An investigation of T cell dysregulation in B-cell chronic lymphocytic leukaemia

SOPHIE GRACE SCRIVENER

Let us know how access to this document benefits you
AN INVESTIGATION OF T CELL DYSREGULATION IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

by

SOPHIE GRACE SCRIVENER

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

Plymouth Postgraduate Medical School Faculty of Science
B-cell chronic lymphocytic leukaemia (B-CLL) is characterized by an accumulation of clonal malignant B cells within lymphoid tissue, the bone marrow and the peripheral blood. Whilst abnormalities of these B cells are the essential cause of this disease, the aim of this research project was to investigate whether the T cell compartment may play a role in the aetiology of this disease by evaluating the expression of key surface antigens involved in both activation of and interaction with B cells and other antigen presenting cells of the immune system. There were marked abnormalities in the expression of certain key activation and interaction antigens on the peripheral blood T cells of patients with B-CLL, in particular, compared to normal controls, there was a significant reduction in the number of circulating T cells expressing CD25, CD28, CD152, CD4, CD5 and CD11a. There was no difference in expression of TCRαβ, CD8, CD54 and CD154. Significantly more T cells from CLL patients expressed HLA-DR. Removal of the malignant clone of cells prior to short-term T cell culture did not affect expression of these markers. Numbers of T cells expressing intracellular CD25 and CD152 were not decreased after activation and a significantly greater number of resting T cells expressed both antigens intracellularly. There was also evidence of a soluble factor present in CLL AB serum which caused increased numbers of normal and CLL T cells to express CD25 and CD152 after culture. Initial results suggest that this may be IFN-γ, levels of which were significantly higher, as measured by ELISA, from resting CLL T cells compared to normals. By studying the expression of these antigens using cell culture, flow cytometric and ELISA techniques, the results suggest a functional state of anergy in these T cells. This anergic state may contribute to the pathogenesis of B-CLL and its related phenomena of immunosuppression and autoimmunity. This was further reflected in the results of the T
cell functional studies and reduced IL-2 expression in the mixed lymphocyte reaction (MLR).
# PLAN

## CHAPTER ONE - INTRODUCTION

<table>
<thead>
<tr>
<th>1.0</th>
<th>Leukaemia</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>B-cell Chronic Lymphocytic Leukaemia (B-CLL)</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Aetiology</td>
<td>4</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Clinical diagnosis</td>
<td>5</td>
</tr>
<tr>
<td>1.1.3</td>
<td>Laboratory diagnosis of CLL</td>
<td>5</td>
</tr>
<tr>
<td>1.1.4</td>
<td>Genetic changes</td>
<td>10</td>
</tr>
<tr>
<td>1.1.4.1</td>
<td>Trisomy 12</td>
<td>10</td>
</tr>
<tr>
<td>1.1.4.2</td>
<td>13q14</td>
<td>11</td>
</tr>
<tr>
<td>1.1.4.3</td>
<td>p53</td>
<td>12</td>
</tr>
<tr>
<td>1.1.4.4</td>
<td>Immunoglobulin gene mutations</td>
<td>12</td>
</tr>
<tr>
<td>1.1.4.5</td>
<td>Additional genetic changes</td>
<td>13</td>
</tr>
<tr>
<td>1.1.5</td>
<td>Telomerase activity</td>
<td>15</td>
</tr>
<tr>
<td>1.1.6</td>
<td>Classification of disease stage</td>
<td>15</td>
</tr>
<tr>
<td>1.1.7</td>
<td>Newer prognostic factors</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1.2</th>
<th>Treatment</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.1</td>
<td>Low risk (modified Rai criteria stage 0), Binet stage A</td>
<td>22</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Intermediate risk (modified Rai criteria stage I - II), Binet stage B</td>
<td>23</td>
</tr>
<tr>
<td>1.2.3</td>
<td>High risk (modified Rai criteria stage III - IV), Binet stage C</td>
<td>23</td>
</tr>
<tr>
<td>1.2.4</td>
<td>Treatment regimes for CLL</td>
<td>24</td>
</tr>
<tr>
<td>1.2.5</td>
<td>Recent advances in the treatment of CLL</td>
<td>28</td>
</tr>
<tr>
<td>1.2.5.1</td>
<td>Autologous and allogeneic stem cell transplants</td>
<td>28</td>
</tr>
<tr>
<td>1.2.5.2</td>
<td>Graft-versus-host and graft-versus-leukaemia</td>
<td>29</td>
</tr>
<tr>
<td>1.2.5.3</td>
<td>Donor lymphocyte infusions</td>
<td>31</td>
</tr>
<tr>
<td>1.2.5.4</td>
<td>Monoclonal antibodies</td>
<td>31</td>
</tr>
</tbody>
</table>
1.2.5.5 Adoptive immunotherapy 33
1.2.6 Disease transformation 37

1.3 Biology of B-CLL 39
1.3.1 B cell development 39
1.3.1.1 Pro-B cells 41
1.3.1.2 Pre-B cells 42
1.3.1.3 Immature B lymphocytes 42
1.3.1.4 Mature B lymphocytes 43
1.3.2 Normal cellular replication and cell cycle control 46
1.3.3 Apoptosis 48
1.3.4 Origins of the CLL cell 50
1.3.5 Morphology 55
1.3.6 Immunophenotype 55
1.3.7 Development and progression of the leukaemia clone 62

1.4 B and T cell interaction and activation in the normal immune response 65
1.4.1 Antigen presentation and recognition 68
1.4.2 Signal transduction and activation - an overview 69
1.4.3 LFA-1 and ICAM-1 70
1.4.4 CD3 and the T-Cell Receptor (TCR) 71
1.4.5 CD4 and CD8 73
1.4.6 CD5 74
1.4.7 CD28 75
1.4.8 CD25 and Interleukin-2 (IL-2) 77
1.4.9 CD154 79
1.4.10 CD152 (CTLA-4) 80
1.4.11 HLA-DR 82
1.5 The T cell in B-CLL  
1.5.1 Normal T cell haemopoiesis  
1.5.1.1 Thymic development  
1.5.1.2 Positive and negative selection  
1.5.1.3 Antigen presentation within the secondary lymphoid tissues  
1.5.1.4 Differentiation of CD4 and CD8 cells  
1.5.2 T cell in B-CLL  
1.5.2.1 CD4 and CD8 subpopulations and total T-cell numbers  
1.5.2.2 T-cell response to mitogen  
1.5.2.3 T-cell colony formation  
1.5.2.4 T-cell cytokine expression  
1.5.2.5 T cell surface antigen expression  
1.6 Summary of T cells in CLL and aims of project

CHAPTER TWO - MATERIALS AND METHODS  
2.1 Patients  
2.1.1 Patient and control groups  
2.1.2 CLL diagnosis  
2.1.3 Patient selection  
2.2 Reagents  
2.2.1 Cell culture reagents  
2.2.2 Flow Cytometry reagents  
2.2.3 B cell depletion reagents  
2.2.4 Permeabilisation reagents
2.2.5 ELISA reagents

2.3 Preparation of solutions

2.3.1 Cell culture

2.3.2 B-cell depletion solutions

2.3.3 ELISA solutions

2.4 Methods

2.4.1 Flow cytometry on the Beckman Coulter Epics Elite

2.4.2 PBMC isolation

2.4.3 Cell culture methods for cell surface expression of antigens CD25, CD28, CD152, CD154 and HLA-DR

2.4.4 Cell preparation methods for surface expression of CD4, CD5, CD8, CD11a, CD54 and TCRαβ

2.4.5 T cell enrichment

2.4.6 Permeabilisation methods

2.4.7 Expression of CD25, CD28 and CD152 in normal controls and CLL patients after B-cell depletion and culture with OKT3 and 50% CLL AB serum

2.4.8 Mixed lymphocyte reaction (MLR)

2.4.9 IL-2 ELISA

2.4.10 IFN-γ ELISA

2.4.11 MLR with IL-15

2.4.12 Expression of CD25, CD28 and CD152 after OKT3 stimulation, IL-15 and permeabilisation

2.5 OPTIMISATION METHODS

2.5.1 Time course for OKT3 adherence to culture plates

2.5.2 The use of CD2 or CD3 for T cell isolation following OKT3 activation

2.5.3 Time course for expression of CD154

2.5.4 Time course for expression of CD25, CD28 and CD152
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5.5 Time course for expression of HLA-DR</td>
<td>134</td>
</tr>
<tr>
<td>2.5.6 Comparison of B-cell depletion methods</td>
<td>135</td>
</tr>
<tr>
<td>2.5.7 Comparison of cell permeabilisation methods - paraformaldehyde and saponin vs DAKO intrastain kit</td>
<td>137</td>
</tr>
<tr>
<td>2.5.8 Time course for expression of CD25, CD28 and CD152 after B-cell depletion and permeabilisation</td>
<td>138</td>
</tr>
</tbody>
</table>

**CHAPTER THREE: RESULTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 Results of optimisation studies</td>
<td>139</td>
</tr>
<tr>
<td>3.1 Time course for OKT3 adherence to culture plates</td>
<td>139</td>
</tr>
<tr>
<td>3.2 CD2/CD3 for identification of T cell populations</td>
<td>140</td>
</tr>
<tr>
<td>3.3 Time course for optimal expression of CD154</td>
<td>142</td>
</tr>
<tr>
<td>3.4 Time course for optimal expression of CD25, CD28 and CD152</td>
<td>143</td>
</tr>
<tr>
<td>3.5 Time course for optimal expression of HLA-DR</td>
<td>144</td>
</tr>
<tr>
<td>3.6 Comparison of B cell depletion methods</td>
<td>145</td>
</tr>
<tr>
<td>3.7 Comparison of cell permeabilisation methods</td>
<td>148</td>
</tr>
<tr>
<td>3.8 Time course for optimal expression of CD25, CD28 and CD152 on stimulated T cells after B cell depletion and permeabilisation</td>
<td>149</td>
</tr>
<tr>
<td>3.9 Optimisation – summary of results</td>
<td>150</td>
</tr>
</tbody>
</table>

**CHAPTER FOUR: RESULTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0 Expression of activation and interaction markers on CLL patients and normals</td>
<td>151</td>
</tr>
<tr>
<td>4.1 Expression of CD25, CD28, CD152, TCRαβ, CD4, CD5, CD8 and HLA-DR on T cells from B-CLL patients and normals</td>
<td>151</td>
</tr>
<tr>
<td>4.2 Expression of CD154 on T cells from B-CLL patients and normals</td>
<td>154</td>
</tr>
<tr>
<td>4.3 Expression of the adhesion molecules LFA-1 (CD11a) and ICAM-1 (CD54) on the T cells from B-CLL patients</td>
<td>154</td>
</tr>
</tbody>
</table>
Expression of CD28 and CD152 on stimulated T cells after B cell depletion

Expression of intracellular CD25, CD28 and CD152 on stimulated and unstimulated T cells after B cell depletion

Expression of CD25, CD28 and CD152 after B cell depletion and OKT3 stimulation in 50% CLL AB serum and 50% normal AB serum.

Expression of CD25, CD28 and CD152 on CLL T cells after B cell depletion and OKT3 stimulation with interleukin-15 (IL-15)

Expression of CD25, CD28 and CD152 on CD2⁺ CLL T cells from a patient in complete clinical and haematological remission

CHAPTER FIVE: RESULTS

5.0 Secretion of cytokines and serum factors

5.1 Secretion of Interleukin - 2 (IL-2) and Interferon-γ (IFN-γ) by T cells in a mixed lymphocyte reaction (MLR)

5.2 Secretion of Interleukin-2 (IL-2) by T cells in a mixed lymphocyte reaction supplemented with Interleukin-15 (IL-15)

CHAPTER SIX: DISCUSSION

6.0 Results summary

6.1 Range of expression in samples

6.1.1 Normal controls

6.1.2 Patient samples

6.1.2.1 Age

6.1.2.2 Prior treatment
6.1.2.3 Stage of disease 178

6.2 Chemotherapy induced T cell dysfunction 178

6.3 Immunosenescence 181

6.3.1 CD25 and CD28 expression, IL-2 secretion 182

6.3.2 CD152, CD154 and LFA-1 expression 183

6.3.3 HLA-DR expression, IFN-γ and TNF-α secretion 184

6.3.4 T cell numbers 185

6.3.5 T cell response to mitogen 185

6.3.6 B cells 186

6.4 Tolerance and clonal anergy 189

6.4.1 CD28, CD25 and LFA-1 expression, IL-2 secretion 189

6.4.2 CD152 expression 191

6.4.3 HLA-DR expression and IFN-γ secretion 192

6.5 Possible immunosuppression by CLL B cells 193

6.6 Conclusions 194

7.0 Future work 196

8.0 Appendices 198

9.0 Abbreviations 217

10.0 References 220

11.0 Publications

TABLES

Table 1: Classification of Leukaemia 2

Table 2: Laboratory diagnosis of CLL using monoclonal antibodies 7

Table 3: Bone marrow infiltration patterns 9

Table 4: Rai's staging criteria for CLL 16

Table 5: Binet’s staging criteria for CLL 17
Table 23: Expression of surface CD25, CD28 and CD152 on normal T cells after B cell depletion and OKT3 stimulation in 50% CLL AB serum and 50% normal AB serum 163

Table 24: Expression of surface CD25, CD28 and CD152 on CLL T cells after B cell depletion and OKT3 stimulation in 50% CLL AB serum and 50% normal AB serum 163

Table 25: Expression of CD25, CD28 and CD152 on CLL T cells after B cell depletion, OKT3 stimulation and culture with Interleukin-15 166

Table 26: Secretion of Interleukin-2 and interferon-γ from normal and CLL T cells from MLR 170

Table 27: Secretion of interferon-γ from normal and CLL T cells: background readings 170

Table 28: Secretion of Interleukin-2 from normal and CLL T cells in an MLR supplemented with Interleukin-15 172

Table 29: Type of treatment received by patients 179

Table 30: Length of time since last treatment regime 179

Table 31: Differences and similarities between lymphocyte subsets in replicative senescence 188
ILLUSTRATIONS

Figure 1: Cellular origins of Leukaemia 3
Figure 2: Laboratory diagnosis of CLL 8
Figure 3: Normal B cell development 40
Figure 4: Normal cellular replication and cell cycle control 47
Figure 5: Possible cellular origins of CLL B cells 54
Figure 6: B and T cell interaction 66
Figure 7: The T cell receptor 72
Figure 8: Normal T cell development 86
Figure 9: Flow cytometry dot plot showing cell populations after staining with negative isotypes 16
Figure 10: Flow cytometry dot plot showing cell population after staining with Leucogate 117
Figure 11: Flow cytometry FS/SS dot plot showing cell population after B cell depletion using MACS CD19 beads 146
Figure 12: Flow cytometry FS/SS dot plot showing cell population after B cell depletion using CD19 Dynabeads 147
Figure 13: Bar chart showing expression of cell surface markers significantly different between patients and normals 198
Figure 14: Bar chart showing expression of intracellular antigens in stimulated T cells from patients and normals 199
Figure 15: Bar chart showing expression of intracellular antigens in resting T cells from patients and normals 200
Figure 16: Bar chart showing expression of cell surface antigens on normal T cells after cell culture in CLL or normal AB serum 201
Figure 17: Bar chart showing expression of cell surface antigens on CLL T cells after cell culture in CLL or normal AB serum

Figure 18: Bar chart showing secretion of cytokines in an MLR from patient and normal T cells

Figure 19: Secretion of IFN-γ from resting patient and normal T cells

Figure 20: Age comparison of CD25, CD28 and CD152 expression in CLL patients

Figure 21: Age comparison of CD25, CD28 and CD152 expression in normal controls

Figure 22: Age comparison of TCR and CD4 in CLL patients

Figure 23: Age comparison of TCR and CD4 in normal controls

Figure 24: Age comparison of LFA-1 and ICAM-1 in CLL patients

Figure 25: Age comparison of LFA-1 and ICAM-1 in normal controls

Figure 26: Mean expression of CD25, CD28 and CD152 in CLL patients according to prior treatment

Figure 27: Mean expression of TCR and CD4 in CLL patients according to prior treatment

Figure 28: Mean expression of LFA-1 and ICAM-1 in CLL patients according to prior treatment

Figure 29: Mean expression of CD25, CD28 and CD152 in CLL patients according to stage of disease

Figure 30: Mean expression of TCR and CD4 in CLL patients according to stage

Figure 31: Mean expression of LFA-1 and ICAM-1 in CLL patients according to stage
ACKNOWLEDGEMENT

I wish to thank the Cornwall Leukaemia Trust and the Plymouth and District Leukaemia Fund for funding this study and also Dr. Anton Kruger, Robin Bradley and Nigel Oakes at The Royal Cornwall Hospital Trust, Treliske, Truro for all their help and support in making this PhD possible in the first place. I wish to thank my supervisors Dr Prentice and Dr Kaminski at Derriford Hospital, and Dr Demaine at the University of Plymouth, for all their advice and support as supervisors. In particular, I would like to thank my parents Sian and Geoff for their continued emotional and financial support throughout my years of study, my sister Jesse for keeping me going in my moments of crisis and my partner Jason for his constant support and enthusiasm throughout all of the PhD.
Declaration

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

This study was financed with the aid of a studentship from the Plymouth and District Leukaemia Fund and the Cornwall Leukaemia Trust.

Relevant scientific seminars and conferences were regularly attended at which work was often presented: external institutions were visited for consultation purposes and several papers were prepared for publication.

Publications:


Invited to write review titled “Evidence of immune dysregulation in B-CLL” for Leukaemia and Lymphoma, in preparation.

Presentations and conferences attended:

December 1998: Abstract presentation 40th Annual ASH meeting, Miami (Blood 92 (10) suppl. 1 429a)


October 1999: Abstract presentation IWCLL, Paris. (Hematology and Cell Therapy 42 (1))

March 2000: Abstract presentation BSH Annual Scientific Meeting, Bournemouth. (British Journal of Haematology 108 suppl.1 74)

November 2000: Abstract publication 42nd Annual ASH meeting, California (Blood 96 (11) supplement 2 508a)

June 2001: Abstract presentation 6th Annual Meeting EHA, Frankfurt (The Hematology Journal 1 suppl.1 38)
**External contacts:**

Dr Anton Kruger Consultant Haematologist  
Haematology Clinic  
Royal Cornwall Hospital Trust Treliske  
Truro  
Cornwall  
01872 252506

Mr Robin Bradley Senior MLSO and Laboratory Manager  
Haematology Laboratory  
Royal Cornwall Hospital Trust Treliske  
Truro  
Cornwall  
01872 252508

SIGNED:  
DATE: 14th January 2003
CHAPTER ONE: INTRODUCTION

1.0 Leukaemia

Leukaemia arises when a single cell in the bone marrow or lymphoid tissue undergoes neoplastic change and starts to proliferate and accumulate. Leukaemia can be classified in two different ways, depending on the type of precursor cell that becomes malignant (either lymphoid or myeloid) and how quickly the disease develops (acute or chronic). Cellular origins of the leukaemias are shown in figure 1. Acute leukaemias consist predominantly of immature cells, usually blast forms and the clinical features of the disease worsen as the number of blasts rapidly increases. Acute leukaemias include acute lymphoblastic leukaemia, the most common type of leukaemia in young children and acute myeloblastic leukaemia, affecting both adults and children. The chronic leukaemias consist of more mature cells, some of which are able to perform some normal functions. Chronic leukaemias include chronic lymphocytic leukaemia which is predominantly found in the elderly, sometimes in younger adults but very rarely in children, and chronic myeloid leukaemia which occurs mainly in adults, but does affect a small number of children. Classifications of the different forms of leukaemia are shown in table 1.

