 1998).

Because ONs are negatively charged they can bind to other positively charged molecules. They are able to bind bovine serum albumin, receptor for platelet derived growth factor, the receptor for basic fibroblast growth factor, gp120 protein of the human immunodeficiency virus as well as VEGF, PKC and protein tyrosine receptors including the epidermal growth factor receptors (Crooke et al 1996, Stein, 1995, Stein et al. 1993, Stein et al. 1994, Ma et al. 1996, Ho et al. 1997, Stein et al 1996, Rockwell et al. 1997). All this happens in a sequence independent manner. It has been also reported to cause non-specific induction of tumour necrosis factor (Hartmann et al. 1996), induction of Sp1 nuclear transcription factor binding activity (Crooke et al. 1996, Perez et al. 1994) and inhibition of transferrin receptor expression (Ho et al. 1991). ON-mediated Sp1 protein induction has been shown to be rapid and independent of NF-kappa B activity (Perez et al. 1994). However non-specific effects of phosphorothioated ONs were usually encountered in the 20-50 μM range which is higher than the concentration used in this study.

A series of experiments specially designed to determine whether various ONs, including phosphorothioated (sODNs) and phosphodiesters (ODNs), could activate human B-lymphocytes directly have shown that sODNs were able to induce significant human B-lymphocyte proliferation (Liang et al. 1996). Proliferating normal B-lymphocytes had increased expression of activation markers such as CD69, CD86 and CD25 and produced IgM, IgG and IgA in the absence of exogenous cytokines and T-lymphocytes (Liang et al. 1996). Another study has shown that when normal B-lymphocytes are exposed to antisense ONs, a large number of B-lymphocytes can be induced to express activation markers despite only a

modest degree of proliferation suggesting that many of the cells were initially activated, but few underwent clonal expansion (Liang et al. 2000). Furthermore, many of the originally activated B-lymphocytes underwent apoptosis rather than clonal expansion. Thus increased percentage of the CD19+ve malignant lymphocytes after incubation with antisense ONs may be initially activated.

6.4.3. The effect of antisense ONs on intracellular IL-2 and IL-4 expression

In order to investigate the effect of ONs on B- and T-lymphocytes from patients with B-CLL, the CD19+ve and CD3+ve lymphocytes were stained for the presence of intracellular IL-2 and IL-4. The results showed that the percentage of unstimulated CD19+ve and CD3+ve lymphocytes expressing intracellular IL-2 and IL-4 decreased after incubation with IL-4c antisense ON. In contrast, in PMA-stimulated cells, the percentage of CD19+/IL-2+, CD3+/IL-2+ and CD3+/IL-4+ cells increased after incubation with IL-4c antisense ON. This increase in the percentage of double positive cells may indicate that PMA-stimulation can overcome suppressive ability of IL-4c antisense ON. IL-4c antisense was specially designed to suppress IL-4 δ2 mRNA. Thus, influencing the percentage of the cells expressing IL-2 appears to be either a side effect of PTO-modified ONs or as a specific effect of a manipulation of IL-4 expression. However if IL-4c antisense ON specifically inhibits the expression of IL-4 δ2 mRNA, and if a positive correlation exists between IL-4 wild type and IL-4 splice variant then possible reduction in IL-4 wild type protein should be seen. This observation is not supported by the increase in IL-4 protein level when PHA-stimulated B-CLL cells were incubated with IL-4c antisense ON. Thus the regulatory network that exists between cytokines and their splice variants is much more complicated and requires further studies.

6.5. FINAL CONCLUSION

The results presented in this thesis firstly suggest that the presence of intracellular IL-4 in malignant B-lymphocytes may be a contributory factor in the pathogenesis of B-CLL. However, the question still remains as to the source of the IL-4. Attempts have been made to evaluate the presence of IL-2 and IL-4 protein and their mRNA transcripts using Western and Northern blotting technique (data not shown). However they were aboundand no conclusisve results were obtained. In addition Northern blotting aldough sensitive is not commonly used to detect low frequency transcripts. In addition higher quantity of oligo-dT separated mRNA are needed (RNA obtained from malignant and normal samplesin this thesis, represents total RNA).

The discrepancy between the absence of protein as measured by ELISA (Kaminski et al. 1998) and Western blotting (data not shown) and the presence of mRNA transcripts in normal and malignant cells could be due to a number of reasons. Firstly, the cells may be unable to translate IL-4 mRNA into protein. Secondly, the protein may be labile and degraded faster in B-CLL compared to normal cells. Thirdly, the protein may be released and immediately bound to malignant B-lymphocytes in an autocrine fashion. Finally there may be a direct interaction between IL-4 and IL-4δ2 transcripts that prevents the translation of the former into protein.

The nested RT-PCR technique used, detected RT-PCR products of cytokines, normally not associated with B-lymphocytes (IL-2 and IL-4), to be significantly increased in malignant B-lymphocytes. Other cytokines such as IL-7 and IL-15 were significantly increased in B-CLL B and T-lymphocytes and normal T-lymphocytes respectively. The nested RT-PCR technique is very sensitive and can detect very small quantities of mRNA. Therefore, in addition to detecting mRNA from the cells of interest, there is a theoretical possibility of amplifying mRNA from small numbers of contaminating cells.

In addition, there are differences in the expression of both IL-2 and IL-4 spliced variants that in turn might influence the expression of the wild type protein. The fact that many of these

cytokines are anti-apoptotic is likely to be of significant importance in the development and or survival of leukaemic B-lymphocytes. Identification of ways of manipulating or interfering with individual cytokines at either the protein or mRNA level could lead to new therapeutic modalities for B-CLL.

The expression of typically T-lymphocyte mRNA transcripts in malignant B-lymphocytes may provide additional information about the biology of those cells. It can be speculated that malignant B-lymphocytes represent a very specific subset of the B–lymphocyte population that, in the process of malignant change, manage to transcribe sets of genes normally active in T-lymphocytes. By doing so they might become self-regulatory and to a certain extent self-sufficient. It is well known that all malignant B-CLL cells are CD5+ve, a marker normally specific for T-lymphocytes and a minority subset of normal B-lymphocytes. Human CD5+ve B-lymphocytes are present in neonatal lymphoid tissue and their frequency decreases with age. In adults, CD5+ve B-lymphocytes are present in the germinal centre and mantle zone. The number of CD5+ve B-lymphocytes is increased in patients with autoimmune disorders and these cells, which are similar to those in CLL, may be induced to produce multi-specific autoantibodies. Some antigens are also able to activate CD5+ve cells (Dutra et al. 2000). However those B-lymphocytes are not malignant. In fact, when CD5+ve and CD5-ve cells were stimulated with surrogate T-dependent and T-independent signals they converged to indistinguishable phenotypes (Gagro et al. 2000). It is thus possible, that CD5+ve B-lymphocytes are at the crossroads of B-cell malignancy and non-organ specific autoimmunity (Youinou et al. 2000). In addition, there are cases of CD8+ve expression on B cells in chronic lymphocytic leukaemia (Islam et al. 2000). In retrospect, much more informative would be the investigation of pure populations of CD5+ve normal B-lymphocytes (10-30% in normal individual) compared with malignant CD5+ve B-lymphocytes.

Finally, as already mentioned, the theory of antisense in suppressing expression of a particular gene is simple, but proving that this is a gene-specific event is much more difficult. Although

all IL-2 and IL-4 antisense ONs used in this study were able to increase the percentage of Annexin V/Propidium as well as CD19+ve/Annexin V positive cells as well as decrease the percentage of intracellular expression of IL-4 and IL-2 in CD3+ve and CD19+ve cells, control antisense had a similar effect. The above effect was obtained without using cationic lipids as a carrier. Thus further studies using IL-2 and IL-4 antisense ONs need to be more specific in designing antisense, as well as further usage of cationic lipids as antisense carriers. Regardless of the fact that antisense ONs work via specific or non-specific mechanisms, they may be of potential therapeutic value in B-CLL.