1.1 B-cell Chronic Lymphocytic Leukaemia (B-CLL)

In 1967, William Dameshek wrote of the "immunoproliferative disorder" B-CLL as "...an accumulative disease of immunologically incompetent lymphocytes" (Dameshek W 1967). This was a more detailed description that followed from Turk's initial identification of the disease in 1903 and Minot and Isaacs clinical explanations in 1924.
# TABLE 1: Classification of Leukaemia

<table>
<thead>
<tr>
<th>TYPE OF LEUKAEMIA</th>
<th>POSSIBLE CELLULAR ORIGIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>common acute lymphoblastic leukaemia</td>
<td>lymphoid progenitor cell</td>
</tr>
<tr>
<td>(cALL)</td>
<td></td>
</tr>
<tr>
<td>pre-B acute lymphoblastic leukaemia</td>
<td>pre-B cell</td>
</tr>
<tr>
<td>(pre-B ALL)</td>
<td></td>
</tr>
<tr>
<td>B-cell acute lymphoblastic leukaemia</td>
<td>B cell</td>
</tr>
<tr>
<td>(B-ALL)</td>
<td></td>
</tr>
<tr>
<td>acute myeloblastic leukaemia</td>
<td>myeloid precursor cells</td>
</tr>
<tr>
<td>(AML)</td>
<td></td>
</tr>
<tr>
<td>chronic lymphocytic leukaemia</td>
<td>mantle zone/follicular mature B cell</td>
</tr>
<tr>
<td>(CLL)</td>
<td></td>
</tr>
<tr>
<td>chronic myeloid leukaemia</td>
<td>pluripotent stem cell</td>
</tr>
<tr>
<td>(CML)</td>
<td></td>
</tr>
<tr>
<td>multiple myeloma</td>
<td>plasma cell</td>
</tr>
<tr>
<td>(MM)</td>
<td></td>
</tr>
<tr>
<td>hairy cell leukaemia</td>
<td>mature B cell</td>
</tr>
<tr>
<td>(HCL)</td>
<td></td>
</tr>
</tbody>
</table>

Table showing the classification of the different forms of leukaemia and the possible origin of the "normal" cellular counterpart from which the leukaemic cell may have arisen.
Figure showing the cellular origin of each different type of leukaemia. Normal cellular counterparts are shown as stem cell, T and B lineage cells, T and B cell precursors and mature T and B cell subsets.

- CML = chronic myeloid leukaemia
- AML = acute myeloid leukaemia
- cALL = common acute lymphoblastic leukaemia
- T-ALL = T cell acute lymphoblastic leukaemia
- T-CLL = T cell chronic lymphocytic leukaemia
- T-PLL = T cell prolymphocytic leukaemia
- HCL = hairy cell leukaemia
- MM = multiple myeloma
- B-CLL = B cell chronic lymphocytic leukaemia
- B-PLL = B cell prolymphocytic leukaemia
Many of the clinical and haematological features of B-CLL described by these physicians remain pertinent.

1.1.1 Aetiology

B-cell Chronic Lymphocytic Leukaemia (B-CLL) is characteristically a disease of the elderly, with approximately 90% of all patients over the age of 50 at diagnosis (Whittaker JA 1992; Montserrat E 2001). This does not rule out younger cases, which have been reported in patients less than twenty years of age and even in childhood (Casey TP 1968). The male: female ratio is highest in this form of leukaemia with two males affected for every one female (Zaknoen SL and Kay NE 1990). B-CLL is the most common form of leukaemia seen in Western countries (Kipps TJ 1997; Thompson AA et al 1999; Sakai A et al 2000), with 30 - 40% of all leukaemias diagnosed being B-CLL (Dierlamm J et al 1997). There are dramatic differences between ethnic groups; B-CLL is virtually absent in Japan and Africa, whilst those of Jewish origin display a two-fold greater risk than other western countries (Caligaris-Cappio F 1997).

There has also been much speculation as to a link between certain occupations and the risk of developing B-CLL. In stark contrast to other types of leukaemia, B-CLL is the only one that has not been related to exposure to radiation (Caligaris-Cappio F and Hamblin TJ 1999). For reasons that remain unclear, B-CLL has been associated with farmers who work with high levels of soybean production, cattle raising, dairy produce and herbicides. A greater risk is also associated with those individuals who work in rubber manufacturing and asbestos production (Dighiero G et al 1991). However, there is no clear link established between CLL and exposure to known environmental agents (Caligaris-Cappio F et al 2001).

Familial CLL, where relatives of patients with CLL have an increased risk of developing the disease, suggests that a subset of CLL cases may have an inherited basis. In familial cases of
CLL, the mean age at diagnosis was shown to be approximately 10 years younger than that seen in sporadic cases (Ishibe N et al 2001). The genetic predisposition towards familial CLL is poorly understood. Familial CLL and sporadic CLL are indistinguishable in their morphology, immunophenotype and $V_H$ gene mutations from non-familial cases (Sakai A et al 2000).

1.1.2 Clinical diagnosis

Patients may have the disease for many years before it is diagnosed. As the name suggests, it is a chronic condition, which is slowly progressive until symptoms become noticeable, or, as with many asymptomatic elderly patients, it becomes apparent during a routine blood test. Symptoms of B-CLL vary amongst individuals, with at least 20% of patients displaying few or none at diagnosis. The commonest symptoms at presentation include anaemia, lymphadenopathy, splenomegaly, hepatomegaly and thrombocytopenia (Montserrat E et al 1997).

Haematologically, the disease is distinguishable from other forms of leukaemia. The expression of the aberrant surface marker CD5 allows for a diagnosis of B-CLL, setting it aside from prolymphocytic leukaemia, hairy cell leukaemia, follicular centre cell lymphoma in leukaemic phase, splenic lymphoma with villous lymphocytes and lymphoplasmacytoid lymphoma which do not express CD5 (DiGiuseppe JA and Borowitz MJ 1998). Whilst mantle cell leukaemia is known to express CD5, it is also CD23 negative.

1.1.3 Laboratory diagnosis of CLL

The NCI-sponsored Working Group on CLL (Cheson BD et al 1996) has published guidelines for the diagnosis of B-CLL using the following criteria: an absolute lymphocyte count of $5 \times 10^9/L$ or more, morphologically mature lymphocytes and an immunophenotype of monoclonal B-cells as described below:
i) surface immunoglobulin (usually IgM or IgM and IgD) of low intensity with either kappa or lambda light chain,

ii) expression of the pan-B cell antigens CD19, CD20 and CD23,

iii) co-expression of CD5 on the leukaemic B cells.

The first line in identification of CLL is a full blood count, measuring not only the total and differential white cell and absolute lymphocyte counts, but also haemoglobin (Hb) concentration and platelet count (fig. 2). The presence of a lymphocytosis and low Hb and platelets requires a blood film, for a more detailed examination of these findings. CLL cells often produce "smudge" or "smear" cells, due to their fragility. Intact clonal cells can be identified more clearly by their morphology. Blood will then be sent for immunophenotyping, which involves the staining of mononuclear white cells for surface markers CD2, CD5, CD22, CD23, FMC7 and a kappa/lambda ratio. A CLL diagnosis can be made at this stage and depending on the type of diagnosis (typical or atypical), the patient may require either a bone marrow biopsy or cytogenetic testing or both. A typical panel for CLL is shown in table 2.
### Table 2: Laboratory diagnosis of CLL using monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Cell population identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>gives an absolute T cell count</td>
</tr>
<tr>
<td>CD5</td>
<td>common to all T cells: positive in B cells in CLL</td>
</tr>
<tr>
<td>CD22</td>
<td>B cell marker: CD22&lt;sup&gt;+&lt;/sup&gt; and CD23&lt;sup&gt;-&lt;/sup&gt; indicative of NHL</td>
</tr>
<tr>
<td>CD23</td>
<td>B cell marker: CD22&lt;sup&gt;-&lt;/sup&gt; and CD23&lt;sup&gt;+&lt;/sup&gt; indicative of CLL</td>
</tr>
<tr>
<td>FMC7</td>
<td>FMC7&lt;sup&gt;+&lt;/sup&gt; indicative of NHL, FMC7&lt;sup&gt;-&lt;/sup&gt; indicative of CLL</td>
</tr>
<tr>
<td>κ/λ ratio</td>
<td>normal ratio 2:1, an imbalance in this ratio indicative of CLL and normally monoclonal</td>
</tr>
</tbody>
</table>

Table showing monoclonal antibodies used to diagnose CLL in the laboratory at Derriford Hospital and how they can be used to differentiate between CLL and other forms of leukaemia and lymphoma.
Figure 2: Laboratory diagnosis of CLL

Full Blood Count (FBC)

Absolute lymphocyte count > 5.0

Blood film
smear cells +ve
pleiomorphic lymphocytes +ve

Immunophenotyping
CD2-ve
CD5+ve
CD22+ve
CD23+ve
FMC7-ve
κ / λ ratio not 2:1

CLL diagnosis made

Typical
Bone marrow biopsy

Atypical
Cytogenetics

CLL diagnosis made

Figure showing the a typical laboratory diagnosis of CLL from blood count to immunophenotyping and bone marrow biopsy
After a clear diagnosis of CLL at this stage, patients with typical morphology may not need any further investigation if no treatment is required. Patients with atypical morphology or those with typical morphology who require treatment may need cytogenetic testing for cyclin D1 to distinguish mantle cell lymphoma (MCL), and a bone marrow biopsy. The biopsy allows for the degree of marrow infiltration to be assessed. Bone marrow infiltration is seen in one of four patterns, interstitial, nodular, mixed interstitial/nodular and diffuse (Rozman C et al 1980; Zwiebel JA and Cheson BD 1998) (Table 3).

**Table 3: Bone marrow infiltration patterns**

<table>
<thead>
<tr>
<th>Pattern of infiltration</th>
<th>Degree of marrow infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial</td>
<td>33% of patients: normal haemopoietic cells are replaced in small quantities by mature lymphocytes, but fat cells and bone marrow structure are preserved</td>
</tr>
<tr>
<td>Nodular</td>
<td>10% of patients: nodules of mature lymphocytes are present in the bone marrow in greater quantities than normal lymphoid follicles and they lack clear centres. Fat cells are still present.</td>
</tr>
<tr>
<td>mixed interstitial/nodular</td>
<td>25% of patients: a combination of both interstitial and nodular patterns of infiltration</td>
</tr>
<tr>
<td>diffuse</td>
<td>25% of patients: a diffuse lymphoid infiltration is seen with massive replacement of normal haemopoietic cells and fat cells. There is more disease progression in patients at stage A and B with this marrow infiltration</td>
</tr>
</tbody>
</table>

Table showing the types of bone marrow infiltration identified in CLL patients and the proportion of patients with that type of infiltration

Attempts have been made to use bone marrow infiltration as an indicator of survival and disease progression. It has been suggested that those patients who present with diffuse marrow infiltration have a shorter survival time than those with non-diffuse infiltration (Rozman C et al 1984). However, it has not been possible to correlate bone marrow infiltration with disease progression and survival and patterns of bone marrow involvement probably do not clearly contribute prognostic information beyond what is available from other staging systems (Pangalis GA et al 1987; Desablens B et al 1989; Mauro FR et al 1994; Geisler CH et al 1996; Zwiebel JA and Cheson BD 1998).
1.1.4 Genetic changes

1.1.4.1 Trisomy 12

Clonal chromosome abnormalities can be detected in 50% - 80% of patients with CLL (Dierlamm J et al 1997). The two most common non-random chromosomal alterations specifically associated with B-CLL are trisomy 12 and 13q14 deletion (Matutes E et al 1996). Approximately 10-20% of CLL patients are diagnosed with this aberration using conventional chromosomal analysis (Oscier DG 1994), whilst the use of fluorescent in situ hybridisation (FISH) will detect trisomy 12 in up to 30% of all patients (Jurlander J 1997; Hamblin TJ et al 1997; Zwiebel JA et al 1998). Trisomy 12 is only found in 21 - 37% of the clonal population of B cells and not in the T cells or non-clonal B cells, suggesting that it may be a genetic abnormality secondary to the leukaemic process (Garcia-Marco J et al 1994; Crossen PE 1997; Dierlamm J et al 1997; Navarro B et al 1998; Juliusson G et al 1998). However, Gahn et al have shown that of 7 patients who expressed the trisomy 12 mutation, 3 had the mutation on CD34+ve stem cells, implying that while trisomy 12 is not primarily involved in the leukaemic process, its expression may not in fact be secondary to the formation of the malignant clone (Gahn B et al 1997).

Studies have shown that the presence of trisomy 12 in CLL patients is linked to a higher frequency of stronger expression of surface membrane immunoglobulin (SmIg) and FMC7 expression (Matutes E et al 1996). It also implies a possibly worse prognosis (Geisler CH et al 1997; Criel A et al 1997; Juliusson G et al 1998; Hogan WJ et al 1999; Dohner H et al 1999). Three genes involved in cell-cycle activation, cyclin D2, CDK4 and MDM2, are all located on chromosome 12. Each is over-expressed in CLL clonal B cells (Jurlander J et al 1998).

A possible association between trisomy 12 and atypical morphology, such as a cleaved nucleus and an increase in the total number of prolymphocytes, has been postulated in several studies (Criel A et al 1994; Matutes E et al 1996; Woessner S et al 1996). However,
the presence of this genetic abnormality has not been linked to the stage of the disease or with patients' responses to treatment. In contrast, disease progression and treatment free survival (TFS) time have been linked to trisomy 12. In a group of 54 untreated patients, each of the seven patients found to have trisomy 12 had a median TFS time significantly less than those patients with a normal chromosome 12 (Hogan WJ et al 1999).

1.1.4.2 13q14

Deletion at 13q14 is indicative of the loss or inactivation of a tumour suppressor gene. Using Fluorescence In-Situ Hybridisation (FISH), approximately 30% of CLL patients show a deletion of chromosome 13 at region q14 (Avet-Loiseau H et al 1996; Starostik P et al 1999). As with trisomy 12, this aberration is only seen in a proportion of the clonal B cells, again suggesting a possible secondary involvement in the leukaemic process (Jabbar SA et al 1995; Navaho B et al 1998). In contrast to other haematological malignancies, 13q14 has been shown to represent a loss of genetic material rather than over-expression (Gardiner AC et al 1996; Dohner H et al 1999). The retinoblastoma (Rb) tumour suppressor gene is located at region 13q14 and while it was thought that this gene might be an important candidate in the biology of CLL, CLL B cells with Rb loss will still express retinoblastoma protein (Oscier DG et al 1990; Liu Y et al 1993). In addition to this gene, it has been speculated that a novel gene exists close to the RB gene, named DBM (deleted in B cell malignancy) (Brown AG et al 1993). Molecular analysis has shown that 80 to 90% of patients with a 13q14 deletion have a monoallelic loss of this region, whilst 34 to 46% of patients who were previously shown to have a cytogenetically normal chromosome at 13q14 actually have the same loss of DBM (Oscier DG et al 1994). At present, the role of this deletion in disease pathogenesis and prognosis remains to be determined.
In contrast to the poorer prognosis associated with trisomy 12, 13q14 deletions are not linked to morphological changes or a worsening prognosis (Finn WG et al 1996; Kroft SH et al 1997; Juliussen G et al 1998; Hogan WJ et al 1999; Dohner H et al 1999).

In addition to the 13q14 deletion, which incorporates the span 13q12-q14, there is also a common deletion found at the region 13q14-q22.

1.1.4.3 p53

A p53 gene abnormality is present in approximately 10 to 25% of patients with B-CLL (Oscier DG 1994; Jurlander J 1998) and its presence has been linked to more aggressive forms of CLL as well as increased chemotherapy resistance and a higher chance of transformation from CLL to Richter's syndrome (El Rouby S et al 1993). p53 is a tumour suppressor gene located on chromosome 17, which induces apoptosis in response to DNA damage (Parham P 2000). It acts as a growth suppressor, either by causing cell cycle arrest at the G1 stage of the cell cycle or by causing apoptosis (Zwiebel JA and Cheson BD 1998). A mutation in this gene could possibly prevent apoptosis and lead to the accumulation of cells characteristic of CLL. The presence of p53 in CLL cases has also been linked to a poorer prognosis and disease progression (Cano I et al 1996; Amiel A et al 1997).

1.1.4.4 Immunoglobulin gene mutations

In normal B cell development, the variable regions of the immunoglobulin heavy (V\textsubscript{H}) and light (V\textsubscript{L}) chains undergo somatic hypermutation as they pass through germinal centres. Early studies on the V\textsubscript{H} genes in CLL suggested that most malignant B cells had unmutated V\textsubscript{H} genes, leading to the hypothesis that the B cell clone could have arisen from a naïve B cell such as CD5\textsuperscript{+} mantle cells which had not passed through the germinal centre (Oscier DG 1999). As larger studies were carried out however, up to 50% of CLL patients V\textsubscript{H} genes were shown to have mutations, indicating that they would have passed through the
germinal centre (Hamblin TJ et al 1999). The finding of unmutated $V_H$ genes was associated with atypical morphology, isolated trisomy 12, advanced stage and progressive disease.

1.1.4.5 Additional genetic changes

Although the main chromosomal and genetic changes of CLL are described above, there are other important alterations in the B-CLL genome.

*Bcl-2 re-arrangements:* a translocation at the region of the Bcl-2 gene can lead to over expression of the Bcl-2 protein. The gene coding for immunoglobulin can translocate with the Bcl-2 gene, leading to over-expression of the protein, which normally functions to prevent premature apoptosis in B cells (Oscier DG 1994). A continual over-expression of this protein could promote an accumulation of B cells unable to undergo programmed cell death. Although the Bcl-2 re-arrangement is rare in CLL, Bcl-2 is over-expressed in approximately 90% of patients (Kipps TJ 1997) and the reason for this is not clear (Gottardi D et al 1995; Osorio L et al 1998). Differences have been observed in expression of Bcl-2 at different stages of the disease. Expression was lower at stage A and B of the disease (33% and 29% respectively), whilst expression at stage C was 80%, suggesting that more advanced cases have a greater involvement of Bcl-2 as the disease progresses (Aviram A et al 2000).

*Chromosome 6:* in CLL, deletions in chromosome 6 have been reported which incorporate the regions 6q15 and 6q23, in approximately 12% of patients with clonal abnormalities (Dierlamm J et al, 1997).
Chromosome 11: approximately 16% of B-CLL patients with a clonal abnormality have structural changes in chromosome 11 and 37% of those have this abnormality as a single aberration. In addition, a translocation at 11:14 has also been demonstrated. Changes at chromosome 11q are most frequent in typical CLL and are associated with progressive disease (Dierlamm J et al 1997; Neilson JR et al 1997). 11q21-q23 deletions involving the ataxia telangeictasia mutated (ATM), radixin (RDX) and FDX genes are also frequently observed using FISH techniques in CLL patients (Stankovic T et al 1999; Oscier DG 1999). This leads to a reduced expression of the ATM protein. The presence of these mutations signifies a very poor prognosis.

Chromosome 14: Changes seen at chromosome 14 involve the immunoglobulin heavy-chain locus at 14q32 and have been observed in 16% of all CLL cases. (Dierlamm J et al 1997). Translocations which result in rearrangement and upregulation of the bcl-3 gene are observed at t(14;19)(q32;q13). This change is often associated with trisomy 12, young age and aggressive disease.

Chromosome abnormalities and surface antigen expression: recent work has investigated the possible relationship between chromosome abnormalities and the expression of surface antigens (Hulkkonen J et al 2002). In particular, deletion of chromosome arm 11q was linked to a reduced expression of the splicing variant CD45RA, but an enhanced expression of CD45RO. Cases of trisomy 12 were associated with increased CD45RA and reduced CD45RO expression, in addition to an increased expression of CD27 and membrane Igkappa. It is possible that these observed links between genetic aberrations and cell surface antigen expression may have an effect on the progression of the disease and the individual patient response to drug treatment.
The finding of chromosomal and genetic changes may provide important information, which increases understanding of the aetiology of CLL and other related lymphoproliferative disorders. Some abnormalities improve the assessment of prognosis and can also contribute to decisions of treatment.

1.1.5 Telomerase activity

When a eukaryotic cell undergoes replication, telomere length is reduced as genetic material is reproduced and divided between the two new cells (Berendes HD and Meyer GF 1968). Somatic cells are able to maintain unlimited replicative potential through the activity of telomerase, an enzyme which prevents telomere shortening and permits continued replication (Ballon G et al 2001; Helder MN et al 2002). Normal peripheral blood cells express low but detectable levels of telomerase activity, which can be upregulated in T and B lymphocytes after *in vitro* stimulation. Approximately 90% of all solid tumours have telomerase activity that is significantly higher than normal cells (Lubbe J et al 1997), yet levels of telomerase activity in CLL are only significantly higher in patients with progressive disease (Bechter OE et al 1998). However, compared to normal controls, telomerase has a significantly higher activity in CLL B cells. The lower levels of telomerase observed in CLL compared to solid tumours and other haematological malignancies, such as hairy cell leukaemia, might reflect the lower number of proliferating cells, the higher degree of cell differentiation and the theory that B-CLL cells may originate from resting B lymphocytes.

1.1.6 Classification of disease stage and prognosis

Whilst detection of genetic aberrations is important in the study of B-CLL, a means of classifying the stage of disease in each individual patient is still more relevant to the identification of those subgroups of patients who require immediate treatment, those who
will need treatment in the near future and those who are able to continue without treatment for some time. In current use today are two methods of staging, which may be used alone or in combination, the Rai staging system and the Binet staging system. There have been many clinical studies that have involved assessing the importance of both systems (Rai KR et al 1975; Binet JL et al 1981) and clinical staging remains the most important prognostic tool (Catovsky D 1997). The staging system described by Rai incorporates a grading of clinical signs and laboratory findings at the time of diagnosis, which segregates patients into five stages from stage 0 through to stage IV. These are detailed in table 4.

Table 4: Rai's staging criteria for CLL

<table>
<thead>
<tr>
<th>CLINICAL STAGING</th>
<th>CLASSIFICATION</th>
<th>SURVIVAL (MONTHS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE 0</td>
<td>Blood and marrow lymphocytosis (absolute blood lymph count ≥15 x 10⁹/L, 40% or more lymph's in the marrow)</td>
<td>&gt;120</td>
</tr>
<tr>
<td>STAGE I</td>
<td>Blood and marrow lymphocytosis with lymph node enlargement</td>
<td>95</td>
</tr>
<tr>
<td>STAGE II</td>
<td>Blood and marrow lymphocytosis with splenomegaly and/or hepatomegaly (with or without lymph node enlargement)</td>
<td>72</td>
</tr>
<tr>
<td>STAGE III</td>
<td>Blood and marrow lymphocytosis with Hb lower than 11g/dL (excluding autoimmune haemolytic anaemia, with or without lymph node, spleen or liver enlargement)</td>
<td>30</td>
</tr>
<tr>
<td>STAGE IV</td>
<td>Blood and marrow lymphocytosis with platelet count lower than 100 x 10⁹/L (with or without lymph node, spleen and liver enlargement and independently of Hb)</td>
<td>30</td>
</tr>
</tbody>
</table>

Table showing the clinical stage of CLL and classification of the symptoms at each stage as defined by Rai (Rai KR et al 1975).

Rai's staging system indicates that patients who can be categorised in stage 0 of the disease need not receive treatment in the form of radiotherapy or chemotherapy and in whom progression of their disease should be monitored regularly. In stages I and II, treatment is
not usually required unless there are significant symptoms relating to more advanced
disease and, if so, a reduction in tumour mass may be advantageous clinically. In stages III
and IV, the objective of treatment is to achieve a complete remission or, if that is not
possible, a good partial remission. Rai has shown in his studies that patients who present
with more severe symptoms and a higher lymphocyte count at the time of diagnosis tend to
have poorer prognosis. Those patients diagnosed at stages 0, I and II lived longer than those
presenting at stages III and IV (Rai KR et al 1975). Patients may progress from one stage to
another but survival times remain specific to the stage at which a prognosis is made. The
staging system as indicated by Binet is shown in table 5.

Table 5: Binet's staging criteria for CLL.

<table>
<thead>
<tr>
<th>CLINICAL STAGING</th>
<th>CLASSIFICATION</th>
<th>SURVIVAL (MONTHS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE A</td>
<td>Hb equal or greater than 10. Platelets equal or greater than 100,000 mm$^3$. Less than three enlarged areas*</td>
<td>&gt;120</td>
</tr>
<tr>
<td>STAGE B</td>
<td>Hb equal or greater than 10. Platelets equal or greater than 100,000 mm$^3$. Three or more enlarged areas*</td>
<td>61</td>
</tr>
<tr>
<td>STAGE C</td>
<td>Hb less than 10 and/or platelets less than 100,000 mm$^3$. Any number of enlarged areas*</td>
<td>32</td>
</tr>
</tbody>
</table>

* = Enlarged areas incorporate cervical, axillary, inguinal, spleen and liver

Table showing the clinical stage of CLL and classification of the symptoms at each stage as defined by Binet (Binet JL et al 1981).

In this system, more emphasis is placed on isolating patients defined as high risk by the
presence of anaemia or thrombocytopenia. It has been postulated by Binet that it is possible
to combine the two systems of classification. Rai stages 0 and III can be assigned to Binet
stage A, Rai stage IV is grouped with Binet stage C and the remaining patients from stage
II can be separated into stage A and B depending on physical signs (Binet JL et al 1981).
Binet stage A can be further sub-divided into Stage A’ and Stage A” (Digheiro G et al 1991). Stage A’ is characterised by a lymphocyte count less than \(30 \times 10^9/L\) and an Hb greater than 12g/dL. 80% of stage A patients fall into this category. Stage A” is characterised with either a lymphocyte count greater than \(30 \times 10^9/L\) or Hb less than 12g/dL. 20% of stage A patients fall into this category.

The use of these staging systems has helped to simplify the classification of B-CLL patients, essentially by separating them into low risk, intermediate risk and high risk (Rai KR et al 1990). Whilst both systems undoubtedly remain at the forefront of B-CLL staging, there is a degree of controversy surrounding both methods as to their usefulness in identifying individual groups of patients who may or may not benefit from therapy (Foon KA et al 1987; Zwiebel JA et al 1998). As new methods of detection emerge, it is envisaged that factors such as CD38 expression, beta2-microglobulin, soluble serum CD23 levels and lymphocyte doubling time may play an important role as independent prognostic factors and indicators of the need for treatment (Vilpo J 1999; Del Poeta G et al 2001; Ibrahim S et al 2001). Some of these methods are being put into practice today.

Whilst the Rai and Binet staging systems are important in the diagnosis of CLL, both rely heavily on clinical and morphological features. In recent years, there has been an increase in the use of current techniques such as immunophenotyping to classify lymphoproliferative disorders. With reference to the classification of CLL, a scoring system has been proposed (Matutes E et al 1994) (table 6). In this system, points are allocated to each patient, depending on the expression of key immunological markers associated with CLL, the final total score indicating the likelihood of a positive diagnosis. In the initial study, the majority of patients (87%) scored the expected total of 4 or 5, indicating a clear case for CLL diagnosis. This system allows a clearer definition of CLL from other morphologically similar diseases such as splenic lymphoma with villous...
lymphocytes (SLVL), follicular lymphoma, mantle cell lymphoma and lymphoplasmacytic lymphomas.

Table 6: Scoring system for CLL

<table>
<thead>
<tr>
<th>Membrane marker</th>
<th>1 point</th>
<th>0 points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smlg</td>
<td>weak</td>
<td>moderate/strong</td>
</tr>
<tr>
<td>CD5</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>CD23</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>FMC7</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>CD22</td>
<td>weak/negative</td>
<td>moderate/strong</td>
</tr>
</tbody>
</table>

Score 4-5: typical CLL  
Score 3-4: atypical CLL  
Score < 3: non-CLL

Table showing the scoring system for CLL. Points are allocated to each patient depending on the presence or absence of the surface antigens shown and the total score can aid a diagnosis of typical or atypical CLL or another form of leukaemia (Matutes E et al 1994).

1.1.7 Newer prognostic factors

B-CLL is a heterogeneous disease, which may constantly change, both within the individual and amongst the population, although it can stay stable until death. A comparison between patients diagnosed in the time span of 1960-1979 against those diagnosed during 1980-1989 has shown striking and statistically different results. In the latter cohort of patients, diagnosis was made at a later age, more patients were diagnosed in low risk groups and survival time was more than double (Rozman C et al 1997). As diagnostic tools have become more widely available and their use has increased amongst the elderly population, this change is likely to reflect not so much a change in the disease itself, but instead more accurate and earlier diagnosis. Today, the median survival time for
patients with CLL is approximately 10 years, but prognosis among individuals is highly variable, with some patients having no reduction in normal life expectancy and others for whom median survival is less than 3 years (Rai K et al 1975; Binet J et al 1981; Dierlamm J et al 1997; Montserrat E 2001). CD38 expression, beta2-microglobulin, soluble serum CD23 levels, CD23 surface expression, interferon-α receptor (IFN-α) expression and lymphocyte doubling time may all play an important role as prognostic factors (Dadmarz R et al 1998; Vilpo J 1999; Popescu D et al 2001; Del Poeta G et al 2001; Ibrahim S et al 2001). Impairment of immune function as demonstrated by hypogammaglobulinaemia, frequent and recurrent infections and neutropenia also suggest a poorer prognosis and disease progression. Low levels of serum IgA and IgG, but not IgM, worsen with disease progression and are indicative of a poor prognosis (Rozman C et al 1988). The expression of adhesion markers in both cellular and soluble form has also been shown to play an important role in disease prognosis. High levels of soluble CD44 and CD54 are both considered adverse prognostic factors (Angelopolou MK et al 1999). The increasing use of molecular techniques has introduced another opportunity to study disease prognosis. It has been possible to show that telomerase, an enzyme which mediates the repair and preservation of telomeres within chromosomes and thus prevents the ageing process normally found in somatic cells, has a significantly higher activity in B cells in CLL patients compared to normal controls. This enhances the ability of CLL cells to live longer and continually repair DNA, not only by preventing the erosion of telomeres, but by actually restoring telomere length (Bechter OE et al 1998). Telomerase activity may become an important independent variable in the new generation of prognostic factors. CD38 may also prove to be a useful tool for disease prognosis (Caligaris-Cappio F et al 1999). CD38 expression has been linked to B cells that show no mutation of their immunoglobulin variable (IgV) genes (Caligaris-Cappio F 1999). Mutation of IgV genes normally occurs when developing B cells encounter antigen in the germinal centres (GC) of
secondary follicles. Point mutations occur in the IgV genes and result in the production of antibody. Examination of IgV re-arrangement in CLL has shown that approximately half of all CLL cases have mutations, implying that the B cell has passed through the GC within the secondary follicles and has had exposure to antigen. In contrast, the other half of the CLL cases which did not reveal IgV mutations would suggest that the clone originates from a naïve B cell that may not have previously encountered antigen within the GC (Hamblin TJ et al 1999; Oscier DG 1999). B cells with unmutated IgV genes have a much higher percentage of CD38-positive cells, so these two features could prove to be useful tool for prognosis of the disease (Damle RN et al 1999). The presence of unmutated IgV genes has been strongly associated with atypical lymphocyte morphology, progressive disease and shorter survival (Oscier D 1999; Caligaris-Cappio F 1999; Damle RN 1999). A more recent report has further confirmed these findings, showing that CD38 expression in association with a secondary 17p deletion are poor prognostic factors, particularly in early stage disease (Chevallier P et al 2002). There have been suggestions that this dichotomy may have clinical implications, as it could provide the basis of why some patients never require treatment while others need intensive therapy (Caligaris-Cappio F 1999). However, it is still not clear exactly what the role of CD38 is in relation to IgV mutations and research is continuing to clarify its role in prognosis of B-CLL (Jelinek DF et al 2001).

The expression of p53 abnormalities and loss of the retinoblastoma (Rb) gene, which is often linked to drug resistance, also indicate a poor prognosis (Morabito F et al 1997; Cordone I et al 1998). In contrast, the deletion at site D13s25, a molecular marker located on chromosome 13 band q14 found in 95% of all clonal CLL cells, gives a more favourable prognosis (Jabbar SA et al 1995; Oscier DG 1999). As molecular techniques expand, an increasing number of genetic sites will be identified allowing for a better and more accurate diagnosis and prognosis for individual CLL patients.
1.2 Treatment

B-CLL is a disease that often follows a path of slow progression particularly for patients diagnosed at an early stage. As many of these patients are elderly, a policy of "wait and see" is often adopted for those who show little or no symptoms or signs related their diagnosis. Many patients can continue for a number of years without symptoms (Montserrat E 2001) and a delay in treatment reduces the risk of drug resistance if the disease should progress. In addition, many of the drugs commonly used for the treatment of B-CLL can have adverse side effects and delaying treatment will avoid these for a time. It is important that the choice of treatment and management of CLL takes account of the patients' age, quality of life and the toxic side-effect of the treatment (Oscier DG 1999).

From the time of diagnosis, it is important to monitor patients closely for a period to observe any noticeable changes indicating progression of disease, such as unexplained weight loss, fever, increasing infective episodes, increasing tiredness, lymph node enlargement, increasing lymphocyte count, falling normal blood counts and abdominal distension or discomfort due to splenomegaly (Whittaker JA 1987; Rai KR et al 1998; Montserrat E 2001).

Once an accurate identification of disease stage has been made, treatment depends on a number of factors including symptoms, prognosis and age as treatment that may be suited to the younger patient with more aggressive disease could be inappropriate and even harmful to the elderly patient with indolent disease (Montserrat E 2001).

1.2.1 Low risk (modified Rai criteria stage 0), Binet stage A

Patients diagnosed at this stage have been shown to obtain no benefit from receiving treatment with either chemotherapy or radiotherapy (Montserrat E 2001). A recent study by the French co-operative group on CLL assigned 609 stage A patients to either no treatment or chlorambucil and compared this group to a further 926 stage A patients who received no
treatment or chlorambucil plus prednisolone. The study took place over ten years with a median follow up of six years and results showed that early intervention with chlorambucil, with or without prednisolone, did not affect the overall survival of the patient groups. This has helped to validate the previous notion that treatment should be deferred until the disease progresses (Catovsky D 1997; Rai KJ 1998; Digheirio G et al 1998). If lymphocyte counts are low and the patient is relatively free from symptoms, they should simply be seen regularly and observed for progression. At this stage, the rate of lymphocyte doubling time is the most important factor in the decision to begin treatment.

1.2.2 Intermediate risk (modified Rai criteria stage I - II), Binet stage B
Therapy may be initiated for patients in this staging group if the patient shows disease related symptoms such as rapid lymphocyte doubling time or enlarged nodes. At present, the aim of therapy within this population is to reduce tumour size and achieve a good level of remission. The cornerstone of treatment is usually chlorambucil (see below) (Rai KJ 1998).

1.2.3 High risk (modified Rai criteria stage III - IV), Binet stage C
Most patients within this group are treated immediately without the "wait-and-watch" phase (Montserrat E 1999; Rai KJ 1999). The aim is to reduce the bulk of the disease and then to improve levels of haemoglobin and platelets and to achieve a complete remission or a good partial remission. Chemotherapy is used first line and increasingly more aggressive regimes are applied in younger patients.
1.2.4 Treatment regimes for CLL

**Chlorambucil**

This agent works by alkylating DNA, attacking nucleophilic sites and causing cell death (Chabner BA et al 1996). It is one of the most frequently used drugs in the treatment of B-CLL as it is usually very effective in reducing the white cell count (WCC). Patients may be treated either with large doses over a short period of time, or smaller doses over a more extended time period (Montserrat E 1999). Continuous, low dose exposure is now discouraged for all alkylating agents as this carries a greater risk of second malignancy (Asten P et al 1999). Chlorambucil induces a good partial response with low toxicity in 60 to 70% of patients, but long-term use invariably leads to drug resistance (Oscier DG, 1999).

**Fludarabine**

This adenosine analogue works by inhibiting DNA polymerase and ribonucleotide reductase within rapidly dividing cells, thus preventing the growth of the leukaemic cells. It is taken up by the cell and becomes incorporated into new nuclear material and once phosphorylated, prevents DNA synthesis. Recently, this drug has become increasingly more important as first line treatment for B-CLL after two large multi-centred trials showed more complete and overall remissions with fludarabine than with previous standard chemotherapy and that the remissions lasted significantly longer (Rai KR et al 1996; Johnson S et al 1996; Bumbea H et al 2001). It is also an important drug in patients with advanced CLL, which prove unresponsiveness to other agents (Giraldo P et al 1999). Toxicities associated with this form of treatment include myelosuppression and a markedly decreased CD4 T cell count, which can lead to an increase in opportunistic infections (Williams WJ 1996). Fever of unknown origin and pneumonia can also occur (Myint H et al 1995; Catovsky D 1997). It may be necessary to protect patients on fludarabine against
specific infections such as pneumocystis carinii pneumonia with oral septrin for up to six months after the last treatment is completed and long-term antibiotic prophylaxis is often recommended (Bergmann L et al 1993). Other potential side effects include auto-immune haemolytic anaemia (AIHA), transfusion related graft versus host disease and, rarely, neurotoxicity. In addition to fludarabine, the other nucleoside analogues cladribine and pentostatin also have activity in CLL. Cladribine can be effective in early stage CLL and has had promising results in the treatment of hairy cell leukaemia. However, the literature and experience with these drugs are not nearly as extensive as with fludarabine. Increasingly, fludarabine is being used in combination with other chemotherapeutic agents with good results (Rai KJ et al 1998). A recent study has shown that cyclophosphamide given in combination with fludarabine gave a 75% complete response in CLL patients (Ferrara F et al 2001; Ferrer AFA 2001). The main complications of this form of treatment include severe neutropenia, myelosuppression and infection.