6.6. FUTURE WORK

In view of the possible therapeutic implications of the findings described in this thesis, and the fact that some of the results are in conflict with those of other authors, certain experiments need to be repeated. These include IL-2, IL-4 and IL-15 mRNA expresion in B-CLL B and T lymphocytes. As mentioned above, it would be more informative to compare pure populations of CD5+ve normal and maliganant B-lymphocytes. In addition, it is necessary to select the most appropiate antisense ON as well as choosing optimal cariers. Once these have been determined, further experiments using these antisense ONs to intefere with the function of the IL-2, IL-4 and IL-15 should be carried out. Finally, to determine the functional effects of antisense ONs, further experiments need to be designed; eg measurement of apoptosis or bcl-2 expression.

APPENDICES

Appendix 1: Intracellular IL-4 expression in CD3+ and CD19+ lymphocytes from patients with B-CLL and normal controls

Number of patients	%CD19+/IL-4+ (unstimulated) (I)	% CD3+/IL-4+ (unstimulated) (II)	Number of controls	%CD19+/IL-4+ (unstimulated) (III)	%CD3+/IL-4+ (unstimulated) (IV)
1	30.0	21.6	1	5.1	2.2
2	21.6	26.2	2	4.7	3.0
3	28.8	13.5	3	5.4	1.8
4	18.3	11.2	4	10.1	1.7
5	53.8	5.4	5	9.9	4.2
6	21.6	2.6	6	1.5	3.8
7	14.8	7.2	7	6.8	3.4
8	6.4	2.6	8	8.2	0.4
9	17.4	3.2	9	11.9	1.0
10	11.3	2.2	10	8.3	0.9
Mean SD+/-	**22.4 SD+/- 13.1**	**9.6 SD+/- 8.5**		**7.2 SD+/- 3.1**	**2.2 SD+/-1.3**
Variable: I/III p=0.03			**Variable: II/IV p=0.01**		

The data is presented as the percentage of CD19+ve and CD3+ve lymphocytes expressing intracellular IL-4. Unstimulated PBMCs were stained with anti-CD19 FITC and anti-IL-4 PE or anti-CD3 FITC and anti-IL-4 PE conjugated monoclonal antibodies and immediately analysed by flow cytometry. In each experiment if possible 10,000 CD19+ve or CD3+ve lymphocytes were collected. IL=Interleukin, %=Percentage, SD=Standard deviation

Appendix 2: Surface IL-4R expression in CD19+ve and CD3+ve lymphocytes in patients with B-CLL and in normal controls

Number of patients	%CD19+/ IL-4R+ (I)	%CD3+/ IL-4R+ (II)	Number of controls	%CD19+/ IL-4R+ (III)	%CD3+/ IL-4R+ (IV)
1	97.1	2.7	1	53.0	1.1
2	42.9	3.7	2	61.5	0.8
3	28.2	0.6	3	82.5	0.6
4	11.2	1.5	4	74.8	2.5
5	70.5	6.8	5	64.9	0.8
6	56.3	3.2	6	88.4	3.3
7	70.0	3.5	7	69.1	1.4
8	83.4	6.1	8	69.6	1.8
9	20.5	5.2	9	82.2	3.7
10	96.9	7.8	10	41.6	1.0
Mean	**57.7**	**4.1**		**68.7**	**1.7**
SD+/-	**SD+/- 31.0**	**SD+/- 2.3**		**SD+/- 14.3**	**SD+/- 1.1**
Variable: I/III			**Variable:II/IV**		
p=ns			**p=0.001**		

The data is presented as the percentage of CD19+ve and CD3+ve lymphocytes expressing surface IL-4-R. Unstimulated PBMCs were stained with anti-CD19 FITC and anti-IL-4R PE or anti-CD3FITC and anti-IL-4R PE conjugated monoclonal antibodies and immediately analysed by flow cytometry. In each individual experiment if possible 10,000 CD19+ve or CD3+ve lymphocytes were collected.
%=Percentage, IL=Interleukin, SD=Standard deviation, ns=Not significant

Appendix 3: Densitometry results of IL-4R α chain RT-PCR products

IL-4 Receptor α chain			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
41.9	45.9	20.1	47.5
46.6	48.6	43.0	47.9
41.4	48.1	31.7	48.6
41.4	42.4	35.9	43.0
41.5	43.8	32.4	43.8
40.4	49.4	39.1	44.1
38.4	41.8	41.7	44.2
32.5	40.3	42.7	43.3
		38.6	45.2
		39.8	42.6
Mean: 40.5	Mean: 45.0	Mean: 36.5	Mean: 45.0
SD+/- 3.9	SD+/- 3.4	SD+/- 6.9	SD+/- 2.2
Variable: B patients/ B control (t-test)		p=ns	
B patients/ B control mean values ratio:		1 : 0.9	
Variable: T patients/ T controls (t-test)		p=ns	
T patients/ T controls mean values ratio:		1: 1	
Variable: B patients/ T patients (t-test)		p=ns	
B patients/ T patients mean values ratio:		1: 0.5	
Variable: B controls/ T controls (t-test)		p=0.009	
B controls/ T controls mean values ratio:		1: 1.2	

Appendix 3 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 3.10. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 4: Densitometry results of γc receptor RT-PCR products

Common gamma chain receptor			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
1740.5	1601.3	2490.6	1749.3
2343.0	2094.0	1381.0	2256.3
2341.1	2060.6	1905.0	1003.9
2547.1	2164.9	1554.0	1404.3
2413.3	1353.2	2214.3	2797.6
2371.0	2372.2	1753.3	2275.3
2619.5	2177.8	2604.3	2595.7
2483.1	2024.0	2473.8	2027.4
881.2	2231.2	2301.7	610.0
830.9	2437.0	1575.9	2212.3
1860.2	2528.2		
Mean: 2039.2	Mean: 2094.9	Mean: 2025.4	Mean: 1893.2
SD+/- 644.0	SD+/- 348.1	SD+/- 446.2	SD+/- 699.7
Variable: B patients/ B control (t-test)			p=ns
B patients/ B control mean values ratio:			1 : 1
Variable: T patients/ T controls (t-test)			p=ns
T patients/ T controls mean values ratio:			1: 0.9
Variable: B patients/ T patients (t-test)			p=ns
B patients/ T patients mean values ratio:			1: 1
Variable: B controls/ T controls (t-test)			p=ns
B controls/ T controls mean values ratio:			1: 0.9

Appendix 4 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.6. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 5: Densitometry results of the IL-2 RT-PCR products

IL-2 (primers spanning exon 1 to 4)			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
1787.0	2272.9	51.2	1987.2
59.7	254.5	42.9	1766.5
77.1	2054.1	12.4	1843.7
10.1	1589.2	21.8	1739.8
75.5	0	53.7	1793.2
1428.7	82.2	10.0	2027.1
1802.3	1734.0	0	1842.4
75.5	1691.2	13.3	252.8
233.6	1745.8	49.1	1916.6
84.0	1860.5	66.4	2001.2
102.7	136.3		
Mean: 521.09	Mean: 1220.0	Mean: 22.9	Mean: 1717.0
SD+/-747.0	SD+/- 894.5	SD+/- 18.71	SD+/- 524.3
Variable: B patients/ B control (t-test)			p=0.04
B patients/ B control mean values ratio:			1 : 0.04
Variable: T patients/ T controls (t-test)			p=ns
T patients/ T controls mean values ratio:			1: 1.4
Variable: B patients/ T patients (t-test)			p=0.35
B patients/ T patients mean values ratio:			1: 2.3
Variable: B controls/ T controls (t-test)			p=0.002
B controls/ T controls mean values ratio:			1: 75

Appendix 5 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.8. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 6: Densitometry results of IL-4 RT-PCR products

IL-4 (primers spanning exon 1 to 4)			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
416.7	404.9	5.2	3.6
7.7	13.9	1.3	605.8
205.0	181.6	7.1	399.5
7.6	3.86	0	689.7
26.1	29.1	221.0	743.6
89.3	43.7	13.7	4.8
118.6	573.0	67.1	418.0
32.8	487.6	18.0	193.1
309.5	284.6	133.3	0
141.2	673.8	45.9	319.2
45.2	36.7		
Mean: 127.2	Mean: 252.1	Mean: 51.3	Mean: 337.7
SD+/- 133.9	SD+/- 249.6	SD+/- 72.6	SD+/- 284.5
Variable: B patients/ B control (t-test)			p=ns
B patients/ B control mean values ratio:			1 : 1
Variable: T patients/ T controls (t-test)			p=ns
T patients/ T controls mean values ratio:			1: 0.9
Variable: B patients/ T patients (t-test)			p=ns
B patients/ T patients mean values ratio:			1: 1
Variable: B controls/ T controls (t-test)			p=ns
B controls/ T controls mean values ratio:			1: 0.9