**Pentostatin**

This potent inhibitor of adenosine deaminase is an anti-tumour antibiotic, which leads to apoptotic death of the leukaemic cells. It has been used successfully in combination with cyclophosphamide to produce complete responses without significant myelosuppression in previously treated patients (Weiss MA et al 2000). It has also been used successfully in combination with theophylline and chlorambucil to down-regulate the anti-apoptotic marker bcl-2 in previously treated CLL patients (Byrd JC et al 2000). The main disadvantage of this drug is its myelotoxicity and immunosuppression, which can lead to an increase in the risk of infection, especially with opportunistic organisms (Margolis J and Grever MR 2000). Treatment with pentostatin can lead to a significant decrease of total lymphocytes, particularly in the CD4 T cell compartment (Kraut EH et al 1990). Levels of both immunosuppression and myelotoxicity however, are lower than with the other purine
analogues (Weiss MA et al 2000). Cladribine, another adenosine deaminase, is believed to work by inducing apoptosis through mitochondrial transmembrane loss and other similar mechanisms (Marzo I et al 2001). It has been used in combination with mitoxantrone and cyclophosphamide in the treatment of previously untreated B-CLL patients (Robak T et al 2001). Studies have shown that cladribine gives similar complete response (CR) rate and overall response (OR) rate as fludarabine in the treatment of CLL (Robak T 2001).

**Cyclophosphamide**

This is another alkylating drug that causes apoptosis of the cell (Haskell CM 1995). Cyclophosphamide may be used as single agent therapy, but it also plays an important role in combination treatments such as COP (Cyclophosphamide, Vincristine, Prednisolone) and CHOP (Cyclophosphamide, Doxorubicin, Vincristine, Prednisolone). However, studies have shown no differences in survival in patients with stage B disease treated with CHOP compared to those treated with chlorambucil plus prednisolone (French Cooperative Group 1994).

**Prednisolone**

This synthetic corticosteroid is used for short periods during treatment to reduce the size of the spleen and lymph nodes and also to alleviate anaemia and thrombocytopenia and other conditions which have an autoimmune basis. Recently, high dose methyl prednisolone has been shown to be an effective treatment regime either alone or in combination with other regimes in salvage treatment for CLL patients with p53 abnormalities and those refractory to previous treatment regimes (Thornton PD et al 2000). They are not used to obtain lasting remission, although they are directly lymphocytotoxic.
**Vincristine**

This drug is used in combination therapy alongside such other agents as prednisolone and cyclophosphamide and may be effective in patients refractory to alkylating agents. Vincristine is a vinca alkaloid that exerts its effect on malignant cells as a spindle poison by inhibiting the mechanisms essential in cell replication. The drug interacts with tubulin subunits within the cell and prevents microtubule assembly, inducing abnormal chromosome segregation in dividing cells (Gonzalez M et al 1999). However, side effects of vincristine treatment can include peripheral neuropathy, as the vinca alkaloids may interfere with normal axon microtubule assembly (Sahenk Z et al 1987).

**Radiotherapy**

The use of radiotherapy for patients has been shown to be beneficial when there are localised bulky lymphoid masses or for an enlarged spleen. Patients often respond well to only small doses of radiation although it is important to monitor blood counts closely as this form of treatment can lead to bone marrow suppression (Whittaker JA 1990; Digheiro G et al 1991). Splenic irradiation will also induce the "abscopal" response, in which malignant cells beyond the filed of irradiation undergo apoptosis. This response has never been fully explained (van Mook WN et al 2001), but mechanisms may include direct cell kill, immune modulation via changes in lymphocyte subsets or the induction of a cytokine response (Weinmann M et al 2001).

**Splenectomy**

Patients with very large spleens or those who are diagnosed with hypersplenism or with problematic autoimmune thrombocytopenia or anaemia may benefit from a splenectomy if localised splenic irradiation is not effective or if the patient is refractory to treatment with steroids. (Digheiro G et al 1991; Whittaker JA 1992). This form of treatment can help to
control the disease for some time but can cause serious morbidity and carries a 7% mortality rate from overwhelming post splenectomy infection (OPSI) (Montserrat E 1999; Mauro FR et al 2000), often form pneumocystis carinii pneumonia.

**Blood Transfusions**

Patients who are anaemic as a result of extensive replacement of bone marrow by leukaemic cells, because of myelosuppression by cytotoxic chemotherapy or because of hypersplenism may benefit from regular or "top-up" blood transfusions. They usually improve general well being and alleviate symptoms such as tiredness and lethargy due to the anaemia, so they are useful in maintaining quality of life. However, repeated transfusions over extended periods of time can lead to difficulties with future transfusions through the development of allo-antibodies, which make cross matching more complicated, and iron overload (Williams W J 1996).

**DiSC Assay**

The differential staining cytotoxicity (DiSC) assay involves *ex vivo* drug panel testing against patient tumour cells to identify optimal therapy (Mason JM et al 1999). To date, there have been numerous studies using this method to identify effective, patient-based treatment regimes in B-CLL. This *ex vivo* method of drug testing has proved useful in the treatment of B-CLL, particularly in relapsed patients where several therapeutic options may exist (Bosanquet AG and Bell BP 1996).

**1.2.5 Recent advances in the treatment of B-CLL**

**1.2.5.1 Autologous and allogeneic stem cell transplants**

The use of stem cell transplants to treat CLL has been increasing over the last few years (Montserrat E 2001). This modality is only applicable to younger patients because of the
toxicity associated with the treatment. The use of allogeneic transplants, when the patient receives stem cells from an HLA-matched (related or unrelated) or mis-matched donor, is also limited by the age of the patient. Although allogeneic transplants, when successful, can carry good durable responses with 40% of patients remaining disease free at 4 years, they also carry a high transplant-related morbidity (TRM) of between 25-50% (Montserrat E 2001). The conditioning therapies carried out before transplant lead to profound pancytopenia, and consequently infections are common. In addition there are therapy related organ-toxicities, especially to the liver and kidneys (Negrin R et al 1998). Autologous transplants, which involve the patient receiving a re-infusion of their own stem cells, has a lower TRM (<2%), but the absence of a plateau in survival curves for these patients suggests that this form of treatment is not a "cure" for CLL. Survival time, however, may be prolonged (Montserrat E 2001). A recent report (Khour I et al 1998) has shown that patients who become refractory to treatment with fludarabine show good responses to stem cell transplantation, with those receiving allogeneic transplants having the most favourable survival times. 87.5% were in complete remission following transplantation and 57% had survival rates of 3 years. Survival times are becoming increasingly more promising, particular in early stage patients (Dreger P et al 2000).

1.2.5.2 Graft-versus-host (GVH) and graft-versus-leukaemia (GVL)

Two immunological barriers need to be crossed for a successful stem cell allograft to take, the host-versus-graft (HVG) and graft-versus-host (GVH). In order to minimise HVG, the immune system of the patient must be eradicated before transplantation occurs to prevent the host system attacking the new graft (Negrin R et al 1998). Conversely, GVH must be minimised after transplantation to prevent the newly infused donor stem cells from recognising the host immune system as foreign. Both of these responses are mediated by alloreactive T cells from either the hosts' immune system or from the donor stem cells.
(Hoffbrand A et al 1999; Burakoff SJ et al 2001). The incidence of GVH is influenced by the number of alloreactive T cells infused, but there is no direct correlation between the total amount of T cell numbers infused and the severity of the GVH reaction.

Evidence from research carried out on murine models and on selective depletion of T-cell subsets in human transplants suggests that the CD4 and CD8 T cell subsets are responsible for the GVH, and that the subset responsible would depend on the class of mismatch that occurred between host and donor (Ferrara JL and Deeg HJ 1991). For instance, if the class I MHC antigens were mis-matched, CD8 T cells would dominate the response and conversely, if the disparity between host and donor was in the class II MHC antigens, CD4 subsets would be the main initiators of GVH (Ferrara JL and Deeg HJ 1991). Both CD4 and CD8 are important when the difference between host and donor is in the minor histocompatibility complex. Cytokine release is also an important factor in GVH. IL-1, IL-2 and Tumour Necrosis Factor (TNF) have all been identified during a GVH response (Hoffbrand AV et al 1999).

However, while the GVH reaction can be fatal if not properly controlled, it has been shown that a type of GVH is responsible for many of the observed cures seen after stem cell transplantation (Weiden PL et al 1979). This response, termed graft-versus-leukaemia (GVL) occurs when donor T cells from the newly infused stem cells recognise and respond to the malignant leukaemic clone. Whether GVL and GVH are either similar phenomena or two distinct and different responses remains to be discovered. The GVL effects indicates that donor T cells can respond to the leukaemic clone whilst the patients own T cells cannot, implying that the malignant clone may be preventing the host T cells from mounting the response observed in the newly infused donor lymphocytes. In comparison, the fact that patients are able to mount a host-versus-graft response suggests that the host T cells can recognise foreign antigen from the donor, but not the malignant clone. This
apparent "anergy" is relevant for this study and for the implications of potential for adoptive immunotherapy using host T cells.

1.2.5.3 Donor lymphocyte infusions

Until recently, the only chance of a cure after a relapsed bone marrow or stem cell transplant was a second transplant (Porter DL and Antin JH 1999). However, it has since been discovered that an infusion of lymphocytes collected from the original stem cell donor back to the patient can induce a direct graft-versus-leukaemia reaction. Using donor lymphocyte infusions (DLI), complete remission can be re-established in many patients whose haematological diseases have relapsed post-allograft, but the exact mechanisms of this effect are not well understood. The effector cells and target antigens that control GVL are poorly defined, but once identified, they may lead to the possibility of harnessing the immune system for a clinical response. The success of DLI again suggests that there is a failure of the patients T cell compartment in responding to the leukaemic clone, whilst the donor lymphocytes are somehow able to identify and respond to the malignant cells and, in some cases, induce a complete remission

1.2.5.4 Monoclonal antibodies

Campath 1-H and Rituximab (also known as Mabthera) are monoclonal antibodies (mAb) which have been used extensively in the treatment of CLL. Campath 1-H is a genetically engineered antibody directed against the CD52 antigen (Rai KR et al 1998) and has recently been licensed in the UK for use in CLL. CD52 is found on the surface of most lymphocytes including normal B and T cells and CLL B cells. It is not expressed on granulocytes, platelets or haemopoietic progenitor cells (Osterborg A 1999).

Rituximab is also a mAb directed against the CD20 antigen found on all B cells and CLL B cells (Montserrat E 2001). It is licensed for the treatment of follicular lymphoma. The
levels of expression of CD20 are much lower on the surface of CLL cells and therefore the clinical effects seen with rituximab in CLL are not as impressive (Dyer MUS 1999).

Both monoclonal antibodies mediate anti tumour activity using immunological effects such as complement-mediated lysis, antibody dependent cellular cytotoxicity (ADCC) and signalling pathways that lead to induction of cell death (Stanglmaier M et al 2000; Pedersen IM et al 2000). Both agents can be used to treat minimal residual disease and can be applied in stem cell transplantation to "purge" any residual malignant cells (Rai KR et al 1998; Binet JL 1999; Rai KR et al 2000). Currently these antibodies are being used in combination with chemotherapeutic agents such as fludarabine in order to improve complete response (CR) rates from 25-30% as seen with fludarabine alone (Rai KR 1999). Recent studies comparing apoptotic rates of fludarabine with Campath 1-H and Rituximab have shown that both are less toxic than fludarabine and that both monoclonal antibodies were efficient at inducing good rates of apoptosis when used in conjunction with fludarabine in vitro (Grdisa M et al 2000).

Both antibodies however, can have serious side effects (Rai KR 1998; Montserrat E 2001). Campath 1-H can cause fever, chills and rash as "first-dose" reactions and an increased susceptibility to opportunistic infections, in particular, reactivation of cytomegalovirus (CMV). Campath 1-H can also deplete T cells, as CD52 is expressed on the T cell surface, which increases the risk of infection to the patient. In particular, patients with prolymphocytic forms of CLL have been shown to express higher levels of CD52 on the T cell surface compared to T cells from normals (Binet JL, 1999). Patients with high white cell counts who are treated with rituximab may develop "cytokine-release syndrome", characterized by fever, rigors, skin rash, nausea, vomiting and hypotension (Montserrat E 2001).

There are a number of newer monoclonal antibodies, which utilise radioactive moieties attached to anti-CD20 to deliver localised targeted radiotherapy to disease sites. These may
improve response rates and play a role in transplant conditioning. A new contender currently undergoing phase I and II trials is the radiolabeled Lym-1, an IgG2a mouse monoclonal antibody conjugated with $^{131}I$, which is directed against one of the HLA-D antigens on human B cells. Results for this drug are still in the early stages (Waldmann TA et al 2000; Montserrat E 2001).

Whilst immunotherapy using monoclonal antibodies appears to be promising, it is clear that the effect of these antibodies is seen not only on the malignant B cells, but also on the non-malignant T cells. This response is undesired as the exact role played by T cells in CLL is still unclear. There is clear evidence, however, that the peripheral malignant B cells are able to express surface antigens such as CD5 which are normally only expressed on peripheral blood T cells and that the expression of other critical activation and interaction T cell antigens is dysregulated (Scrivener S et al 2001). Until more is known about the dialogue that takes place between B and T cells during this response, the use of monoclonal antibodies to control the disease could have unwanted side effects. Although monoclonals such as Campath 1-H have shown good partial responses in patients refractory to treatment, the duration of response is relatively short (Rai KR et al 1998). Monoclonal antibodies have not provided a "cure" for CLL.

1.2.5.5 Adoptive immunotherapy

Recently, there have been a number of advances made in adoptive immunotherapy, ranging from the transfection of DNA into tumour cells to the generation of cytotoxic T cells raised against specific antigens expressed on the cell surface of the malignant cells. In haematological malignancies much of the work has centred on lymphoma, chronic myeloid leukaemia and multiple myeloma, but the field is rapidly expanding and an increasing number of studies have been using these methods in CLL.
DNA vaccines

Three conditions are required for successful antitumoral vaccination: the existence of a tumour antigen, an efficient vaccine delivery system and a relatively intact immune system (Binet JL 1999). Recent work has focused on inducing anti-idiotypic antibodies in mice which recognise mutated and un-mutated immunoglobulin V gene sequences (Stevenson FK et al 1999) using a fusion gene design. Using this method, antigens fused to an "alert" signal such as Fragment C of Tetanus Toxin are delivered via DNA and these fusion gene designs can induce antibodies against the malignant clone. The same design has also been able to induce antibodies against alternative tumour antigens such as adhesion molecules. The prospect of such treatment suggests that a vaccine against specific proteins found in leukaemic clones could be used to treat CLL patients, either alone or in combination with conventional drug therapy or to clear residual disease (Binet JL 1999). However, CLL is a heterogeneous disease, with patients displaying a wide range of different cellular and molecular markers, many of which are also found in normal B and T cells. To identify one particular marker which is specific to CLL may be problematic and cause destruction of normal, healthy cells in addition to the malignant cells.

Pre-existing but incomplete evidence and the work that forms the basis of this PhD research suggest that T cells may be dysfunctional in CLL. Such a finding implies an immune system that is not "intact" and until such findings are considered, DNA vaccines are unlikely to provide significant forms of treatment or a cure for CLL.

Gene Therapy

Gene therapy is another rapidly expanding field in which cellular antigens are transferred into leukaemic B cells in an attempt to stimulate a host anti-leukaemia immune response (Kipps TJ 1999). The theory behind this suggests that despite expressing MHC molecules, CLL B cells are ineffective antigen presenting cells (APCs) and are therefore unable to
stimulate T cells. If this could be reversed and B cells could present antigen, the T cells would become activated and recognise the malignant cells. Using high-titre, replication-deficient adenovirus vectors housing a recombinant CD154 transgene (Ad-CD154) for transfection, CLL B cells were made to express CD154, an activation and adhesion marker normally only found transiently expressed on the surface of T cells.

In a normal immune response, CD154 on the T cells binds to CD40, which is expressed on B cells and dendritic cells. By transfecting CD154 into the leukaemic B cells, it was envisaged that the CLL cells would be able to up-regulate critical co-stimulatory molecules on the infected leukaemia cell surface by interacting with other leukaemia cells and inducing these changes in bystander leukaemic cells as well. In vitro experiments have shown that these transfected leukaemia cells can generate cytotoxic T cells specific for the non-infected leukaemia cells (Kato K et al 1998).

In a phase I trial, patients with intermediate or high-risk disease received infusions of autologous, Ad-CD154-infected leukaemia cells (Kipps TJ et al 1999). The patients did not experience any significant toxicity, apart from the minor side effects of fever, fatigue and nausea, which resolved spontaneously after a few days. Observed changes in the leukaemic and T cells included expression of co-stimulatory molecules on bystander CLL B cells, significant plasma levels of IFN-γ and IL-12, a 2-3 fold increase in absolute T cell numbers associated with a 21-72% reduction in absolute lymphocyte counts and a 33-90% reduction in lymph node size within 1-4 weeks of treatment. However, there was no report of clearance of the disease from the patient or of the duration of the observed responses.

Transfecting leukaemic B cells with CD154 may enable them to communicate with each other exclusively and the long-term effect of this on the patients' immune system is unknown, but it could further enhance T cell defects that may prevent B and T cells from communicating with each other. While this research suggests a reduction in lymphocyte count and a possible form of increasing biological activity in CLL, longer term
observations will be needed both in vitro and in vivo for evidence of durable and cytotoxic T cell responses to the leukaemic clone. Again, the possibility of existing T cell dysfunction in this disease is not taken into account and without the "intact" immune system, results are unlikely to be satisfactory in the treatment of CLL.

**Dendritic cell immunotherapy**

Dendritic cells (DCs) were discovered in mice 28 years ago (Steinman RM and Cohn ZA 1973) and since that time, they have become recognised as the most potent cell type for initiating cellular immune responses (Brenner M et al 2000). They are specialized antigen presenting cells which express all the relevant accessory cell surface antigens such as MHC I and II and the adhesive and co-stimulatory markers necessary to stimulate an immune response. After capturing foreign antigen, DCs migrate to the secondary lymphoid organs to complete their activation process while stimulating T cells, a process they can carry out without any additional cytokine release, hence they have been termed "professional antigen presenting cells" (Ridge JP et al 1998). There has been a surge of interest in DCs and their potential for creating a vaccine which harnesses the patients' own immune response to malignant cells. This form of immunotherapy has already shown encouraging results in both haematological and non-haematological malignancies.

In CLL patients, autologous CLL T cells have been cultured alone or with B-CLL lysate-pulsed and unpulsed autologous dendritic cells. Those T cells cultured with the autologous CLL lysate-pulsed dendritic cells showed a significant increase in the secretion of IFN-γ and also a specific cytotoxicity to autologous B-cell targets (Goddard RV et al 2001). Those T cells cultured with non-CLL B cell lysate showed no response. The majority of the responding T cell population were CD4+ve. Such results suggest that there is potential for an in vitro system that can prime the in vivo immune response to recognise and respond to the malignant cells. However, while some patients show a good cytotoxicity response
following culture with the pulsed DCs, this was not observed in every patient, suggesting that this procedure is not suitable for all CLL cases. For those patients that did show a response, it was necessary to present the CLL B cell lysate in a different way to normal B cell antigen before the T cells were able to recognise it as foreign and elicit a cytotoxic response. A proportion of the patients were unable to respond at all, suggesting that the T cells may not be capable of either recognizing the malignant lysate as foreign or that they were unable to react due to a dysfunctional immune response. The maximum CTL response observed was only 30%, even at a very high effector:target ratio (40:1), which again suggests that there may be an underlying T cell dysfunction preventing a full response.

The theory of requiring an "intact" immune system for effective adoptive immunotherapeutic techniques is demonstrated in each of these new approaches to treating CLL. In each case, while initial results have been promising, the techniques have so far failed to prove clinically successful and are not equally tolerated by each patient. Until T cell function in CLL is more thoroughly investigated and any dysfunction addressed and corrected, it is unlikely that adoptive immunotherapy will be successful in treating or curing CLL.

1.2.6 Disease Transformation

A small group of patients have been shown to respond poorly to treatment and can go on to develop transformation of B-CLL (Enno A et al 1979). Disease progression correlates with refractiveness to treatment. The blood of these patients contains more immature-appearing cells with the morphological characteristics of a prolymphocyte. In a patient with stable, early B-CLL, approximately 15% of cells are of this sort (Oscier D 1999), but in disease progression, between 20 and 50% of all cells appear as prolymphocytes. Progression to this
stage signals more aggressive disease, responding poorly to chemotherapy with a very poor prognosis (Kipps TJ 2000).

Disease transformation in B-CLL is not necessarily linked to exposure to treatment regimes. Between 3 and 10% of patients will develop Richter's syndrome (Montserrat E 2001) and a third of these patients will not have received any treatment. This syndrome was described (Richter MH 1928) as an aggressive, high-grade, large-B-cell lymphoma, occurring in patients around 2 years after the initial diagnosis of CLL. Symptoms of Richter's syndrome include rapid lymph node enlargement, fever and weight loss accompanied by the laboratory findings of a monoclonal gammopathy and an increase in p53 genetic mutations (Giles FJ et al 1998). Median survival time is 5 months after transformation and chemotherapy treatment produces only (Kipps TJ 2000).

A very rare transformation occasionally observed in patients is from CLL to ALL (acute lymphoblastic leukaemia), where the B cells originate from the same CLL B cell clone. This is associated with high levels of expression of c-myc, a proto-oncogene which positively regulates cell proliferation (Ceballos E 2000). Malignant transformation frequently occurs in cells which over-express c-myc and its expression results in continuous proliferation (Larsson LG et al 1991).
1.3 Biology of B-CLL

The CLL B cell clone is an accumulation of mature long-lived B cells, which express many surface markers distinguishing them from normal B cells (Caligaris-Cappio F 1996; Dierlamm J et al 1997; Jurlander J 1998; Soderberg O 1998; Caligaris-Cappio F and Hamblin TJ 1999). It is likely that these cells have arisen from one progenitor cell as indicated by the presence of identical light chains and other key markers, but uncertainty remains as to the genetic origin of the clone. While many clonal cells express genetic abnormalities, phenotypically identical leukaemic cells are found with different genotypes in the same patient (Jurlander J 1998). These genetic abnormalities may be pathogenetic or secondary phenomena. Of equal importance to the development of this disease is the potential loss of homeostasis in B cell ontogeny which may not be related to specific genetic abnormalities. It is necessary, therefore, to understand how normal B cell development progresses in order to understand the possible ways in which the malignancy may arise (Fig. 3).

1.3.1 B cell development

B cell development begins when lymphoid stem cells in the bone marrow differentiate into the earliest distinctive B-cell lineage, the progenitor B cell (pro B cell). Each stage in the development pathway of B cells is accompanied, and often defined by, re-arrangements of the B cell immunoglobulin (Ig) genes and is often under the tight control of cytokines and the microenvironment (LeBien TW et al 2000).
Fig. 3 Normal B cell development

PLURIPOTENT STEM CELL
Both heavy and light Ig genes are germline.
No surface Ig expression

BONE MARROW

PRO-B CELL
Light chain genes remain germline, heavy chain genes re-arrange. Cell surface expression of CD10, CD19, CD45R, VLA-4, RAG-1, RAG-2, TdT.
IL-7 required, released from stromal cells.

PRE-B CELL
Light chain genes re-arrange. Cell surface expression of CD10, CD19, CD20, CD22, CD45R, RAG-1, RAG-2. IL-7 still required for growth

PERIPHERAL BLOOD AND ORGANS

IMMATURE B CELL
Heavy and light chain genes are both re-arranged. IgM is expressed on cell surface. Cell surface expression of CD19, CD20, CD21, CD22, CD45R, MHC II, negative selection occurs.

MATURE B CELL
IgD and IgM are both expressed on the cell surface. Cell surface expression of CD5, CD19, CD20, CD21, CD22, CD23, LFA-1, ICAM-1, MHC II. B and T cells interact. Somatic mutation occurs.

ACTIVATED B CELL
CD5, CD19, CD20, CD21, CD22, CD23, CD40 and CD25 surface expression.

IMMUNOBLAST

LYMPH NODE

LYMPHOPLASMACYTOID CELL

PLASMA CELL

Figure showing a schematic representation of B cell development from the pluripotent stem cell in the bone marrow, progression through the peripheral blood and organs and into the lymph nodes. Surface markers expressed at each stage are shown.
1.3.1.1 Pro B cells

Pro B cells are the most primitive recognizable cells in the B cell lineage (Stiles DP et al 1997). They express the surface proteins CD10, CD19 and the nuclear proteins terminal deoxynucleotidyl transferase (TdT) and recombinant activating genes 1 and 2 (RAG-1 and RAG-2) (Schatz DG et al 1989; Oettinger MA et al 1990). RAG-1 and RAG-2 both play an important role in immunoglobulin gene re-arrangement as they have the ability to recognize and cleave DNA at specific re-combination sequences. Antibody diversity is increased by the insertion of N regions at the points of joining between variable (V), diversity (D) and joining (J) segments (Oettinger MA et al 1992; Spanopoulou E et al 1995). TdT is responsible for these insertions. The first immunoglobulin re-arrangements to take place in the B cell are heavy chain genes (Tonegawa S 1983). Once this has occurred, the cell can be classified as a pre-B cell. Pro-B cells which fail to make a functional VDJH rearrangement undergo apoptosis.

The bone marrow stromal environment plays a critical role in the development of pro-B cells to pre-B cells (Hahn BK et al 2000; Ansel KM and Cyster JG 2001). Pro-B cells are able to bind to the stromal cells through very late antigen-4 (VLA-4), which binds with vascular cell adhesion molecule (VCAM-1) on the stromal cell. This interaction promotes the binding of another receptor pair, c-Kit on the pro-B cell with stem cell factor, which in turn triggers the expression of interleukin-7 (IL-7) receptors on the pro-B cell surface. IL-7 released by the stromal cells binds to the IL-7 receptors (IL-7R), inducing the pro-B cell to mature into a pre-B cell. Studies have shown that mice bred with disruptions in the genes for IL-7 and the IL-7 receptor α chain all lead to severe impairment in B cell development (von Freeden-Jeffry U et al 1995). Other important chemokines in B cell development include CXCL12, produced by bone marrow stromal cells that provide a stimulatory factor for pre-B cells (Ansel KM and Cyster JG 2001).
1.3.1.2 Pre-B cell

Pre-B cells are found almost exclusively in the bone marrow and are representative of a transient phase in B cell development that lasts approximately 2 days (Stiles DP et al 1997). IL-7 continues to be secreted by the stromal cells which drive the maturation process by inducing down-regulation of the adhesion marker VLA-4 on the pre-B cell surface. The pre-B cell can now detach from the stromal cell, as direct contact is no longer required for growth. However, IL-7 released from the stromal cells is still required for growth and maturation. Re-arrangement of the light chain genes begins in the pre-B cell once heavy chain gene re-arrangements have stopped (Levine M et al 2000). TdT is no longer expressed and as a result, there is no insertion of N-regions into light chain genes (LeBien TW 2000). Re-arrangement continues until a functional (kappa) κ or (lambda) λ light chain is produced. Once either light chain protein is produced, it associates with the existing heavy chain unit and together they are transported to the cell surface as membrane immunoglobulin M (mIgM). Expression of RAG-1 and RAG-2 is downregulated and the cell therefore loses the ability to further re-arrange its light chain genes. This successful assembly of one heavy chain and one light chain gene prevents any further gene re-arrangements. Clonal restriction arises from this process, known as allelic exclusion. Should a specific lymphocyte divide after this stage, all daughter cells will express the same heavy and light chains, as there can be no further immunoglobulin re-arrangements. One B cell can therefore give rise to a whole population of clonal cells in response to antigenic stimulation.

1.3.1.3 Immature B lymphocyte

Once the B cell begins to express surface IgM it becomes an immature B lymphocyte. At this stage, it will remain in the bone marrow for approximately 1-3 days. Bone marrow B cells with functional heavy and light chains express B cell receptors (BCRs). At this point,
immature B-lymphocytes will undergo negative selection. Any B cell that can cross-link its IgM receptors or bind to self-antigen on the cell surface will undergo apoptosis. Large numbers of B cells are "lost" within the bone marrow, either as a consequence of self-reactive IgM or through non-functional immunoglobulin production (Duchosal MA 1997; Rudin CM and Thompson CB 1998). Of the $2 \times 10^7$ IgM+ B cells that develop each day within the bone marrow, 10% will reach the spleen and only 1-3% will enter the mature B cell pool (Loder F et al 1999). Without this fail-safe mechanism, the immune system would be constantly under threat from B cells recognising their own proteins as foreign. Alternative splicing of RNA transcripts from the already re-arranged heavy chain leads to the production of immunoglobulin D (IgD), which occurs before the cell becomes mature and leaves the periphery (Levine MH et al 2000). Once a cell has successfully completed this process, it can exit the bone marrow and enter the peripheral organs as a mature B lymphocyte.

1.3.1.4 Mature B cell

Mature B cells, now expressing the surface receptors CD23, lymphocyte function associated antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1), major histocompatibility complex II (MHC II) and CD40 among others, will now enter the peripheral blood and organs. Expression of these key surface antigens allows for the interaction with APCs such as T cells. The in vivo activation and differentiation of B cells occurs within designated lymphoid follicles found within the lymph nodes (Thorbecke GJ et al 1994; Kuby J 1997). Antigen is filtered through the lymphatic system and is delivered to the lymph node via afferent lymphatic vessels. The antigen is taken up by macrophages and DCs within the paracortex of the lymph node, where it is presented to naïve T cells. This results in T helper ($T_{HL}$) cell activation and proliferation. B cells which have successfully bound antigen on their smlg will process and present it in association with
MHC II, enter the paracortex and interact with an activated $T_H$ cell specific for the antigen. A T cell-B cell conjugate is formed and T cell dependent-B cell activation takes place. The activated, proliferating B cells now start to form small complexes at the edge of the paracortex where they begin to differentiate and grow, a process that takes 3-4 days. At this point, some of the B cells will continue to develop into plasma cells, which will start to secrete antibody which, until now, has only been expressed as cell surface protein (Duchosal MA 1997; Rudin CM and Thompson CB 1998).

The majority of plasma cells will home to the bone marrow, where they will mature. These cells express little or no CD40, CD19, CD20, CD22, MHC II or surface Ig ($slg$) and no longer require T cell help (Burrows PD and Cooper MD 1993). The remaining B cells, which do not differentiate into plasma cells, migrate with $T_H$ cells to primary follicles, which will develop to become secondary follicles. These secondary follicles are specialised microenvironments where interactions can take place between B cells, T cells and follicular DCs (FDCs).

As cell proliferation continues, activated B and T cells move towards the centre of the follicle to form the germinal centre (GC). GC formation relies heavily on B and T cell interactions through CD40 and CD154. Blockade of this receptor pair prevents GC formation, B cell activation and memory B cell development. (Gray D et al 1994; Grewal IS and Flavell RA 1997).

As the GC forms, the activated B cells undergo intense proliferation and are termed centroblasts. These centroblasts will now move to one side of the follicle, where they form the dark zone (Thorbecke GJ et al 1994) and continue to divide, giving rise to small cells expressing membrane Ig, known as centrocytes. As these centrocytes proliferate in the dark zone, they will undergo somatic mutation of the heavy and light chain variable regions. The molecular mechanisms which underlie somatic mutation are poorly understood, but require the presence of both $T_H$ cells and GCs, suggesting that interaction with T cells in this
specialised environment is critical for B cell development to continue. The process of somatic mutation will produce a small number of cells which have a high affinity for antigen and these cells will undergo positive selection by binding to the antigen presented by the FDCs, delivering a signal that protects them from apoptosis.

Centrocytes that successfully bind to antigen on the FDCs will undergo differentiation to become either small memory B cells or larger plasma cells (Agematsu K 2000). Plasmablasts are induced by interleukin-1 (IL-1) and CD23 produced by the FDCs. The CD23 is expressed in a membrane form and secreted as a soluble form. The plasmablasts will develop into plasma cells within the medulla of the lymph node, where they will begin to secrete antibody.

Memory B cells are formed when the centrocytes bind antigen which is released from the FDCs (MacLennan IC et al 1997). After this antigen has been processed, it is presented in association with MHC II to activated T\(^\text{H}\) cells. The up-regulation of the surface marker CD154 on the T cell surface binds to CD40 on the centrocyte, the signal necessary for the formation of a memory B cell.

Follicular centres are therefore critical for the selection of B cells with the best fit for antigen after somatic mutation and for the differentiation of B cells into plasma cells or memory cells. Each process requires the presence of T cells to provide stimulatory signals such as CD154 for signalling within a microenvironment. Plasma cells can also develop outside of follicular centres, in either lymph nodes or spleen. Activated B cells proliferate within these areas and can differentiate into plasma cells, which release antibody, a secreted form of membrane-bound immunoglobulin. Plasma cells do not divide, have a life span of approximately 4 weeks and do not express cell surface immunoglobulin (Parham P, 2000).
1.3.2 Normal cellular replication and cell cycle control

The cell cycle can be divided into four distinct stages, growth 1 (G1), synthesis (S), growth 2 (G2) and mitosis (M) (Alberts B et al 1994) (fig 4). At the end of each of these stages, certain cell cycle requirements must have been fulfilled before progression to the next stage (Stiles DP et al 1997). The M phase, which incorporates the whole process of mitosis, involves the division of nuclear material. The time between one M phase and the next is known as interphase, during which G1, S and G2 occur.

After a phase of mitosis has taken place, the cell will enter G1, when it will undergo synthesis and accumulation of structures and molecules necessary for cellular and DNA replication. During G1, the cell monitors its environment and size for favourable replication conditions. As it progresses through G1, proteins including Fos, Jun and c-myc accumulate as transcription factors and act on other genes required for DNA synthesis. Cells which have p53 mutations are much more susceptible to genetic instability and the potential to become malignant. Cells in G1 that have not committed themselves to DNA replication can temporarily or permanently cease dividing, a stage termed G0. These cells are capable of remaining in this state for days, weeks or even years before proliferation resumes.

If feedback signals from the environment and the cell are favourable, the cell enters S phase, during which all nuclear DNA is replicated. G2 represents a phase where the cell produces sufficient material for the mitotic division (Wolfe SL et al 1993) and ensures that DNA replication is complete before entering the M phase.
Fig 4: Normal cellular replication and cell cycle control

M = Mitosis
G1 = Gap 1 prior to DNA synthesis
S = DNA synthesis
G2 = Gap 2, between DNA synthesis and mitosis
The length of each segment depends on the cell type and conditions of growth.
1.3.3 Apoptosis

Under certain environmental or cellular signals, cells will undergo a programmed cell death known as apoptosis, from the Greek word apo meaning "off" and ptosis, meaning "falling". Apoptosis is a common occurrence in many cell types, including those of the haemopoietic system and it is essential for maintaining a stable population of cells by ensuring that the rate of new cell production is balanced by an equal rate of cell death (Stites DP et al 1997). Any disruption to this equilibrium may cause either an accumulation of cells resistant to apoptosis or a loss of cells that are more susceptible to it.

One of the most critical factors controlling apoptosis is the oncoprotein family Bcl-2 (Stites DP et al 1997; Osorio LM and Aguilar-Santelises 1998). Most members of the Bcl-2 family, which includes Bax, Bcl-x, Bak and Mcl-1, are integral membrane proteins that are found associated with organelles that include the endoplasmic reticulum (ER), mitochondria and the plasma membrane (Kuby J 1997). Each member of this family will fall into one of two groups; those that actively induce apoptosis and those that inhibit it. Whether or not a cell will undergo apoptosis appears to be related to the levels of expression of these proteins. Cells expressing high levels of Bcl-2 will be more resistant to apoptosis, whilst those with a greater expression of Bax will be more susceptible to it.

Other membrane proteins involved in the regulation of apoptosis in lymphocytes include the tumour necrosis factor receptor (TNFR) family, which incorporates TNFR1 and TNFR2, Fas and CD40 (Rudin CM et al 1996). Binding of Fas with Fas ligand or cross-linking Fas with anti-Fas antibodies and receptor ligand binding of TNFR1 induce apoptosis in lymphocytes, including autoreactive and cytotoxic T cells via the "death domain", a cytoplasmic tail region through which programmed cell death signals can be initiated (Rudin CM et al 1996; Osorio LM and Aguilar-Santelises M 1998). CD40, expressed on activated B cells, prevents B cell apoptosis after binding with its ligand, CD154 on activated T cells (Rudin CM et al 1996).
Caspases play a vital role in apoptosis. They are a family of cysteine proteases produced in cells as catalytically inactive zymogens (Chai J et al 2000). Effector caspases, such as caspase-3, are activated by initiator caspases, such as caspase-9, through proteolytic cleavage at specific internal residues. Once these become activated, the effector caspases are responsible for proteolytic cleavage of many different cellular targets, which ultimately leads to death of the cell.

A family of cysteine proteins, the caspases, also play a critical role in apoptosis. When activated, caspases act in a cascade and cleave specific substrates, mediating many of the biochemical and morphological changes that take place in a cell undergoing apoptosis, such as cell shrinkage, chromatin condensation, DNA fragmentation and and plasma membrane blebbing (Kohler C et al 2002).

Once a cell has received the appropriate signal that triggers apoptosis, it will undergo a series of morphological changes. Over the course of several hours, the cell will shrink as its nucleus, nuclear chromatin and cytoplasm become condensed (Osorio LM and Aguilar-Santelises M 1997). The chromosomal DNA is cleaved and fragments of the cytoplasm are pinched off as the cytoskeleton starts to degrade, a process known as "blebbing". After these processes have occurred, the cell will shed tiny membrane-bound bodies that contain intact-organelles. These organelles are quickly phagacytosed by surrounding macrophages, preventing the release of any intracellular cell contents into the surrounding tissue, thus preventing an inflammatory response.

In contrast to this process, cells can also die by necrosis, from the Greek word meaning death. Necrosis is not programmed or triggered and is usually the result of accidental injury (Kuby J 1997). It results in the cell swelling and bursting, releasing intracellular contents into the surrounding tissues. These intracellular contents are cytotoxic to bystander cells and as a result, an inflammatory response is usually triggered.
1.3.4 Origins of the CLL cell

In an attempt to identify the possible origins of the CLL B cell, it is necessary to first examine the cellular and genetic features that are both unique to the malignant cell and those that it has in common with normal B cells in the developmental pathway.