Appendix 6 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.10. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 7: Densitometry results of IL-7 RT-PCR products

IL-7			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
2061.0	2171.2	1218.9	1547.9
2248.5	2111.0	934.5	225.4
2511.1	2844.2	423.8	20.1
465.1	800.2	1885.7	2250.2
1335.4	3180.0	2610.8	971.7
268.3	3103.1	113.1	2827.3
1244.9	3128.2	1092.1	555.1
1409.4	2975.0	2549.7	482.0
1619.8	2743.6	1570.8	3349.7
2693.0	3229.5	0	2617.1
1750.0	1000.0		
Mean: 1600.6	Mean: 2480.5	Mean: 1239.9	Mean: 1484.6
SD+/- 772.8	SD+/- 868.9	SD+/- 925.5	SD+/- 1202.6
Variable: B patients/ B control (t-test)		p=0.03	
B patients/ B control mean values ratio:		1 : 0.8	
Variable: T patients/ T controls (t-test)		p=0.04	
T patients/ T controls mean values ratio:		1: 0.6	
Variable: B patients/ T patients (t-test)		p=0.02	
B patients/ T patients mean values ratio:		1: 1.6	
Variable: B controls/ T controls (t-test)		p=ns	
B controls/ T controls mean values ratio:		1: 1.2	

Appendix 7 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.12. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 8: Densitometry results of IL-15 RT-PCR products

IL-15			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
2293.6	1974.9	1813.5	2529.8
1949.4	1702.2	1861.4	2470.6
1886.6	1613.3	1788.1	2242.7
1660.6	1618.9	1731.9	1698.4
1990.7	1688.7	1823.8	2267.6
1736.6	1730.3	1855.1	2290.4
1746.0	1208.9	1739.5	2362.1
1759.5	1603.5	1626.9	2506.4
1964.2	1169.6	1626.4	2396.0
1545.8	986.5	1905.1	2032.4
1945.4	459.1		
Mean: 1861.7	Mean: 1432.4	Mean: 1777.2	Mean: 2279.6
SD+/- 201.1	SD+/- 433.9	SD+/- 95.4	SD+/- 251.9
Variable: B patients/ B control (t-test)		p=ns	
B patients/ B control mean values ratio:		1 : 1	
Variable: T patients/ T controls (t-test)		p=0.001	
T patients/ T controls mean values ratio:		1: 0.6	
Variable: B patients/ T patients (t-test)		p=0.007	
B patients/ T patients mean values ratio:		1: 1.8	
Variable: B controls/ T controls (t-test)		p=0001	
B controls/ T controls mean values ratio:		1: 1.3	

Appendix 8 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.14. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 9: Densitometry results of IL-2 and IL-2 δ2 RT-PCR products

IL-2 (primers spanning exon 1 to 3)							
IL-2 wild type band				IL-2 spliced variant (δ 2) band			
Patients		**Controls**		**Patients**		**Controls**	
B lymph.	T lymph.	B lymph.	T lymph.	B lymph.	T lymph.	B lymph.	T lymph.
249.6	241.2	15.2	229.0	46.6	138.2	0	135.0
215.9	67.2	31.3	240.4	0	0	0	123.6
109.5	231.5	45.2	238.5	0	65.9	0	70.1
168.5	197.5	73.5	268.1	0	44.4	5.3	122.8
96.3	142.9	53.5	202.1	21.8	5.3	0	121.9
200.5	147.4	109.6	236.8	34.2	6.7	0	172.4
217.3	274.4	81.1	174.5	0	85.4	0	18.7
138.9	216.3	91.4	180.1	0	41.1	0	22.2
229.6	135.4	0	157.2	22.4	39.8	0	19.6
21.4	225.9	29.2	30.3	0	0	0	0
249.2	48.3			19.2	0		
Mean: 172.4 SD+/- 73.2	**Mean:** 175.3 SD+/- 79.0	**Mean:** 53.0 SD+/- 35.3	**Mean:** 195.7 SD+/- 67.9	**Mean:** 13.1 SD+/- 16.7	**Mean:** 38.8 SD+/- 44.1	**Mean:** 0.53 SD+/-1.6	**Mean:** 80.6 SD+/-61.7
t-test **Variable: B patients/ B controls** p=0.0001 Ratio: 1: 0.31 **Variable: B patients /T patients** p=ns Ratio: 1 : 1				**t-test** **Variable: B patients/ B controls** p=0.03 Ratio: 1: 0.04 **Variable: B patients/T patients** p=ns Ratio: 1: 3			
t-test **Variable: T patients/ T controls** p=ns Ratio: 1: 1.1 **Variable: B controls/ T controls** p=0.001 Ratio: 1: 3.7				**t-test** **Variable: T patients/ T control** p=ns Ratio 1: 2 **Variable: B controls/T controls** p=0.003 Ratio: 1 : 152			
Spearmans' correlation test **Variable: B patients wild type/B patients splice variant** p=ns **Variable: T patients wild type/T patients splice variant** r=761 p=0.006 **Variable: B controls wild type/B controls splice variant** p=ns **Variable: T controls wild type/T controls splice variant** r=758 p=0.01							

Appendix 9 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.19. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant, lymp=Lymphocytes

Appendix 10: Densitometry results of IL-4 and IL-4 δ2 RT-PCR products

IL-4 (primers spanning exon 1 to 3)							
IL-4 wild type band				IL-4 spliced variant (IL-4 δ2) band			
Patients		Controls		Patients		Controls	
B lymph.	T lymph.	B lymph	T lymph	B lymph	T lymph	B lymph	T lymph
29.6	13.5	19.0	18.5	12.9	1.9	9.5	24.3
27.4	17.9	22.9	34.4	10.7	2.6	10.7	19.8
28.9	37.1	20.0	20.5	10.4	3.1	12.9	12.1
25.9	35.3	22.8	11.4	9.0	1.7	14.0	7.1
23.9	10.9			10.3	1.0		
18.7	34.5			10.3	1.6		
31.9	32.6			13.8	1.8		
29.9	16.9			14.1	2.8		
Mean: 27.0 SD+/-4.1	Mean: 24.8 SD+/-10.9	Mean: 21.2 SD+/-1.9	Mean: 21.2 SD+/-9.6	Mean: 11.4 SD+/-1.9	Mean: 2.1 SD+/-0.7	Mean: 11.8 SD+/-2.0	Mean: 15.8 SD+/-7.6
Variable: B patients/ B controls p=0.03 Ratio: 1: 0.8 **Variable: B patients/T patients** p=ns Ratio: 1: 0.9				**Variable: B patients/ B controls** p=ns Ratio: 1: 1 **Variable: B patients/T patients** p=0.001 Ratio: 1: 0.2			
Variable: T patients/ T controls p=ns Ratio: 1: 0.9 **Variable: B controls/T controls** p=ns Ratio: 1: 1				**Variable: T patients/ T controls** p=0.0001 Ratio: 1: 7.6 **Variable: B controls/T controls** p=ns Ratio: 1: 1.3			
Spearmans' correlation test **Variable: B patients wild type/B patients splice variant** r=874 p=0.005 **Variable: T patients wild type/T patients splice variant** p=ns **Variable: B controls wild type/B controls splice variant** p=ns **Variable: T controls wild type/T controls splice variant** p=ns							

Appendix 10 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.21. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant, lymph=Lymphocytes

Appendix 11: Densitometry of IL-5 RT-PCR products

IL-5			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
885.9	1004.9	671.5	0
148.0	774.8	61.5	974.5
649.7	1058.7	335.7	8.8
905.7	449.4	0	848.6
16.7	116.3	666.3	0
1012.5	0	790.7	910.2
933.3	879.7	0	754.7
872.9	764.2	630.3	0
1173.0	610.5	854.0	917.7
1018.5	1076.7	855.3	963.9
1052.8	1013.7		
Mean: 788.0	Mean: 704.4	Mean: 486.2	Mean: 537.8
SD+/-373.8	SD+/-374.8	SD+/-353.9	SD+/-465.0
Variable: B patients/ B control (t-test)			p=ns
B patients/ B control mean values ratio:			1 : 1.6
Variable: T patients/ T controls (t-test)			p=ns
T patients/ T controls mean values ratio:			1: 0.8
Variable: B patients/ T patients (t-test)			p=ns
B patients/ T patients mean values ratio:			1: 0.9
Variable: B controls/ T controls (t-test)			p=ns
B controls/ T controls mean values ratio:			1: 1.1

Appendix 11 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.25. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 12: Densitometry of IL-13 RT-PCR products

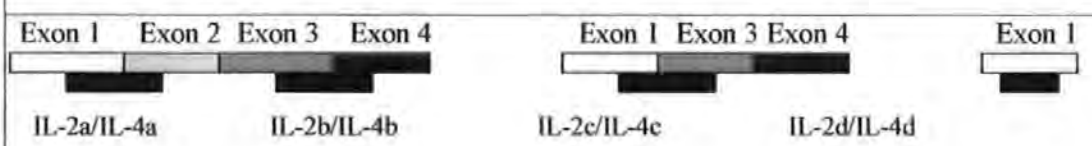
IL-13			
Patients		Controls	
B lymphocytes	T lymphocytes	B lymphocytes	T lymphocytes
536.2	362.2	220.5	414.6
39.4	428.8	466.6	477.2
468.0	88.9	467.0	320.0
309.7	439.3	34.8	520.7
334.0	416.4	346.6	510.8
350.2	419.3	350.9	211.5
325.0	455.7	511.9	306.3
390.1	48.5	403.7	0
196.0	190.6	152.1	361.9
363.6	259.8	541.3	261.3
296.8	289.9		
Mean: 328.2	Mean: 308.4	Mean: 349.5	Mean: 349.0
SD+/-130.6	SD+/-145.8	SD+/-166.0	SD+/-158.6
Variable: B patients/ B control (t-test)			p=ns
B patients/ B control mean values ratio:			1 : 1
Variable: T patients/ T controls (t-test)			p=ns
T patients/ T controls mean values ratio:			1: 1.1
Variable: B patients/ T patients (t-test)			p=ns
B patients/ T patients mean values ratio:			1: 0.9
Variable: B controls/ T controls (t-test)			p=ns
B controls/ T controls mean values ratio:			1: 1.1

Appendix 12 shows the densitometry results of the IL-4R α chain RT-PCR product shown in Figure 5.27. The Students t test was used to measure statistical significance.
SD=Statistical significance, Mean=Mean optical density values, ns=Not significant

Appendix 13: Flow cytometric analysis of the effect of PTO-modified IL-2 antisense ONs on unstimulated PBMCs separated from patients with B-CLL

Antisense	%CD19+ total	%CD19+/A+	%PI+/A +
Negative control	66.5	58.1	46.9
	69.9	50.8	17.0
	57.5	32.6	30.0
	94.4	34.1	15.9
	80.4	52.7	36.9
Mean	**73.5**	**45.6**	**27.4**
IL-2b	56.8	61.5	-
	74.6	80.4	46.6
	55.1	51.8	39.9
	73.2	58.4	32.4
	81.3	57.4	38.3
Mean	**62.8**	**61.9**	**39.3**
IL-2c	54.3	67.4	54.3
	69.5	75.8	47.5
	57.1	48.7	30.2
	81.9	58.2	32.6
	80.1	55.9	34.4
Mean	**68.5**	**61.2**	**39.8**
IL-2d	66.8	64.7	43.2
	62.3	64.5	33.0
	52.4	40.8	18.9
	75.3	47.0	19.4
	74.4	67.1	44.8
Mean	**66.2**	**56.8**	**31.8**

Position of the individual antisense oligonucleotides



PBMCs isolated by density gradient centrifugation from five patients with B-CLL and from five normal controls were used. PBMCs were diluted in 5% FCS culture medium to a concentration of $0.5x10^6$ and incubated for 48 hours with PTO-modified IL-2 ONs at a total concentration of 3 x 2.5 µM in 24 well plates. Collected PBMCs were stained with anti-CD19 PE, double stained with anti-CD19 PE/Annexin V FITC (CD19+/A+) and double stained with Propidium iodine/Annexin V FITC (PI+/A+) and analysed immediately by flow cytometry. Ant.= Antisense, PI = Propidium iodine, A = Annexin V

Appendix 14: Flow cytometric analysis of the effect of PTO-modified IL-4 antisense ONs on unstimulated PBMCs separated from patients with B-CLL

Antisense	%CD19 +total	%CD19+/A+	%PI+/A+
Negative control	66.5	58.1	46.9
	69.9	50.8	17.0
	57.5	32.6	30.0
	94.4	34.1	15.9
	80.4	52.7	36.9
Mean	**73.5**	**45.6**	**27.4**
IL-4a	58.3	53.8	45.0
	75.0	79.3	9.6
	51.6	45.1	39.9
	95.4	48.2	22.4
	78.5	61.0	42.6
Mean	**71.7**	**57.6**	**31.9**
IL-4b	63.4	61.7	41.5
	66.5	63.8	45.0
	46.7	52.2	35.3
	72.0	45.9	22.3
	80.5	55.4	38.5
Mean	**65.8**	**55.8**	**36.5**
IL-4c	61.0	55.2	46.1
	74.0	75.8	48.1
	55.0	39.7	27.6
	77.4	57.6	34.7
	84.3	52.2	41.7
Mean	**70.3**	**56.0**	**39.6**
IL-4d	59.6	51.1	40.2
	66.8	48.1	33.1
	42.8	52.7	48.3
	74.2	50.3	17.7
	75.5	59.6	41.8
Mean	**63.7**	**52.3**	**36.2**
Control antisense	63.6	65.9	50.5
	70.9	79.1	49.6
	41.1	61.6	-
	71.5	57.9	32.8
	81.9	36.0	40.5
Mean	**65.8**	**60.0**	**43.3**

PBMCs isolated from five patients with B-CLL and from five normal controls were used. PBMCs were diluted to a concentration of $0.5x10^6$ and incubated for 48 hours with PTO-modified IL-4 ONs at a total concentration of 3 x 2.5 µM in 24 well plates. PBMCs were stained with anti-CD19 PE; double stained with anti-CD19 PE/Annexin V FITC (CD19+/A+) and double stained with Annexin V FITC/Propidium Iodine (PI+/A+) and analysed immediately by flow cytometry. Ant.= Antisense, PI = Propidium iodide, A = Annexin V

Appendix 15: Flow cytometric analysis of the effect of PTO-modified IL-2 antisense ONs on unstimulated PBMCs separated from normal controls

Antisense	%CD19+ total	%CD19+/A+	%PI+/A+
Negative control	2.6 7.8 5.2 7.2 12.5	1.4 3.3 1.2 1.7 4.8	0.5 4.8 7.2 3.6 8.0
Mean	**7.0**	**2.4**	**4.8**
IL-2a	2.7 5.7 5.4 6.6 10.3	0.3 3.4 2.1 2.2 0.7	0.9 6.4 3.7 3.5 5.6
Mean	**6.1**	**1.7**	**4.0**
IL-2b	1.6 4.8 5.7 7.0 11.1	0.6 6.4 1.2 1.4 3.1	1.0 8.4 1.0 5.3 9.3
Mean	**6.0**	**2.5**	**5.0**
IL-2c	1.6 5.8 4.4 6.9 10.0	0.4 3.1 1.4 2.0 4.4	2.2 8.6 4.7 6.7 15.4
Mean	**5.7**	**2.2**	**7.5**
IL-2d	2.0 7.8 4.8 7.8 10.2	0.4 3.1 1.3 1.0 3.3	2.6 6.5 8.5 4.7 8.3
Mean	**6.4**	**1.8**	**6.1**