CLL B cells have a low to undetectable expression of surface immunoglobulins and, if detected, they are usually IgM or IgM and IgD. Only rarely are they IgG or IgA (Caligaris-Cappio F 1996; Soderberg O 1998; Caligaris-Cappio F and Hamblin TJ 1999). The immunoglobulins detected on the cell surface often have a polyreactive, autoantibody activity and can behave as rheumatoid factor (RF), specifically identifying the Fc region of human IgG (Parham P 2000). IgM autoantibodies against RF are frequently found on both the cell surface and in the serum of patients with CLL (Borche L et al 1990). Autoantibody activity can also be directed against haemopoietic antigens expressed on the surface of red blood cells and, occasionally, platelets, which can lead to autoimmune haemolytic anaemia (AIHA) and autoimmune thrombocytopenic purpura (AITP) respectively (Caligaris-Cappio F 1996).

The CLL B cells accumulate in the peripheral blood and bone marrow in the G0 phase of the cell cycle, yet these apparently resting cells also express membrane markers of cellular activation such as CD80 and CD86, which are normally restricted to activated normal B cells (Caligaris-Cappio F et al 2001). Under normal conditions, the CLL B cells appear anergic. They have a reduced capacity to act as efficient antigen presenting cells (APCs), whilst normal B cells are highly effective APC (Caligaris-Cappio F 1996). However, if the CLL B cells are stimulated through CD40, either using monoclonal antibodies or activated T cells expressing the CD40 ligand CD154, they can become highly efficient APCs, suggesting a restricted dialogue between CLL B and T cells during a normal immune response.
CD5 expression is found on all malignant CLL B cells, but is also expressed on T cells and a subset of normal B cells (Lydyard PM et al 1999). Normal B cells expressing CD5 are also known as B1 cells, and they represent the most prominent B cells in early foetal life, decreasing with age. Circulating B1 cells in the adult are normally found in the follicular mantle zone (FMZ) of lymph nodes, but a limited number of normal B1 cells can also be found in the bone marrow. Normal B1 cells produce polyreactive autoantibodies, including those directed against blood group antigens (Lydyard PM et al 1999). B1 cells are also the main source of IgM derived autoantibodies. Patients with rheumatoid arthritis have increased levels of B1 cells (Plater-Zyberk C et al 1985). It is unclear what controls the levels of normal B1 cells in the bloodstream, but children with DiGeorge syndrome (lacking a thymus) have decreased levels of B1 cells, suggesting a role for the thymus in maintaining B1 cell numbers in the periphery (Kourtis AP et al 1997). Normal B1 cells express low to undetectable amounts of Bcl-2 (Caligaris-Cappio F 1996).

Early studies on the V_{H} genes in CLL suggested that most malignant B cells had unmutated V_{H} genes (Kipps T et al 1989). As larger studies were carried out however, up to 50% of CLL patients V_{H} genes were shown to be mutated, indicating that they would have passed through the germinal centre (Caligaris-Cappio F et al 2001; Hamblin T J et al 1999).

As the nature of the CLL B cell has become clearer, attempts to identify its origin have combined the evidence above (Figure 5, pg 53). Initially, much work focused on the similarities between the CLL CD5+ B cell and the normal CD5 B cell, B1 (table 7).
Table 7: Similarities and differences between CLL CD5 B cells and normal CD5 B1 cells

<table>
<thead>
<tr>
<th>Feature</th>
<th>CD5+ CLL B cell</th>
<th>CD5+ normal B1 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyreactive autoantibodies</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>somatic hypermutation</td>
<td>yes/no (50% of patients)</td>
<td>no</td>
</tr>
<tr>
<td>formation of mouse erythrocyte rosettes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Bcl-2 expression</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>low surface Ig expression</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

Table showing the similarities and differences between CD5+ve CLL B cells and the normal subset of B cells that naturally express CD5.

A number of cellular and molecular similarities between the two cells have emerged. CLL patients, CD5+ B cells and normal B1 cells produce polyreactive IgM autoantibodies directed against RF and haemopoietic cell antigens (Caligaris-Cappio F 1996). Both are also able to produce these antibodies in the absence of somatic mutation, suggesting that neither subset has passed through the germinal centre. In addition, both CLL B cells and B1 cells express CD20 and both are able to form rosettes with mouse erythrocytes (Caligaris-Cappio F 1996). The CD5 normal B cell is considered to be one of the possible candidates for the origin of the CLL clone. Normal CD5 B1 cells are located in the mantle zone of secondary lymphoid follicles and it is from here that it is suggested CLL B cells may arise (Caligaris-Cappio F 1996; Soderberg O 1998; Caligaris-Cappio F and Hamblin TJ 1999; Caligaris-Cappio Fetal 2001). One possible theory suggests that CLL is a malignancy of a mantle-zone-based subpopulation of anergic self-reactive CD5+ B cells devoted to the production of polyreactive natural autoantibody (Caligaris-Cappio F et al 2001).

However, there are also striking differences between CLL B cells and normal B1 cells. While CLL B cells express low to undetectable levels of surface Ig, normal B1 cells express normal levels of surface Ig. CLL B lymphocytes are stuck in G0, yet are able to express cell surface markers and cytokines that are indicative of an activated B cell.
B1 cells do not follow this pattern. The B1 cell cycle correlates with expression of cell surface markers and there is no discrepancy between their activated and resting state. They also express low levels of Bcl-2, whilst CLL B cell Bcl-2 expression is high. Normal B1 cells do not demonstrate somatic hypermutation, yet 50% of CLL B cells do (Hamblin TJ et al 1999; Caligaris-Cappio F et al 2001). In addition, the production of the autoantibodies in CLL is believed to be from the residual "normal" B cell population (Hamblin TJ and Oscier DG 1997).

Whilst it is appealing to try to identify a normal cellular counterpart for the CLL B cell, attempts have so far failed. Such a hypothesis would need to encompass all aspects of the disease, such as the autoimmunity, hypogammaglobulinaemia, low surface Ig, CD5 expression, accumulation in G0 but expression of activation markers and cytokines, the discrepancy between somatic mutation in only half the patients and an inconsistent genetic repertoire, and the identified dysregulation in T cell function observed in CLL patients.
Figure 5: Possible cellular origins of CLL B cell

<table>
<thead>
<tr>
<th>BONE MARROW</th>
<th>MATURE B LYMPHOCYTE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LYMPH NODE</strong></td>
<td></td>
</tr>
<tr>
<td>B CELL BINDS ANTIGEN AND INTERACTS WITH T CELL IN PARACORTEX</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PLASMA CELL</strong></td>
<td><strong>MIGRATE WITH T₃ CELLS TO PRIMARY FOLLICLES</strong></td>
</tr>
<tr>
<td></td>
<td><strong>B AND T CELLS MIGRATE TO GERMINAL CENTRE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>B CELLS BECOME CENTROBLASTS/CENTROCYTES MOVE TO DARK ZONE UNDERGO SOMATIC MUTATION AND AFFINITY MATURATION</strong></td>
</tr>
<tr>
<td><strong>PLASMA CELLS</strong></td>
<td><strong>MEMORY B CELLS</strong></td>
</tr>
<tr>
<td></td>
<td><strong>REMAIN IN LYMPH NODE</strong></td>
</tr>
<tr>
<td></td>
<td><strong>REMAIN IN FOLLICULAR MANTLE OR CIRCULATE IN PERIPHERAL BLOOD</strong></td>
</tr>
</tbody>
</table>

* Indicates possible place of origin of CLL B cell

Illustration showing possible cellular origins of the leukaemic clone. As the B cell develops, positions marked with an asterix indicate where it is possible for the clone to develop.
1.3.5 Morphology

CLL B cells appear as small, mature, unstimulated peripheral blood B cells which are easily damaged on preparation of a blood film, giving a characteristic smear cell (Calligaris-Cappio F and Hamblin TJ 1999). Within the peripheral blood, the malignant cells can be distinguished by heavily clumped basophilic chromatin within the nucleus and a small, agranular, pale blue cytoplasm (Dadmarz R and Cawley JC 1987; Dierlamm J et al 1997). Approximately 15% of patients have larger B cells with a more prominent nucleolus (prolymphocytes) (Oscier D 1999) and others may display a cleaved nucleus or lymphoplasmacytoid features. The bone marrow and lymph nodes are infiltrated by identical lymphocytes.

1.3.6 Immunophenotype

One of the key distinguishing features of the CLL cell is its low to undetectable levels of surface immunoglobulins (slgs) (Dohner H and Stilgenbauer S 2001) (table 8). The majority of CLL cases express surface immunoglobulin M (slgM) alone or with slgD. The presence of slgA or slgG is rare. The presence of either slgM and/or slgD is detected only very faintly by immunofluorescent methods. However, experiments involving detection of total cellular immunoglobulin content (i.e. both internal and external) have shown that levels of total IgM are comparable with those of normal B cells, whilst IgD varies greatly, with patients varying in expression from very low to near normal. The low level of expression of slgM has only been seen in normal B cells which have been anergized via interaction with self-antigen (Goodnow CC et al 1989).

The malignant cells will all express either kappa or lambda as a light chain, this being indicative of a clonal population of cells (Jurlander J 1998). In CLL there is usually a clear imbalance in the kappa/lambda ratio, which in a normal patient should be 2:1.
CLL B cells have been shown to express the interaction and activation markers CD80 and CD86 (Caligaris-Cappio F et al 2001) (table 8).

Expression of the B cell receptor (BCR) is altered in CLL patients. The BCR consists of surface Ig non-covalently linked with Igα/Igβ (CD79a/CD79b) and CD5. In most CLL patients, the extracellular domain CD79b is absent (Alfarano A et al 1999; Hamblin TJ et al 1999; Oscier DG et al 1999; Garcia-Vela J et al 1999; Caligaris-Cappio F et al 2001). However, CD79b has been shown to be functionally normal in all CLL patients, regardless of whether it is expressed at the cell surface (Rassenti LZ and Kipps TJ 2000). Normal B cells use CD79b after activation to downregulate BCR expression, suggesting that the CLL B cells may be activated. The use of CD79b as a marker for accurate diagnosis of CLL is now becoming more common.

CD5, CD19, CD20, CD21, CD22 and CD23 are expressed in a characteristic manner by the malignant B cells and are now described.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Molecular weight</th>
<th>Normally found on:</th>
<th>Role:</th>
<th>± on CLL B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5</td>
<td>67kDa</td>
<td>T cells, B-1 B cells</td>
<td>part of the BCR, activation and differentiation</td>
<td>+</td>
</tr>
<tr>
<td>CD19</td>
<td>95kDa</td>
<td>B cells</td>
<td>T-B cell interactions, activation, differentiation</td>
<td>+</td>
</tr>
<tr>
<td>CD20</td>
<td>33-37kDa</td>
<td>B cells</td>
<td>B cell activation and signal transduction</td>
<td>+</td>
</tr>
<tr>
<td>CD21</td>
<td>145kDa</td>
<td>mature B cells, mature T cells (low), immature T cells (high)</td>
<td>complement, antigen presentation to T cells, CD23R</td>
<td>+</td>
</tr>
<tr>
<td>CD22</td>
<td>135kDa</td>
<td>B cells</td>
<td>accessory signalling through the BCR</td>
<td>--</td>
</tr>
<tr>
<td>CD23</td>
<td>45kDa</td>
<td>B cells, monocytes, macrophages</td>
<td>marker of B cell activation</td>
<td>+</td>
</tr>
<tr>
<td>CD79b</td>
<td>33-40kDa</td>
<td>B cells</td>
<td>part of the BCR, spliced after activation to downregulate BCR</td>
<td>--</td>
</tr>
<tr>
<td>CD80/CD86</td>
<td>60kDa/80kDa</td>
<td>B cells, dendritic cells, some T cells</td>
<td>B cell activation, T-B cell interaction</td>
<td>+</td>
</tr>
<tr>
<td>slg</td>
<td>--</td>
<td>B cells</td>
<td>antigen binding</td>
<td>±</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>25kDa</td>
<td>cells protected from apoptosis</td>
<td>protects against apoptosis</td>
<td>+</td>
</tr>
</tbody>
</table>

Table showing molecular weight, normal cell expression and function of key B cell antigens. The table indicates those antigens which are found on the CLL B cell and those that are absent.
**CD19**

CD19 is a 95 kDa pan B cell antigen and a member of the immunoglobulin supergene family that plays a critical role as a response regulator, influencing activation, differentiation and survival of the B cells (Gardby E and Lycke NY 2000). Mice deficient in CD19 lack B-1 cells and B-2 cells respond poorly to thymus dependent antigens. As a result of this poor interaction with T cells, generation of Th2 T cells is impaired because of reduced signalling through CD40 on CD19 deficient B cells, identifying a key role of CD19 in T and B cell interactions (Gardby E et al 2001).

After B-cell antigen receptor (BCR) cross linking with antigen, CD19 becomes rapidly phosphorylated, triggering critical activation pathways that lead to B cell receptor-mediated survival *in vivo* (Otero DC et al 2001). CD19 also plays an important role in the balance between humoral immunity and autoimmunity by regulating signalling thresholds from antigen stimulation of the BCR. Transgenic mice, which overexpressed the CD19 antigen by 3-fold, generated spontaneous autoantibodies in an environment that otherwise did not predispose them to autoimmunity (Sato S et al 2000; Tedder TF et al 2000). CD19 signals as a complex with CD21 in the recognition of microbial antigens through the complement system (Fearon DT and Carroll MC 2000) and also a complex with CD22 in governing mature B cell activation (Tedder T et al 2000).

CD19 is expressed on CLL B cells, but expression is significantly lower on the CLL B cell surface, compared to expression on normal B cells (Ginaldi L et al 1998; Cabezudo E et al 1999).

**CD20**

CD20 is a 33-37 kDa non-glycosylated pan B cell integral membrane protein which plays a critical role in B cell activation, modulation of growth signals and signal transduction (Deans JP et al 1998; Shan D et al 1998; Riley JK and Sliwkowski MX 2000). CD20 has
been shown to be both physically and functionally coupled to MHC II and CD40 and may have an important role in modulating effects on their functions (Leveille C et al 1999). CD20 is expressed on all CLL B cells but expression is significantly lower on the B cell surface compared to normals (Almasri NM et al 1994; Ginaldi L et al 1998). A small subset of patients has been identified with high CD20 expression, but it has been speculated that they may represent a biologically different disease (Almasri NM et al 1997).

**CD21**

CD21 is a 145kDa antigen, which interacts with CD23 and IFN-α, is the receptor for the Epstein Barr Virus (EBV) and binds the complement fragments iC3b, C3dg and C3d (the low affinity receptor for IgE) (Braun M et al 1998). CD21 is tightly regulated during B lymphocyte differentiation and is only expressed on mature B cells, not pro-, pre-, or plasma B cells (Schwab J and Illges H 2001). CD21 is also expressed at low density on mature T cells and at high density on immature thymocytes (Braun M et al 1998; Fischer EM et al 1999). Although mature T cells only express low levels of CD21, both B and T cells express equal amounts of CD21 mRNA (Braun M et al 1998). CD21 is critical for antigen presentation to T cells and other APC. CD21 captures immune complexes on the surface of follicular dendritic cells and presents them to activated B cells in germinal centres (Zabel MD and Weis JH 2001). CD21 expression is up-regulated on CLL B cells, but expression on the B cell surface varies between patients, with one study citing a link between high CD21 cell surface expression and soluble CD23 levels which correlated with a good prognosis (Gagro A et al 1997).
**CD22**

CD22, a member of the Ig superfamily, is a 135 kDa B-cell transmembrane glycoprotein which acts as an accessory antigen in signalling through the BCR (Hatta Y et al 1999). It plays a critical role by down modulating signals through the BCR and preventing autoimmune responses, as demonstrated by CD22 knockout mice (O'Keefe TL et al 1999). After eight months, CD22 deficient mice develop high levels of monoclonal, somatically mutated, high affinity serum IgG antibodies directed against double stranded DNA, cardiolipin and myeloperoxidase, suggesting that a single gene defect is sufficient to trigger autoantibody production in aged populations. CD19 and CD22 influence each other's functions; CD22 negatively regulates CD19 tyrosine phosphorylation and CD22 function is dependent on CD19 expression (Fujimoto M et al 1999). CD22 expression is down regulated in CLL and is often not expressed on the cell surface at all (Matutes E and Polliack A 2000). However, cytoplasmic CD22 has been identified in small numbers of patients who have no CD22 surface expression (Kanikova E et al 1994).

**CD23**

CD23 is a 45 kDa transmembrane protein expressed on the surface of B cells, monocytes and macrophages. It binds IgE, CD21, CD11b and CD11c and is classed as a lectin (Gu B et al 1998; Rogola B and Rymarczyk B 1999; Payet ME et al 1999; Lampert IA et al 1999). CD23 is a marker of B cell activation, normally induced after activation by other cell types such as T cells (Karagiannis SN et al 2001). CLL patients have an increased expression of surface CD23 (Lampert IA et al 1999; Kneitz C et al 2000) and soluble CD23 (sCD23) in their serum, with 3 to 500 times greater expression than normals (Molica S et al 1999; Chen JR et al 1999). The level of sCD23 in patients' serum may be a prognostic marker, which is more accurate than clinical staging (Sarfati M et al 1996). Those patients who
express higher amounts of sCD23 have a poorer prognosis and earlier disease progression. CD23 expression is upregulated variably on CLL B cells.

**CD5**

CD5 is a 67kDa transmembrane glycoprotein (Freedman AS et al 1987) normally expressed on the surface of T cells, but has been shown to be present on a sub-set of normal B cells (Lozano F et al 2000; Bondada S et al 2000). B cells expressing CD5 are classified as B1, whilst normal CD5 negative cells are identified as B2 cells (Lydyard PM et al 1999). CLL is classed as a CD5 malignancy (Caligaris-Cappio F 1996; Soderberg O 1998; Geiger KD et al 2000) with mantle cell lymphoma (MCL). Almost all CLL B cells express CD5, although it remains unknown whether CD5 expression on malignant cells represents transformation from a normal B1 cell or whether CD5 is an acquisition of a previously transformed B cell (Hamblin TJ and Oscier DG 1997; Lydyard PM et al 1999). The expression of CD5 on malignant B cells is one of the main diagnostic features of CLL and its presence is important with relation to the aetiology of the disease (Lydyard PM et al 1999).

CD5 is part of the BCR (Lankester AC et al 1994) and an important co-receptor in antigen-specific receptor-mediated activation and differentiation (Lozano F et al 2000). It has been suggested that CD5 plays an important role in keeping normal B1 cells in a partial state of activation. The high prevalence of autoimmunity in CLL has led to different speculations as to the role of CD5. One such hypothesis supports the idea that CLL is a tumour of a separate lineage of CD5⁺ve B cells mainly involved in the production of polyclonal autoantibodies (Bondada S et al 2000), supported by the fact that some CLL cells have been stimulated in vitro to produce these polyclonal autoantibodies (Caligaris-Cappio F 1996). However, it has also been shown that CD5⁻ve B cells can be induced to express autoantibodies in response to stimulation. Finally, the autoantibodies produced in CLL
have been shown to be from the "normal" B cells as opposed to the leukaemic clone. CD5 is expressed on CLL B cells and intensity is significantly higher compared to other B cell malignancies and normal B cells (Cabezudo E et al 1999).