PBMCs from five normal controls were used. PBMCs were diluted in culture medium to a concentration of $0.5X10^5$/ml and incubated for 48 hours with different IL-2 PTO-modified antisense ONs in 24 well plates. Collected cells were stained with anti-CD19 PE; double stained with anti-CD19 PE and Annexin V FITC (CD19+/A+) and double stained with Annexin V FITC and Propidium iodine (PI+/A+) and analysed by flow cytometry. Ant.= Antisense, PI = Propidium Iodide, A = Annexin V

Appendix 16: Flow cytometric analysis of the effect of PTO-modified IL-4 antisense ONs on unstimulated PBMCs separated from normal controls

Antisense	%CD19+ total	%CD19+/A+	%PI+/A+
IL-4a	2.2	0.3	0.9
	6.2	3.7	6.4
	5.2	0.6	2.8
	8.2	2.7	3.5
	10.7	2.0	7.3
Mean	**6.5**	**1.8**	**4.1**
IL-4b	3.3	0.3	2.4
	7.5	4.2	7.7
	5.5	1.3	1.5
	7.5	1.9	6.2
	10.0	1.7	5.3
Mean	**6.7**	**1.8**	**4.6**
IL-4c	2.5	0.4	2.4
	5.5	2.9	6.8
	5.2	1.1	2.4
	7.1	1.4	5.8
	9.2	2.5	6.3
Mean	**5.9**	**1.6**	**4.7**
IL-4d	1.9	0.5	3.4
	7.0	3.1	10.6
	8.4	2.1	2.6
	7.8	1.6	6.1
	12.0	3.7	12.0
Mean	**7.4**	**2.2**	**6.9**
Control antisense	2.0	0.2	1.2
	4.0	2.8	6.5
	5.2	1.8	3.4
	7.2	2.1	4.4
	10.3	4.2	9.6
Mean	**5.7**	**2.2**	**5.0**

PBMCs isolated by density gradient centrifugation from normal controls were used. PBMCs were diluted in 5% FCS culture medium to a concentration of $0.5X10^5$/ml. Cells were incubated for 48 hours with different IL-4 PTO-modified antisense ONs and control antisense ON. Collected cells were stained with anti-CD19 PE, double stained with anti-CD19 PE and Annexin V FITC (CD19+/A+) and double stained with Annexin V FITC and Propidium Iodine (PI) (PI+/A+) and analysed by flow cytometry.
Ant.= Antisense, PI = Propidium Iodide, A = Annexin V

Appendix 17: Flow cytometric analysis of the effect of PTO-modified IL-2 and IL-4 antisense ONs on unstimulated CD3+ve lymphocytes from normal controls

Antisense	% CD3+	% CD3+/A+	Antisense	% CD3+	% CD3+/A+
Neg. control	47.2	3.5	Cont. ant.	42.2	4.4
	36.9	10.1		42.3	5.0
	31.9	0.6		38.9	4.8
	51.8	8.3		46.2	14.4
Mean	**41.9**	**5.6**	**Mean**	**42.4**	**7.1**
IL-2a	45.2	6.1	IL-4a	50.8	3.4
	32.7	8.7		38.8	5.8
	46.4	0.9		37.4	1.5
	24.0	3.6		48.2	7.0
Mean	**37.0**	**4.8**	**Mean**	**43.8**	**4.4**
IL-2b	46.6	5.9	IL-4b	47.9	3.5
	35.5	6.5		34.1	7.7
	42.8	1.3		34.1	0.7
	41.9	9.0		46.8	10.6
Mean	**41.7**	**5.6**	**Mean**	**40.7**	**5.6**
IL-2c	50.5	3.6	IL-4c	47.7	1.9
	37.3	7.0		34.4	7.0
	39.7	2.5		41.2	1.7
	46.7	10.2		53.2	8.3
Mean	**43.5**	**5.8**	**Mean**	**44.1**	**4.7**
IL-2d	45.7	10.2	IL-4d	40.7	4.2
	36.4	5.8		35.9	9.7
	41.6	2.2		28.1	1.2
	51.7	6.3		39.9	8.5
Mean	**43.8**	**6.1**	**Mean**	**36.1**	**5.9**

PBMCs from normal healthy controls were incubated with various PTO-modified IL-2 and IL-4 antisense ONs at a concentration of 3x 2.5μM for 48 hours. After incubation, the PBMCs were stained with anti-CD3 PE monoclonal antibody or double stained with anti-CD3 PE and Annexin V FITC (CD3+/A+) and immediately analysed by flow cytometry. Ant.=Antisense, A = Annexin V, ONs=oligonucleotides

Appendix 18: Flow cytometric analysis of B-CLL PBMCs after addition of control, IL-2d PTO-modified and unmodified antisense ONs

Antisense	%CD19+ totaL	%CD19+/A+	%PI+/A+
Negative control			
	81.0	13.3	1.7
	50.5	9.4	1.5
	87.3	3.2	0.4
Mean	**72.3**	**8.6**	**1.2**
IL-2d PTO modified ON			
	81.9	13.0	2.9
	48.1	15.4	2.0
	87.3	1.2	2.2
Mean	**72.4**	**9.8**	**2.3**
IL-2d non-modified ON			
	79.5	13.3	2.4
	41.8	20.1	3.6
	84.6	3.5	1.3
Mean	**68.3**	**12.3**	**2.4**
Control antisense			
	80.7	14.3	2.1
	57.9	2.0	4.6
	83.0	2.5	0.3
Mean	**73.8**	**6.2**	**2.3**

PBMCs from patients with B-CLL obtained following a Lymphoprep gradient separation were used. PBMCs were incubated with control antisense, unmodified IL-2d antisense ON and PTO-modified IL-2d antisense ON for 48 hours. Antisense ONs were added at the concentration of 3x2.5μM.

Appendix 19: Flow cytometric analysis of intracellular IL-2 and IL-4 expression in unstimulated and PMA-stimulated CD19+ve and CD3+ve PBMCs from normal controls

A) Normal control unstimulated PBMCs

Antisense	Time	%CD19+/IL-4+	%CD19+/IL-2+	%CD3+/IL-4+	%CD3+/IL-2+
Negative control					
	2 hr	6.8	0.3	26.1	2.6
	4 hr	0.6	0.1	23.3	0.1
	6 hr	1.1	0.4	27.6	1.7
IL-4c antisence ON					
	2 hr	14.0	1.5	19.6	1.1
	4 hr	0.1	0	12.7	0
	6 hr	0.4	0.1	9.4	1.0

B) Normal control PBMCs -stimulated with PMA (20 ng/ml)

Antisense	Time	%CD19+/IL-4+	%CD19+/IL-2+	%CD3+/IL-4+	%CD3+/IL-2+
Negative control					
	2 hr	2.7	4.7	22.2	47.4
	4 hr	0.5	0.3	15.7	48.1
	6 hr	2.8	0.7	50.6	57.7
IL-4c antisense ON					
	2hr	2.5	0.7	15.5	44.5
	4 hr	0.1	0.3	18.8	54.0
	6 hr	1.0	0.7	44.3	58.9

Table A show the percentage of CD19+ve cells expressing intracellular IL-2 and IL-4 after incubation with IL-4c antisense ON. Table B shows that incubation of PMA-stimulated PBMCs from normal controls with IL-4c antisense ON.
Neg. cont.= Negative control, hr=Hours

Appendix 20: Flow cytometric analysis of unstimulated and PMA-stimulated CD19 and CD3+ve B-CLL cells expressing intracellular IL-2 and IL-4

Unstimulated PBMCs from patients with B-CLL

Antisense	%CD19+/IL-4+	%CD19+/IL-2+	%CD3+/IL-4+	%CD3+/IL-2+
Negaive control	28.7 4.5 4.7 3.3	12.7 1.8 2.9 1.0	7.7 1.0 2.7 0.4	2.8 0.1 2.2 0.6
Mean	**10.3**	**4.6**	**2.9**	**1.4**
IL-4c antisense ON	0.4 5.7 5.7 1.5	0 2.0 2.6 1.6	0.5 0.2 2.6 1.1	0.1 0 3.9 1.2
Mean	**3.3**	**1.5**	**1.1**	**1.1**

PMA-stimulated PBMCs from patients with B-CLL

Antisense	%CD19+/IL-4+	%CD19+/IL-2+	%CD3+/IL4+	%CD3+/IL-2+
Negative control	3.3 9.9 7.3 5.7	- 7.4 7.0 5.1	0.1 0.3 5.2 1.4	0.1 0.7 5.8 1.8
Mean	**6.5**	**6.5**	**1.7**	**2.1**
IL-4c antisense ON	6.6 8.7 2.7 6.2	15.0 - 6.0 5.9	19.5 0.4 5.3 1.6	10.8 0.8 4.9 1.7
Mean	**5.9**	**8.9**	**6.7**	**4.5**

Unstimulated and PMA-stimulated PBMCs from patients with B-CLL were investigated for the percentage of CD3+ve and CD19+ve lymphocytes expressing intracellular IL-2 or IL-4 after incubation with PTO-modified IL-4c antisense ON for 6 hours.

Appenix 21: ELISA readings of PHA stimulated PBMCs isolated from patients with B-CLL and incubated for 48 hours with IL-2 and IL-4 antisense ONs

IL-2 ELISA readings (in pg)

Patient number	**Negative control**	**Control antisense**	**IL-2a**	**IL-2b**	**IL-2c**	**IL-2d**
1	41.85	9.02	40.41	23.57	14.44	51.77
2	9.84	0.98	10.24	3.46	3.99	7.32
3	22.61	0	7.76	0.98	3.99	13.71
4	174.06	27.00	62.50	17.28	47.45	23.57
Mean	**62.09**	**9.25**	**30.23**	**11.32**	**17.47**	**24.09**

IL-4 ELISA readings (in pg)

Patient number	**Negative control**	**Control antisense**	**IL-4a**	**IL-4b**	**IL-4c**	**IL-4d**
1	1.44	0.14	1.92	1.27	0.65	2.00
2	0	0.05	0.06	0.09	0.04	0.14
3	1.10	0.62	1.03	1.68	0.93	2.31
4	4.27	13.79	11.65	9.62	7.64	11.01
Mean	**1.70**	**3.65**	**3.66**	**3.17**	**2.31**	**3.86**

PHA stimulated (1μg/ml) PBMCs from four patients with B-CLL were incubated for 48 hour with various PTO-modified IL-2 and IL-4 antisense ONs as well as control antisense.

Appendix 22: Beta actin sequencing results (Wilbur-Lipman DNA alignment)

bacts.seq	HSAC07	Index	Number	Length	Length
1>962	164>846	100.0	0	0	456

```
      v10       v20       v30       v40       v50       v60       v70       v80
GGGCGTGATGGTGGGCATGGGTCAGAAGGATTCCTATGTGGGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGA
GGGCGTGATGGTGGGCATGGGTCAGAAGGATTCCTATGTGGGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGA
GGGCGTGATGGTGGGCATGGGTCAGAAGGATTCCTATGTGGGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGA
      ^170      ^180      ^190      ^200      ^210      ^220      ^230      ^240
      v90       v100      v110      v120      v130      v140      v150      v160
AGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGACATGGAGAAAATCTGGCACCACACCTTCTACAATGAGCTG
AGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGACATGGAGAAAATCTGGCACCACACCTTCTACAATGAGCTG
AGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGACATGGAGAAAATCTGGCACCACACCTTCTACAATGAGCTG
      ^250      ^260      ^270      ^280      ^290      ^300      ^310      ^320
      v170      v180      v190      v200      v210      v220      v230      v240
CGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAACCGCGAGAAGATGACCCA
CGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAACCGCGAGAAGATGACCCA
CGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAACCGCGAGAAGATGACCCA
      ^330      ^340      ^350      ^360      ^370      ^380      ^390      ^400
      v250      v260      v270      v280      v290      v300      v310      v320
GATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTTGCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTA
GATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTTGCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTA
GATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTTGCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTA
      ^410      ^420      ^430      ^440      ^450      ^460      ^470      ^480
      v330      v340      v350      v360      v370      v380      v390      v400
CCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCACACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCC
CCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCACACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCC
CCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCACACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCC
      ^490      ^500      ^510      ^520      ^530      ^540      ^550      ^560
      v410      v420      v430      v440      v450
ATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACTGACTACCTCATGAAGATCCT
ATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACTGACTACCTCATGAAGATCCT
ATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACTGACTACCTCATGAAGATCCT
      ^570      ^580      ^590      ^600      ^610
```

bacta.seq	HSAC07	Index	Number	Length	Length
1>1004	204>775	100.0	0	0	382

```
      v400      v390      v380      v370      v360      v350      v340      v330
GGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGAAGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGA
GGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGAAGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGA
GGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGAAGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGA
      ^210      ^220      ^230      ^240      ^250      ^260      ^270      ^280
      v320      v310      v300      v290      v280      v270      v260      v250
CATGGAGAAAATCTGGCACCACACCTTCTACAATGAGCTGCGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGG
CATGGAGAAAATCTGGCACCACACCTTCTACAATGAGCTGCGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGG
CATGGAGAAAATCTGGCACCACACCTTCTACAATGAGCTGCGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGG
      ^290      ^300      ^310      ^320      ^330      ^340      ^350      ^360
      v240      v230      v220      v210      v200      v190      v180      v170
CCCCCCTGAACCCCAAGGCCAACCGCGAGAAGATGACCCAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTT
CCCCCCTGAACCCCAAGGCCAACCGCGAGAAGATGACCCAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTT
CCCCCCTGAACCCCAAGGCCAACCGCGAGAAGATGACCCAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTT
      ^370      ^380      ^390      ^400      ^410      ^420      ^430      ^440
      v160      v150      v140      v130      v120      v110      v100      v90
GCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTACCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCA
GCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTACCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCA
GCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTACCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCA
      ^450      ^460      ^470      ^480      ^490      ^500      ^510      ^520
      v80       v70       v60       v50       v40       v30
CACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCCATCCTGCGTCTGGACCTGGCTG
CACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCCATCCTGCGTCTGGACCTGGCTG
CACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCCATCCTGCGTCTGGACCTGGCTG
      ^530      ^540      ^550      ^560      ^570      ^580
```

Appendix 23: IL-2 wild type sequencing results (Wilbur-Lipman DNA alignment)

2s.seq	Seq2 HUMIL2B	Similarity Index	Gap Number	Gap Length	Consensus Length
1>921	2880>3099	100.0	0	0	147

```
   v160       v170       v180       v190       v200       v210       v220       v230
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
^2880      ^2890      ^2900      ^2910      ^2920      ^2930      ^2940      ^2950
   v240       v250       v260       v270       v280       v290       v300
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACTAAAGG
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACTAAAGG
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACTAAAGG
^2960      ^2970      ^2980      ^2990      ^3000      ^3010      ^3020
```

2as.seq	Seq2 HUMIL2B	Similarity Index	Gap Number	Gap Length	Consensus Length
1>947	2880>3099	100.0	0	0	147

```
      v220       v210       v200       v190       v180       v170       v160       v150
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
^2880      ^2890      ^2900      ^2910      ^2920      ^2930      ^2940      ^2950
      v140       v130       v120       v110       v100       v90
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACTAAAGG
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACTAAAGG
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGAACTAAAGG
^2960      ^2970      ^2980      ^2990      ^3000      ^3010      ^3020
```

Appendix 24: IL-2 delta 2 (IL-2δ2) sequencing results (Wilbur-Lipman DNA alignment)

il2ex1-3 (2) ex.SEQ	HUMIL2	Index	Number	Length	Length
1>903	509>681	100.0	0	0	116

```
        v260       v250       v240       v230       v220       v210       v200       v190
CACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGCATTTA
CACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGCATTTA
CACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGCATTTA
 ^510       ^520       ^530       ^540       ^550       ^560       ^570       ^580
        v180       v170       v160
CTGCTGGATTTACAGATGATTTTGAATGGAATTAAT
CTGCTGGATTTACAGATGATTTTGAATGGAATTAAT
CTGCTGGATTTACAGATGATTTTGAATGGAATTAAT
 ^590       ^600       ^610       ^620
```

il2ex 1-3 (2).SEQ	HUMIL2	Index	Number	Length	Length
1>938	2974>4033	98.3	0	0	115

```
        v170       v180       v190       v200       v210       v220       v230       v240
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAANAAGAACTCAAACCTNTGGAGGAAGTGCTAAATTTAGCTCAAAGC
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAA AAGAACTCAAACCT TGGAGGAAGTGCTAAATTTAGCTCAAAGC
AGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGC
   ^3070      ^3080      ^3090      ^3100      ^3110      ^3120      ^3130      ^3140
        v250       v260       v270
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAG
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAG
AAAAACTTTCACTTAAGACCCAGGGACTTAATCAG
   ^3150      ^3160      ^3170      ^3180
```