1.3.7 Development and progression of the leukaemic clone

The CLL B cell originates in the bone marrow, where it is believed to arise from a single progenitor cell which becomes malignant when its replication and survival escape from normal regulation. One of the key factors thought to be involved in the accumulation of the malignant cells is a resistance to the normal processes of cell death and apoptosis. The anti-apoptotic protein Bcl-2 is overexpressed in 90% of CLL patients B cells (Kipps TJ 1997). The mechanisms behind this are poorly understood, but it is not a result of Bcl-2 gene rearrangement, gene amplification or a prolonged half-life of Bcl-2 mRNA (Gottardi D et al 1995). Expression of Bcl-2 protein in CLL does not correlate with stage of disease or prior treatment (Binet JL et al 1996), but it has been suggested that it may not only inhibit apoptosis and prolong survival, but may also lead to the accumulation of the malignant cells stuck in G_0 (Gottardi D et al 1995). CLL B cells, while resistant to apoptosis in vivo, spontaneously apoptose and down-regulate Bcl-2 expression when cultured in vitro, suggesting a critical role of the micro-environment in the prolonged survival of these cells (Osorio LM et al 1998).

In contrast to the up-regulation of Bcl-2 in CLL, levels of the pro-apoptotic protein Fas is low or undetectable (Panayiotidis P et al 1995). Even cells induced to express Fas are still resistant to apoptosis, suggesting a more complex process is at work. However, although cell surface expression of Fas is low, levels of soluble Fas are increased in CLL patients.

The role of cytokines in apoptosis in CLL is unclear, with some groups reporting cytokine-mediated prevention of B-CLL apoptosis, whilst others suggest Bcl-2 independent mechanisms (Osorio LM et al 1998). Cytokines believed to be involved in suppression of
apoptosis in CLL include IFN-α, IFN-γ, TNF-α, basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), IL-1, IL-2, IL-4, IL-6, IL-8 and IL-13. Recent reports have suggested that two cytokines may be capable of inducing B-CLL B cell apoptosis. IL-5 appears to increase spontaneous apoptosis (Mainou-Fowler T et al 1994) and IL-10 may be involved in both the induction and prevention of B-CLL apoptosis, although reports are conflicting (Osorio LM et al 1998)

CLL cells may also avoid cell death by expression of CD6 on the cell surface, which protects against apoptosis after ligation and is expressed on a large proportion of B-CLL cells (Osorio LM et al 1998).

As the leukaemic cell numbers, normal bone marrow function becomes compromised, although often the disease progresses very slowly. It is likely that this process within the marrow may begin many years before the patient becomes aware of any symptoms or the disease is diagnosed. Tumour load increases steadily and presentation is at least partly dependent on total body tumour load. It is impossible to say how long the tumour load has been present. CLL is a chronic disease, making it possible for the tumour dysfunction to begin at a very early age, although there is as yet no evidence to support this assumption.

Once the clone begins to expand, normal B-cell haemopoiesis will become compromised. Red cell, granulocyte and platelet production are reduced as the B-cells take up more space within the marrow and the subsequent cytopaenias result in the key symptoms of CLL. Reduced red cell production and autoimmune haemolysis will eventually lead to anaemia and its associated tiredness and breathlessness (Caligaris-Cappio F and Hamblin TJ 1999). Reduced granulocyte production and low levels of normal gamma globulins lead to an increased risk of infection. Thrombocytopenia can lead to an increased risk of bruising and bleeding.

A prominent finding in CLL presentation is large (sometimes tender or painful) lymph nodes and an enlarged spleen and liver. These symptoms and signs form the basis of
clinical staging. Total T cell numbers were initially thought to be reduced in CLL, but this has since been shown to be a result of the dilution effect from the large numbers of B cells (Catovsky D et al 1974; Totterman H et al 1989). Absolute T cell numbers actually increase as the disease progresses. In addition, an imbalance in the normal CD4/CD8 ratio can be seen, with an increase in total CD8 numbers (Kay NE et al 1979; Kay NE 1981; Semenzato G et al 1983; Totterman TH et al 1989; Burger T et al 1990). This increase may be linked to the stage of the disease, as the further the disease has progressed, the greater the number of CD8 cells.
1.4 B and T cell interaction and activation in the normal immune response

The development, differentiation, growth and immune responses of B cells are intricately linked with T cells, for instance in the formation of germinal centres and the activation of naïve B cells within secondary lymphoid follicles. Once the T cell dependent development of B cells is complete, B and T cells continue to play vital roles in the activation of each other. To study the immune response of a CLL patient, in particular the role of the T cell, it is necessary to understand how B and T cells are able to activate and interact with each other (fig 6). The activation of T cells is complex, incorporating many processes and interactions. Activation is regulated by other key immune responders, cytokines and the T cells themselves (Noelle JR and Snow EC 1990; Clark EA and Ledbetter JA 1994; Robey E and Allison JP 1995). T cells respond to antigen presented to them from antigen presenting cells (APC) such as B cells, dendritic cells, macrophages and monocytes. They are unable to respond to antigen that is not presented in association with the MHC (Owens T 1996). This chapter will detail the processes involved in activation of T cells and their interaction with B cells during an immune response. Properties of the key cell surface markers are detailed in table 9.
### TABLE 9: T cell surface antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Class</th>
<th>Molecular weight</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a (LFA-1)</td>
<td>transmembrane glycoprotein</td>
<td>170kDa</td>
<td>adhesion</td>
</tr>
<tr>
<td></td>
<td>intergrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD54 (ICAM-1)</td>
<td>transmembrane glycoprotein</td>
<td>90kDa</td>
<td>adhesion</td>
</tr>
<tr>
<td></td>
<td>(!CAM-I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCR</td>
<td>Ig like glycoprotein</td>
<td>α chain 43-53kDa</td>
<td>antigen binding</td>
</tr>
<tr>
<td></td>
<td>β chain 43kDa</td>
<td></td>
<td>signal transduction</td>
</tr>
<tr>
<td>CD4</td>
<td>transmembrane glycoprotein</td>
<td>59kDa</td>
<td>adhesion and signal</td>
</tr>
<tr>
<td></td>
<td>protein</td>
<td></td>
<td>transduction</td>
</tr>
<tr>
<td>CD8</td>
<td>disulfide-linked dimer exists as α homodimer or αβ heterodimer</td>
<td>34kDa</td>
<td>adhesion and signal transduction</td>
</tr>
<tr>
<td>CD5</td>
<td>transmembrane glycoprotein</td>
<td>67kDa</td>
<td>signal transduction from TCR/CD3</td>
</tr>
<tr>
<td>CD25 (IL-2R)</td>
<td>glycoprotein</td>
<td>55kDa</td>
<td>activation</td>
</tr>
<tr>
<td>CD28</td>
<td>immunoglobulin homodimer</td>
<td>44kDa</td>
<td>adhesion and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>activation</td>
</tr>
<tr>
<td>CD154 (CD40L)</td>
<td>type II transmembrane protein</td>
<td>32kDa</td>
<td>activation</td>
</tr>
<tr>
<td>CD152 (CTLA-4)</td>
<td>immunoglobulin</td>
<td>44kDa</td>
<td>negative regulator of activation</td>
</tr>
<tr>
<td>HLA-DR (MHC II)</td>
<td>Ig like glycoprotein</td>
<td>α chain 34kDa</td>
<td>activation and antigen presentation</td>
</tr>
<tr>
<td></td>
<td>β chain 29kDa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table showing molecular weight, type and role of key T cell surface markers
1.4.1 Antigen presentation and recognition

An immune response starts when an APC such as a B cell, dendritic cell (DC) or macrophage meets antigen. Macrophages derive from circulating monocytes. Circulating macrophages phagocytose and engulf dead cells and cell debris and are responsible for killing and breaking down microorganisms (Parham P 2000). They can recognize and react to pathogens and cooperate with lymphocytes during immune responses. Dendritic cells are the most efficient antigen presenters and their sole function is to present antigen to T cells, whereas macrophages and B cells have other additional effector functions. Within secondary lymphoid tissue, B cells can be found in the lymphoid follicles, macrophages are found throughout the lymph node whilst dendritic cells are only present in T cell areas. B cells in the lymph nodes will bind specific antigens via surface immunoglobulin (sIg) and internalise them via receptor-mediated endocytosis. The antigen is transported away from the plasma membrane and as it travels into the cell, it becomes acidified, aiding the breakdown of the pathogen into residual peptides, proteins and glycoproteins. These smaller peptide units bind to MHC class II molecules and are carried back to the cell surface as a peptide:MHC II unit. The B cell is now able to present this peptide to a T cell which is specific for the particular antigen and is retained within the lymph node where it will come into contact with many passing T cells. The T cell specific for a given pathogen represents only one in $10^4$ to one in $10^6$ of the total number of circulating T cells (Parham P 2000).

At this stage, the role of interaction and adhesion molecules is critical. These cell surface molecules allow T cells to bind to the B cell and test for receptor specificity for the given pathogen (Clark EA and Ledbetter JA 1994). Adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) on T cells and intercellular adhesion molecule-1 (ICAM-1) on APCs are critical for allowing contact to take place (Makgoba MW et al 1989). These contact driven molecules are also vital in allowing the T cell to leave the
lymph node and re-enter the circulation. LFA-1 on the T cell surface binds strongly to ICAM-1 which is also expressed on the vascular endothelium, thus allowing the T cell to pass through. The T cell will move through the lymph node, coming into contact with many APC and testing each antigen:MHC II complex for recognition. When the T cell binds successfully with a peptide:MHC II complex, it will make contact with both the peptide and the surface of the MHC molecule and the activation process can begin (Friedl P and Gunzer M 2001).

1.4.2 Signal transduction and activation - an overview

Once a T cell recognises its specific antigen and binds with the MHC complex, a signal is sent through the T cell receptor (TCR) to change conformation of the adhesion molecules and increase cell to cell contact binding activity between LFA-1 and associated ICAM molecules, stabilising the interactions. A newly emerging theory suggests that within 0.5 - 3 minutes after T and B cell contact through the MHC/TCR, the T cell polarizes towards the B cell through cytoskeletal engagement and TCR, LFA-1, ICAM-1, CD28 and CD2 among others are all recruited towards this crucial interaction (Friedl P and Gunzer M 2001). After 3-10 minutes, an immunological synapse is formed between the T and B cell (Holdorf AD et al 2000), incorporating more antigen ligand pairs. The central ligand consists of the TCR with CD4 or CD8 binding to the MHC. CD2 binds CD58 and CD28 binds the B7 components CD80 and CD86. CD28 is constitutively expressed on the T cell surface where it binds with either B7.1 (CD80) or B7.2 (CD86), both of which are up-regulated at different time points following B cell activation.

The ligation of CD28 signals to the nucleus of the T cell to up-regulate the production of the critical T cell cytokine IL-2 and its receptor (IL-2R), the cell surface marker CD25. IL-2 provides signals for both the T cell producing it and recruits surrounding T cells to
continue activation, whilst CD25 provides the receptor for IL-2. Key cytoplasmic components, such as Lck, Fyn and ZAP-70, will also be triggered at this point. LFA-1 and ICAM-1 expression are increased on an outer plane, allowing for stronger binding of the T cell to the APC and also to form a protective zone to allow stabilising of TCR signalling (Dustin ML and Cooper JA 2000).

The next stage in the activation process involves the expression on the T cell of CD154. This is another key cell marker which binds with CD40 on the B-cell surface to further enhance the production of cytokines such as IL-2 and to prolong the expression of receptors such as IL-2R (Clark LB et al 1996). The final stage involves a definitive "off" signal, to ensure that the T cell response is controlled and does not involve any prolonged activation or non-specific responses. CD152 (CTLA-4) is expressed on the T cell and binds to the B7 proteins on the B cell which thus signals to the T cell once the activation process has been completed (Oosterwegel MA et al 1999) (Table 9).

1.4.3 LFA-1 and ICAM-1

LFA-1 plays a crucial role in cell-cell contact at the start of immune recognition, allowing T-cells to come into close proximity with APCs to test for antigen specificity (Rothlein R and Springer TA 1986; Rothlein R et al 1986). LFA-1 is a member of a large family of adhesion molecules known as the integrins (Makgoba MW et al 1989) and it binds to the 80-114kDa integral membrane glycoprotein ICAM-1. LFA-1 shares a structure with its other family members and consists of two non-covalently linked polypeptides, the α and β subunits (Keizer GD et al 1985). Cloning of the genes for these two subunits has revealed three potential sites for binding that would facilitate cell-cell adhesion (Figdor CG et al 1990).

LFA-1 exists in both an active and inactive form. Resting lymphocytes express inactive LFA-1 and therefore do not adhere spontaneously. Stimulation with phorbol myristate
acetate (PMA), or monoclonal antibodies directed against CD2 and CD3, has been shown to strongly induce cell adhesion, suggesting that these surface molecules may activate LFA-1 via intracellular signalling pathways. Once a T cell encounters an APC, LFA-1 binds to ICAM-1 for initial cell-cell contact.

In the first instance, cell binding is non-specific and of low affinity. If cell recognition by the TCR occurs, a signal is delivered via CD3, activating LFA-1. The activated form can now facilitate high affinity binding between the two cells and antigen recognition can occur. Binding through LFA-1 is mediated through re-arrangements of the cytoskeleton that creates a specialised domain on the T cell surface and subsequently directs T cell effector functions towards the APC (Sedwick CE et al 1999).

1.4.4 CD3 and the T-Cell Receptor (TCR)

The TCR is structurally similar in many ways to immunoglobulin receptors found on B-cells (Davis MM et al 1984; Samelson LE and Schwartz RH 1984; Clevers H et al 1988) (Fig 7). Each T cell expresses a unique receptor on its cell surface allowing it to bind a specific antigen. The TCR comprises of an acidic α chain of 43-53kDa and a basic β chain of 43kDa (Fitch FW et al 1984; Clark SP et al 1984). The TCR is a disulfide-linked heterodimer, consisting of two forms, the αβ or the γδ. An individual T cell will express one pair or the other, never both (Borst J et al 1990). The majority of T-cells express the αβ chain. Each receptor complex will express one functional α chain (encoded on chromosome 14) and one functional β chain (chromosome 7). Amino acid analysis of these two chains shows them to have sequence homology with immunoglobulin and both consist of constant and variable regions, with antigen binding taking place at the variable regions (Kavaler J et al 1984). In comparison to its counterpart receptor CD3, the TCR only has a short cytoplasmic tail, as shown in figure 7 (Parham P 2000).
Figure showing the T cell receptor. The different regions are identified as the αβ antigen binding site with both constant and variable regions and the CD3 receptor, both on the surface of the cell, and the εδ subunits located within the T cell.
The CD3 molecule is a T cell specific marker, which is always found closely associated with the TCR and its related structures. CD3 is a complex consisting of three separate polypeptide chains, γ (25kDa), δ (20kDa) and ε (20kDa) (Terhorst C and van den Elsen P 1985), each of which is encoded on human chromosome 11 (Tunnacliffe A et al 1987). CD3 and the TCR are also associated with either a double ζ (zeta) chain (16kDa), or a single η (eta) chain that is linked to a single ζ chain (Letourneur F and Klausner RD 1992). Together, these components make up the TCR complex. Neither TCR nor CD3 is found without expression of the other (figure 7). The CD3 polypeptides are found evenly distributed across the T cell membrane. Approximately 30,000-40,000 are found on the surface of each T cell (Reinherz EL et al 1986), with an intracellular immunoglobulin-like domain which is tightly linked to a kinase. CD3 and ζ are not responsible for binding antigen.

As a part of the TCR complex, they transduce signals into the cell, which trigger transcriptional events within the nucleus once the TCR has formed a complex with the presented MHC: antigen (Letourneur F and Klausner RD 1992). This binding of MHC: antigen to the TCR induces conformational changes and triggers signals through the CD3. The T cell activation process is complex, but key events conclude in the release of calcium from intracellular cytoplasmic stores, which, together with other signals, activates transcription of the interleukin-2 gene and the interleukin-2 receptor gene. This results in release of IL-2 from the cell and expression of the IL-2 receptor, both of which result in activation of T cells.

1.4.5 CD4 and CD8

CD4 is a 56kDa protein, tightly but non-covalently linked to the TCR complex and its role in signal initiation and transduction (Janeway CA Jr et al 1988). CD4, and its counterpart
CD8, a 34kDa glycoprotein, can be found on mature αβ expressing T cells as well as on monocytes and macrophages. CD4 consists of four extracellular immunoglobulin-like domains, a hydrophobic transmembrane region and a long cytoplasmic tail and is encoded on human chromosome 2 (Barclay AN et al 1993). A T cell will express either CD4 or CD8, dividing T cells into two distinct groups. CD4* T cells will only bind to and recognise antigen in association with MHC II on APC, whilst CD8* T cells will only interact with MHC I on APC (Fleury SG et al 1991; Janeway CA Jr 1991). CD4 will bind to the membrane-proximal β2 domain of MHC II and its role is two-fold. During binding between the MHC II:antigen and the TCR, CD4 acts as an adhesion molecule by binding to the MHC II, helping to tighten the bond between T cell and APC. CD4 also acts as a separate signal transducer, with the intracellular domain linked to kinases which become phosphorylated following TCR engagement due to the close proximity of CD4 to the TCR. These phosphorylated kinases can then activate the cytoplasmic protein tyrosine complex ZAP-70, which is critical for initiating subsequent intracellular T cell signalling.

CD8 works in a similar way, strengthening the bond between the TCR complex and the MHC class I protein bound with antigen. It also binds intracellular kinases (Weiss A et al 1994). Antibodies against CD4 or CD8 are capable of blocking the activation of T cells, demonstrating their importance in the activation pathway.

1.4.6 CD5

CD5 is a 67kDa glycoprotein, expressed on the cell surface of all T cells and a small subset of normal B cells (Verwilghen J et al 1990; Luo W et al 1992). CD5 is thought to play an important role in the regulation of signals transduced from the TCR/CD3 complex after activation (Carmo AM et al 1999) and to positively costimulate mature T cells activated through the TCR. It plays a pivotal role in inducing resting B-cells to proliferate after T and B-cell interaction (Bikah G et al 1998). CD5 is also associated with intracellular casein
kinase 2 (CK), which is a major regulator of cell growth and signalling (Raman C and Kimberley RP 1998). CD5 can induce additional key cell factors for growth in mature T cells. Anti-CD5 stimulation in conjunction with anti-CD28/PHA ligation can enhance production of IL-2 mRNA by 1.6 fold and secretion of IL-2 protein by 2.2 fold (Gringhuis SI et al 1997).

1.4.7 CD28

CD28 (also known previously as Tp44), characterised first as the human T lymphocyte surface antigen reacting with monoclonal antibody 9.3 (Hansen JA et al 1980), is a 44kDa transmembrane glycoprotein and a member of the immunoglobulin gene superfamily (Harper K et al 1991; Freeman GJ et al 1992; Yin D et al 1999). Many members of this family play important roles in adhesion and signal transduction. Other subfamily members include CD7 and CD152 (Harper K et al 1991). CD28 is expressed on the surface of 80% of CD4 T cells and 50% of CD8 T cells (Lenschow DJ et al 1996) and it binds with two distinct receptors CD80 (B7.1) and CD86 (B7.2) expressed on B cells, dendritic cells and other APCs (Holdorf AD et al 2000). The co-stimulatory signal provided through the ligation of these receptors is believed to be critical for providing activation signals to the T cell.