Appendix 25: IL-4 wild type sequencing results (Wilbur-Lipman DNA alignment)

Seq1	Seq2	Similarity	Gap	Gap	Consensus
test1.seq	HUMIL4	Index	Number	Length	Length
1>357	144>614	99.7	0	0	350

```
   v10        v20        v30        v40        v50        v60        v70        v80
CGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAAGACTCTGTGCACCGAGTTGACCGTAA
CGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAAGACTCTGTGCACCGAGTTGACCGTAA
CGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAAGACTCTGTGCACCGAGTTGACCGTAA
      ^150       ^160       ^170       ^180       ^190       ^200       ^210       ^220
   v90        v100       v110       v120       v130       v140       v150       v160
CAGACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCGACTGTGCTCCGGCAGTTCTAC
CAGACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCGACTGTGCTCCGGCAGTTCTAC
CAGACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCGACTGTGCTCCGGCAGTTCTAC
      ^230       ^240       ^250       ^260       ^270       ^280       ^290       ^300
   v170       v180       v190       v200       v210       v220       v230       v240
AGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGCTGATCCGATTCCT
AGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGCTGATCCGATTCCT
AGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGCTGATCCGATTCCT
      ^310       ^320       ^330       ^340       ^350       ^360       ^370       ^380
   v250       v260       v270       v280       v290       v300       v310       v320
GAAACGGCTCGANAGGAACCTCTGGGGCCTGGCGGGCTTGAATTCCTGTCCTGTGAAGGAAGCCAACCAGAGTACGTTGG
GAAACGGCTCGA AGGAACCTCTGGGGCCTGGCGGGCTTGAATTCCTGTCCTGTGAAGGAAGCCAACCAGAGTACGTTGG
GAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGGGCTTGAATTCCTGTCCTGTGAAGGAAGCCAACCAGAGTACGTTGG
      ^390       ^400       ^410       ^420       ^430       ^440       ^450       ^460
   v330       v340       v350
AAAACTTCTTGGAAAGGCTAAAGACGATCA
AAAACTTCTTGGAAAGGCTAAAGACGATCA
AAAACTTCTTGGAAAGGCTAAAGACGATCA
      ^470       ^480       ^490
```

	Seq2	Similarity	Gap	Gap	Consensus
il4as.seq	HUMIL4	Index	Number	Length	Length
1>315	118>575	99.7	0	0	306

```
   v310       v300       v290       v280       v270       v260       v250       v240
GGCAACTTTGTCCACGGACACAAGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAA
GGCAACTTTGTCCACGGACACAAGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAA
GGCAACTTTGTCCACGGACACAAGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAA
     ^120       ^130       ^140       ^150       ^160       ^170       ^180       ^190
   v230       v220       v210       v200       v190       v180       v170       v160
GACTCTGTGCACCGAGTTGACCGTAACAGACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGG
GACTCTGTGCACCGAGTTGACCGTAACAGACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGG
GACTCTGTGCACCGAGTTGACCGTAACAGACATCTTTGCTGCCTCCAAGAACACAACTGAGAAGGAAACCTTCTGCAGGG
     ^200       ^210       ^220       ^230       ^240       ^250       ^260       ^270
   v150       v140       v130       v120       v110       v100       v90        v80
CTGCGACTGTGCTCCGGCAGTTCTACAGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCAC
CTGCGACTGTGCTCCGGCAGTTCTACAGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCAC
CTGCGACTGTGCTCCGGCAGTTCTACAGCCACCATGAGAAGGACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCAC
     ^280       ^290       ^300       ^310       ^320       ^330       ^340       ^350
   v70        v60        v50        v40        v30        v20        v10
AGGCACAAGCAGCTGATCCGATTCCTGAAACGGCTCGANAGGAACCTCTGGGGCCTGGCGGGCTTG
AGGCACAAGCAGCTGATCCGATTCCTGAAACGGCTCGA AGGAACCTCTGGGGCCTGGCGGGCTTG
AGGCACAAGCAGCTGATCCGATTCCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGGGCTTG
     ^360       ^370       ^380       ^390       ^400       ^410       ^420
```

Appendix 26: IL-4 delta 4 (IL-4 δ2) sequencing results (Wilbur-Lipman DNA alignment)

ex1#1.seq	HSIL4SV	Index	Number	Length	Length
91	75>210	100.0	0	0	91

```
      v10        v20        v30        v40        v50        v60        v70        v80
AGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAAGAACACAACTGAGAAGGAAA
AGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAAGAACACAACTGAGAAGGAAA
AGTGCGATATCACCTTACAGGAGATCATCAAAACTTTGAACAGCCTCACAGAGCAGAAGAACACAACTGAGAAGGAAA
   ^80        ^90        ^100       ^110       ^120       ^130       ^140       ^150
      v90
TTCTGCAGG
TTCTGCAGG
TTCTGCAGG
   ^160
```

Appendix 27: IL-5 sequencing results (Wilbur-Lipman alignment)

...il5s.seq	HUMIL5	Index	Number	Length	Length
1>1017	1883>2075	100.0	0	0	129

```
      v80        v90        v100       v110       v120       v130       v140       v150
CACCAACTGTGCACTGAAGAAATCTTTCAGGGAATAGGCACACTGGAGAGTCAAACTGTGCAAGGGGGTACTGTGGAAAG
CACCAACTGTGCACTGAAGAAATCTTTCAGGGAATAGGCACACTGGAGAGTCAAACTGTGCAAGGGGGTACTGTGGAAAG
CACCAACTGTGCACTGAAGAAATCTTTCAGGGAATAGGCACACTGGAGAGTCAAACTGTGCAAGGGGGTACTGTGGAAAG
        ^1890      ^1900      ^1910      ^1920      ^1930      ^1940      ^1950      ^1960
      v160       v170       v180       v190       v200
ACTATTCAAAAACTTGTCCTTAATAAAGAAATACATTGACGGCCAAAAA
ACTATTCAAAAACTTGTCCTTAATAAAGAAATACATTGACGGCCAAAAA
ACTATTCAAAAACTTGTCCTTAATAAAGAAATACATTGACGGCCAAAAA
        ^1970      ^1980      ^1990      ^2000      ^2010
```

il5as.seq	HUMIL5	Index	Number	Length	Length
1>1001	1885>2074	86.5	0	0	126

```
      v150       v140       v130       v120       v110       v100       v90        v80
CCAACTGNGCNCTGAANAAATCTTTCAGGGAATAGGCNCNCTGGANCCCCCAACTGNGCAANGGGGNACTGTGGAAANAC
CCAACTG GC CTGAA AAATCTTTCAGGGAATAGGC C CTGGA    C AACTG GCAA GGGG ACTGTGGAAA AC
CCAACTGTGCACTGAAGAAATCTTTCAGGGAATAGGCACACTGGAGAGTCAAACTGTGCAAGGGGGTACTGTGGAAAGAC
      ^1890      ^1900      ^1910      ^1920      ^1930      ^1940      ^1950      ^1960
      v70        v60        v50        v40
TATTCAAAAACTTGTCCTTAATAAANAAANACATTGGCGGCCAAAA
TATTCAAAAACTTGTCCTTAATAAA AAA ACATTG CGGCCAAAA
TATTCAAAAACTTGTCCTTAATAAAGAAATACATTGACGGCCAAAA
      ^1970      ^1980      ^1990      ^2000      ^2010
```

Appendix 28: IL-7 sequencing results (Wilbur-Lipman alignment)

il72a.seq	HUMIL7A	Index	Number	Length	Length
1>941	279>1203	99.4	1	1	617

```
      v610      v600      v590      v580      v570      v560      v550      v540
TCGGCAACTCCGCGNAAAGACCAGGGTCCTGGGAGTGACTATGGNCGGTGAGAGCTTGCTCCTGCTCCAGTTGCGGTCAT
TCGGCAACTCCGCG  AAGACCAGGGTCCTGGGAGTGACTATGG CGGTGAGAGCTTGCTCCTGCTCCAGTTGCGGTCAT
TCGGCAACTCCGCGG-AAGACCAGGGTCCTGGGAGTGACTATGGGCGGTGAGAGCTTGCTCCTGCTCCAGTTGCGGTCAT
 ^280      ^290      ^300      ^310      ^320      ^330      ^340      ^350
      v530      v520      v510      v500      v490      v480      v470      v460
CATGACTACGCCCGCCTCCCGCAGACCATGTTCCATGTTTCTTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTTGT
CATGACTACGCCCGCCTCCCGCAGACCATGTTCCATGTTTCTTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTTGT
CATGACTACGCCCGCCTCCCGCAGACCATGTTCCATGTTTCTTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTTGT
  ^360      ^370      ^380      ^390      ^400      ^410      ^420      ^430
      v450      v440      v430      v420      v410      v400      v390      v380
TCTGTTGCCAGTAGCATCATCTGATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTTCTAATGGTCAGCA
TCTGTTGCCAGTAGCATCATCTGATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTTCTAATGGTCAGCA
TCTGTTGCCAGTAGCATCATCTGATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTTCTAATGGTCAGCA
  ^440      ^450      ^460      ^470      ^480      ^490      ^500      ^510
      v370      v360      v350      v340      v330      v320      v310      v300
TCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTGAATAATGAATTTAACTTTTTTAAAAGACATATC
TCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTGAATAATGAATTTAACTTTTTTAAAAGACATATC
TCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTGAATAATGAATTTAACTTTTTTAAAAGACATATC
  ^520      ^530      ^540      ^550      ^560      ^570      ^580      ^590
      v290      v280      v270      v260      v250      v240      v230      v220
TGTGATGCTAATAAGGAAGGTATGTTTTTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAATAGCACTGG
TGTGATGCTAATAAGGAAGGTATGTTTTTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAATAGCACTGG
TGTGATGCTAATAAGGAAGGTATGTTTTTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAATAGCACTGG
  ^600      ^610      ^620      ^630      ^640      ^650      ^660      ^670
      v210      v200      v190      v180      v170      v160      v150      v140
TGATTTTGATCTCCACTTATTAAAAGTTTCAGAAGGCACAACAATACTGTTGAACTGCACTGGCCAGGTTAAAGGAAGAA
TGATTTTGATCTCCACTTATTAAAAGTTTCAGAAGGCACAACAATACTGTTGAACTGCACTGGCCAGGTTAAAGGAAGAA
TGATTTTGATCTCCACTTATTAAAAGTTTCAGAAGGCACAACAATACTGTTGAACTGCACTGGCCAGGTTAAAGGAAGAA
  ^680      ^690      ^700      ^710      ^720      ^730      ^740      ^750
      v130      v120      v110      v100      v90       v80       v70       v60
AACCAGCTGCCCTGGGTGAAGCCCAACCAACAAAGAGTTTGGAAGAAAATAAATCTTTAAAGGAACAGAAAAAACTGAAT
AACCAGCTGCCCTGGGTGAAGCCCAACCAACAAAGAGTTTGGAAGAAAATAAATCTTTAAAGGAACAGAAAAAACTGAAT
AACCAGCTGCCCTGGGTGAAGCCCAACCAACAAAGAGTTTGGAAGAAAATAAATCTTTAAAGGAACAGAAAAAACTGAAT
  ^760      ^770      ^780      ^790      ^800      ^810      ^820      ^830
      v50       v40       v30       v20       v10
GACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAAACTTGTTGGAATAAAATT
GACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAAACTTGTTGGAATAAAATT
GACTTGTGTTTCCTAAAGAGACTATTACAAGAGATAAAAACTTGTTGGAATAAAATT
  ^840      ^850      ^860      ^870      ^880      ^890
```

Appendix 29: IL-13 sequencing results (Wilbur-Lipman alignment)

13s.seq	HUMIL13B	Index	Number	Length	Length
1>929	2265>2422	100.0	0	0	81

```
        v100       v110       v120       v130       v140       v150       v160       v170
ATCAACGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGG
ATCAACGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGG
ATCAACGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGG
^2290      ^2300      ^2310      ^2320      ^2330      ^2340      ^2350      ^2360

G
G
G
^2370
```

il13a.seq	HUMIL13B	Index	Number	Length	Length
1>218	2265>2422	100.0	0	0	81

```
        v100       v90        v80        v70        v60        v50        v40        v30
ATCAACGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGG
ATCAACGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGG
ATCAACGTGTCAGGCTGCAGTGCCATCGAGAAGACCCAGAGGATGCTGAGCGGATTCTGCCCGCACAAGGTCTCAGCTGG
^2290      ^2300      ^2310      ^2320      ^2330      ^2340      ^2350      ^2360

G
G
G
^2370
```

Appendix 30: IL-15 sequencing results (Wilbur-Lipman alignment)

il15s.seq	HSIL15MR2	Index	Number	Length	Length
1>970	251>643	100.0	0	0	393

```
 v100       v110       v120       v130       v140       v150       v160       v170
GCTGTTTCAGTGCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTT
GCTGTTTCAGTGCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTT
GCTGTTTCAGTGCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTT
          ^260       ^270       ^280       ^290       ^300       ^310       ^320       ^330
 v180       v190       v200       v210       v220       v230       v240       v250
ATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACAGCAATGAAGTG
ATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACAGCAATGAAGTG
ATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACAGCAATGAAGTG
          ^340       ^350       ^360       ^370       ^380       ^390       ^400       ^410
 v260       v270       v280       v290       v300       v310       v320       v330
CTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCC
CTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCC
CTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCC
          ^420       ^430       ^440       ^450       ^460       ^470       ^480       ^490
 v340       v350       v360       v370       v380       v390       v400       v410
TAGCAAACAACAGTTTGTCTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAAAT
TAGCAAACAACAGTTTGTCTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAAAT
TAGCAAACAACAGTTTGTCTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAAAT
          ^500       ^510       ^520       ^530       ^540       ^550       ^560       ^570
 v420       v430       v440       v450       v460       v470       v480       v490
ATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTCTTGATTGCAATTGATTC
ATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTCTTGATTGCAATTGATTC
ATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTCTTGATTGCAATTGATTC
          ^580       ^590       ^600       ^610       ^620       ^630       ^640
```

il15a.seq	HSIL15MR2	Index	Number	Length	Length
1>974	251>643	100.0	0	0	393

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 v420       v410       v400       v390       v380       v370       v360       v350
GCTGTTTCAGTGCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTT
GCTGTTTCAGTGCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTT
GCTGTTTCAGTGCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATTGAAGATCTT
          ^260       ^270       ^280       ^290       ^300       ^310       ^320       ^330
 v340       v330       v320       v310       v300       v290       v280       v270
ATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACAGCAATGAAGTG
ATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACAGCAATGAAGTG
ATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCACCCCAGTTGCAAAGTAACAGCAATGAAGTG
          ^340       ^350       ^360       ^370       ^380       ^390       ^400       ^410
 v260       v250       v240       v230       v220       v210       v200       v190
CTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCC
CTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCC
CTTTCTCTTGGAGTTACAAGTTATTTCACTTGAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCC
          ^420       ^430       ^440       ^450       ^460       ^470       ^480       ^490
 v180       v170       v160       v150       v140       v130       v120       v110
TAGCAAACAACAGTTTGTCTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAAAT
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TAGCAAACAACAGTTTGTCTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAGGAAAAAAAT
          ^500       ^510       ^520       ^530       ^540       ^550       ^560       ^570
 v100       v90        v80        v70        v60        v50        v40        v30
ATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTCTTGATTGCAATTGATTC
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ATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAACACTTCTTGATTGCAATTGATTC
          ^580       ^590       ^600       ^610       ^620       ^630       ^640
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