Anergy, a term used to describe a state of T cell unresponsiveness, is prevented during T cell activation by the successful binding of CD28 to CD80 and CD86 after the initial antigen driven contact through the TCR (Lenschow DJ et al 1996; Chambers CA and Allison P 1999; Boulougouris G et al 1999). A concept termed the immunological synapse has been used to describe the specific reorganisation of proteins in the contact surface between the T cell and the APC during antigen presentation to T cells and plays a crucial role in antigen receptor signalling (Holdorf AD et al 2000). In conjunction with other surface antigens, CD28 plays a critical role in adhesion between APC and T cells, as CD28
bound to B7 spans a distance identical to the TCR bound to MHC II/peptide. CD28 can also transduce signals to change cytoskeleton conformation to further enhance T cell adhesion to APC. This is demonstrated clearly by T cells which lack CD28 and the adhesion molecule, CD2, as they are defective in their ability to interact with APCs (Green JM et al 2000).

As the activation process of the T cell continues, CD28 is able to regulate sensitivity of signalling through the TCR. Once CD28 has bound its ligands, the number of engaged TCRs required for T cell activation decreases by approximately six-fold (Viola A and Lanzavecchia A 1996). CD28 is also involved in co-ordinating production of IL-2, via costimulatory signalling with CD3 to stabilise the IL-2 transcription mRNA and by activating nuclear factor-kappa B (NF-kappa B), a transcription factor involved in direct transcription of interleukin-2 (Jenkins MK et al 1991; Powell JD et al 1998; Boulougouris G et al 1999; Boonen GJJC et al 1999; Holdorf AD et al 2000). In addition to augmenting production of IL-2, ligation of CD28 also up-regulates CD25, the receptor for IL-2, enhancing T cell responses to IL-2 (Holdorf AD et al 2000).

Other critical signals, which are up-regulated after CD28 ligation, include anti-apoptotic survival proteins such as bcl-xL (Boise LH et al 1995). Because CD28 ligation leads to increased T cell helper function, this also correlates with increases in CD154 (CD40L) cell surface expression, further enhancing the T cell immune response (Klaus SJ et al 1994). Mice deficient in CD28 are unable to form germinal centres, cannot sustain T cell proliferation, do not produce T cell growth factors and provide poor support for B cell activation, suggesting that CD28 plays a critical role in T and B cell interactions (Shahinian A et al 1993; Vallejo AN et al 1999; Sansom DM 2000). Expression of CD28 is reduced during the ageing process of lymphocytes, known as replicative senescence, which is
defined as a generalised, age-related decline in immune responses (Effros RB and Pawelec G 1997; Vallejo AN et al 1999; Globerson A and Effros RB 2000).

1.4.8 CD25 and Interleukin-2 (IL-2)

CD25, the IL-2 receptor, is made up of at least three distinct receptor chains; a 55kDa α chain (IL-2Rα, Tac, p55, CD25), a 70-75kDa β chain (IL-2Rβ, p70/p75, CD122) and a 64kDa γ chain (IL-2Rγ, γc, p64) (Taniguchi T et al 1986; Waldmann TA 1991; Taniguchi T and Minami Y 1993). The IL-2R, CD25, was first identified by a monoclonal antibody which did not bind to normal resting T cells but did react strongly with mitogen-activated T-cells and was initially named Tac as a result (T activated) (Uchiyama T et al 1981). Interleukin-2 is a 15.5kDa glycoprotein produced by activated T cells and in small amounts by B cells (Taniguchi T et al 1983; Gaffen SL et al 1996).

Synthesis and secretion of IL-2 and the expression of CD25 occur as early consequences of activation of mature T cells. The expression of the IL-2 receptor CD25 is up-regulated following activation. The binding of IL-2 to its receptor causes rapid clonal expansion of the T cell population originally activated by antigen. It is also a growth factor that is required for T cells to progress from G1 into S phase (June CH et al 1989). Naïve, resting T cells will only express the β and γ chains of the IL-2 receptor. Activation through TCR:CD3 and co-stimulatory molecules leads to the up-regulation of the α chain and together the three units comprise to create a high affinity receptor for IL-2 (Waldmann TA 1986; Waldmann TA et al 1993). IL-2 then binds the IL-2 receptor, triggering the cell to undergo cell division.

A T cell that is activated in this way can undergo two to three cell divisions a day, eventually producing thousands of daughter cells. T cells which are unable to produce IL-2 or to express its receptor CD25 and cells which are unable to respond to IL-2 will not proliferate or respond and the immune response will fail. CD25 knockout mice have
provided evidence that IL-2 and its receptor CD25 are critical to prevent autoimmunity and maintain homeostasis of the immune system. Although IL-2 deficient mice demonstrate normal T cell development (Schorle H et al 1991), there are markedly reduced in vitro T cell responses and significantly increased levels of IgG1 and IgE, suggesting an important role of IL-2 in B cell differentiation. However, many immune responses take place normally during early life, suggesting that other cytokines may be able to compensate for IL-2. In comparison, mice lacking CD25 develop autoimmune disorders such as haemolytic anaemia and massive enlargement of peripheral lymphoid organs (Willerford DM et al 1995).

Expression of IL-2 and its receptor CD25 are closely linked to ligation of CD28 and the TCR:CD3 complex, for without these signals, T cells fail to produce IL-2. Production of IL-2 begins when signals delivered through the TCR:CD3 receptors activate the transcription factor NF-kappa B which in turn activates transcription of the IL-2 gene. Regulation of IL-2 mRNA is under the tight control of both CD28 and the TCR which stabilises transcription and increases IL-2 production 20-30 fold (Umlauf SW 1995; Parham P 2000;).

IL-2 shares many functional properties with IL-15 (Giri JG 1995; Bulfone-Paus S et al 1997). Both cytokines are able to stimulate the proliferation of T cells, up-regulate expression of CD25 and CD95 (FasL) and downregulate CD27 and both can increase the mRNA transcription of IL-2 and CD25. IL-2 and IL-15 also share receptor components, with both cytokines utilising the β and γ chains of the IL-2 receptor.

Expression of CD25 is not restricted purely to T-cells. It has also been identified on normal B cells, CLL B cells, hairy cell leukaemia B cells, Burkitts lymphoma B cells and all B cell lines carrying the HTLV-1 genome. Other haematological cells expressing this receptor include cultured monocytes, Kupffer cells of the liver, cultured lung macrophages,
Langerhans cells of the skin and Reed-Sternberg cells in Hodgkin's Lymphoma (Waldmann TA 1986).

**1.4.9 CD154**

CD154 (CD40L, gp39), the ligand for CD40, is a type-II transmembrane protein of 33-39 kDa protein expressed on activated T cells, the majority of which are CD4+, in both the peripheral blood and lymph nodes (Ochs HD et al 1994; Clark LB et al 1996). It shares significant sequence homology with TNF-α and has been classified as a member of the tumour necrosis factor cytokine family (Hollenbaugh D et al 1992). Basophils, B-cells, monocytes, NK cells, CD8+ T cells and mast cells also express low levels of surface CD154. The CD154 ligand, CD40, is expressed on the surface of APCs including B-cells, dendritic cells, activated macrophages, fibroblasts, endothelial cells and follicular dendritic cells (Grewal IS et al 1997). Expression of CD154 on T cells is detectable 1-2 hours after activation and reaches maximum expression between 4-8 hours, declining back to resting levels at 24 - 48 hours (Clark LB et al 1996).

CD154 is often classed as the second critical signal in T-cell activation after TCR ligation, the first involving the ligation of CD28 with CD80 (B7.1) and CD86 (B7.2). Activated T cells expressing CD154 are able to induce resting B cells to express CD80 and CD86 (Fanslow WC et al 1994). This interaction between CD154 on the T cells and CD40 on B cells provides critical signals to B-cells for IL-4 production, Ig class switching, B cell activation and progression through the G1-G2 stages of the cell cycle and upregulation of receptors for IL-2, IL-4 and IL-15 (Banchereau J et al 1994; Clark LB et al 1996; Grewal IS and Flavell RA 1997). Monoclonal antibodies which block this CD154-CD40 receptor pair prevent B cell activation, memory B cell development, entry of B cells into the cell cycle and germinal centre formation (Durie FH et al 1994; Gray D et al 1994; Han S et al 1995; Grewal IS and Flavell RA 1997).
A classic example of the role of CD154 is seen in patients who suffer from the rare genetic disorder hyper-IgM syndrome. An X-linked immunodeficiency, hyper-IgM syndrome is characterised by mutations in CD154 expression. Patients display normal to high levels of the immunoglobulin IgM, but no IgG, IgA or IgE, demonstrating an inability of the B cells to switch immunoglobulin production away from IgM (Banchereau J et al 1994; Clark LB et al 1996; Grewal IS and Flavell RA 1997). When the B cells from such patients are cultured in the presence of normal T cells, immunoglobulin production returns to normal (Grewal IS and Flavell RA 1997).

Important information about the role of this antigen can also be obtained by the study of CD154 knockout mice and blocking antibodies, which have demonstrated that CD154 is also important in the regulation of costimulatory activity on APCs, T-cell activation and auto-immune responses. CD4 T cells from CD154 deficient mice show poor proliferation and express little or no IL-4 and IFN-γ compared to normal mouse CD4 T cells (Grewal IS and Flavell RA 1997). Blocking antibodies against the CD40-CD154 interaction produce symptoms in the mouse similar to hyper-IgM syndrome (Gray D et al 1994) with increased IgM responses and production and decreased IgG production. Further, the blockade of this pathway demonstrated that this interaction is critical for B cell activation and proliferation, B cell differentiation into antibody-secreting plasma cells, isotype switching and the generation of memory B cells.

1.4.10 CD152 (CTLA-4)

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) or CD152 is a 44-kDa member of the immunoglobulin supergene family and it is very similar to CD28 (Brunet J et al 1987). Genetically it maps close to CD28 on chromosome 2 (Freeman GJ et al 1992) and structurally, there are many similarities. Both glycoproteins show extensive amino acid and nucleotide homology and share a similar exon and intron structure (Harper KJ et al 1991;
Lindsten T et al 1993). Functionally, both CD28 and CD152 bind to the receptors CD80 and CD86. However, CD152 binds to CD80 and CD86 with 10-100 fold greater affinity than CD28 (Liu Y 1997; Oosterwegel MA et al 1999).

CD152 is not expressed on the cell surface of naïve, resting T cells, in comparison to CD28 (Sansom DM 2000). Whilst CD28 mRNA can be found constitutively in all resting T cells, increasing only moderately after activation, CD152 mRNA can only be identified following T cell activation, and CD152 surface expression does not occur until 48 hours post activation (Walunas TL et al 1994; Krummel MF and Allison JP 1995; Kosmaczewska A et al 2001). Surface expression, however, remains limited, even under optimal stimulating conditions, due to its unique storage conditions. CD152 protein is produced in high quantities post activation, when it is transiently expressed on the cell surface before being rapidly endocytosed to be stored in intracellular vesicles (Alegre M et al 1996; Sansom DM 2000; Kosmaczewska A et al 2001). This storage of CD152 suggests that it is present in significant levels and may play an important role in T cell regulation before being expressed on the cell surface.

Whilst CD28 plays a critical role in the activation of T cells, CD152 provides the counterpart signal, inducing T cells to down-regulate their responses (da Rocha Dias S and Rudd CE 2001). Experiments crosslinking CD152 during activation have shown a reduction in IL-2 production and a decrease in up-regulation of the IL-2 receptor CD25 (Brunner MC et al 1999; Sansom DM 2000). This ultimately leads to G1 cell cycle arrest, through inhibition of the production of the cell cycle proteins cyclin D3 and cdk4 (Alegre M et al 1998). Antibody crosslinking of CTLA-4 also prevents CD28 induced IL-2 mRNA production and CD69 expression, further inhibiting the T cell response (Brunner MC et al 1999).

The role of CD152 can be observed clearly in CTLA-4 knock-out mice, which develop splenomegaly, lymphadenopathy, multi-organ lymphocytic infiltration and tissue
destruction, dying within 3-4 weeks (Tivol EA et al 1995). These findings suggest that CTLA-4 plays a critical role in down-regulating T cell activation and preventing autoimmunity. Blocking antibodies to CD152 during activation produced a 4 to 5 fold reduction in the level of expression of CD95 (FasL), which induces antigen-induced cell death (AICD), a process that ensures the deletion of self-reactive T cells (da Rocha Dias and Rudd CE 2001). This suggests that under normal conditions of CD152 expression during T cell activation, ligation of CD152 with CD80/CD86 induces termination of the immune response by reducing IL-2 and IL-2 R production, arresting cell cycle progression and inducing AICD to prevent the initiation of autoimmunity.

1.4.11 HLA-DR

The major histocompatibility complex (MHC), discovered in 1975 (Nabholz M et al 1975) consists of two subtypes – MHC class I and MHC class II. MHC molecules are coded by genes located on chromosome six (Sompayrac L, 1999). The three families of class II antigens, DR, DQ, and DP, are encoded within the HLA-D region of chromosome 6 as a series of discrete gene clusters. (Kappes DJ and Strominger 1986; Roucard C et al 2001). All MHC II molecules are transmembrane with extracellular domains resembling immunoglobulin molecules, and cytoplasmic tails. All belong to the immunoglobulin gene superfamily. MHC II is expressed constitutively on B cells, dendritic cells, thymic epithelium cells, monocytes, macrophages and Langerhans cells but is also upregulated on T cells following activation by such factors as IFN-γ (Amaldi I et al 1989). HLA-DR, along with its fellow members DP and DQ, consist of an α chain (34kDa) and a β chain (29kDa), both of which are surface glycoproteins (Kappes DJ et al 1984). The MHC II molecules are highly polymorphic and there are multiple alleles of each subtype.

Normal immune surveillance depends on the ability of MHC class II molecules, expressed on cells such as B cells and dendritic cells, to bind antigens and carry them to the cell
surface for display to T cells (Nelson CA and Fremont DH 1999). MHC II molecules are able to bind peptides from a large array of antigens and then hold them at the cell surface long enough for T cell recognition to occur. This is done with the help of the MHC associated invariant chain and HLA-DM, which is a non-peptide binding class II molecule. In fulfilling their role in the presentation of processed antigen to T cells each class of the MHC is restricted. MHC I can only present antigen to CD8+ T cells and MHC II can only present to CD4+ T cells (Scholl PR and Geha RS 1994). CD4 T cells recognize peptides bound to MHC II, which are selectively expressed in specialized antigen presenting cells that take up extracellular microbial antigens (Jensen PE 1999). CD8 T cells recognize peptides bound to MHC class I, which are widely expressed and will bind peptides derived from intracellular sources ie viral peptides and tumour peptides, allowing cytotoxic T cells to monitor for foreign antigen. HLA-DR can be found expressed on the surface of the T cell following activation and, in conjunction with anti-CD3 stimulation, is responsible for inducing proliferation and expression of IL-2, IL-3, IFN-γ and TNF-α (Spertini F et al 1992).
1.5 The T cell in B-CLL

1.5.1 Normal T cell haemopoiesis

1.5.1.1 Thymic development

T cells start their developmental life, like all other haemopoietic cells, in the bone marrow, or fetal liver, where they originate from a pluripotent stem cell (Spits H et al 1998; Robson MacDonald H et al 2001) (fig. 8). They undergo very little maturation within the marrow, travelling early in the growth stage to the thymus, the primary lymphoid organ, hence the term T lymphocyte, derived from thymus-dependent (Green DR and Schuler M 2000; Moroy T and Karsunky H 2000). T cell development in the thymus can be broadly divided into two main areas. In the early developmental stage, common T-lineage-restricted precursors give rise to an αβ or γδ T lineage cell. In the later stage, αβ CD4+8+ double positive cells commit to become either CD4+ or CD8+ single positive T cells (Berg U and Kang J 2001). T cell precursors undergo an orderly series of movements between thymic subcompartments as they develop into mature T cells (Ansel KM and Cyster JG 2001). In the early stages of development, on entering the thymus, the immature T cell precursors, at this stage known as thymocytes, interact closely with thymic stromal cells and extracellular matrix components (ECM) which consist of fibronectin and soluble growth factors such as IL-7, as well as dendritic cells and macrophages. (Banwell CM et al 2000; Anderson G et al 2000). Stromal cells and ECM components play a critical role in the development of T cell precursors, through soluble and cell surface molecules. Several different chemokines are produced by the thymus, such as macrophage inflammatory protein-2 (MIP-2), stromal cell-derived factor 1 (SDF-1) and thymus-expressed chemokine (TECK). Immature thymocytes have been shown to express receptors for many of these chemokines (Anderson G et al 2000). The colonization of the thymus and the continued development of the immature thymocytes are likely to rely heavily on chemokines produced by the thymus. Immature thymocytes will also express a range of cell surface receptors such as very late
antigen-4 (VLA-4), VLA-5 and CD44, which are capable of binding strongly to ECM components, supporting the hypothesis that ECM components play an important role in thymocyte maturation. Several studies have suggested that the ECM may act as a "scaffold" to concentrate and localize growth factors needed for thymocyte development (Anderson G et al 2000). At this point in thymocyte development, the production of IL-7, most notably from MHC class II thymic epithelial cells, plays an important role in the maturation of double negative T cells. Mice deficient in IL-7R function have severely reduced numbers of thymocytes (Berg JL and Kang J 2001).

Rearrangement of the T cell receptor (TCR) genes is a complex and highly regulated process (Davis MM et al 1984; Sim GK et al 1984; Born W et al 1985; Hedrick SM et al 1985). T-cells can express two different receptor types, classed as \( \alpha:\beta \) and \( \gamma:\delta \) (Terhorst C and van den Elsen P 1985; Robey E and Fowlkes BJ 1998; Rothenberg EV 2000). There is no commitment to either lineage at this stage, as receptor expression simply depends on which re-arrangement is made first. Thymocytes will start to re-arrange their \( \beta, \gamma \) and \( \delta \) genes at approximately the same time. Although there is no pre-determined lineage, \( \beta \)-chain genes will more often than not become functionally re-arranged before those of a \( \gamma:\delta \) receptor (Robey E and Fowlkes BJ 1994). Once this has happened, \( \alpha \)-gene re-arrangement begins, and once completed, leads to expression of an \( \alpha:\beta \) receptor on the cell surface (de Villartay JP et al 1991; Lacorazza HD et al 2001). It is now impossible for the cell to become \( \gamma:\delta \), as the \( \delta \)-chain gene locus is found in the middle of the \( \alpha \)-chain locus and once this has re-arranged, the \( \delta \)-chain locus is deleted from the chromosome. Evidence suggests that lineage fate of the TCR may depend on a mutation of the Notch 1 gene (Washburn T et al 1997; Robey E and Fowlkes BJ 1998). T cells that have a heterozygous mutation of Notch 1 are more likely to develop as \( \gamma:\delta \) T cells than \( \alpha:\beta \). If a T-cell fails to produce either an \( \alpha:\beta \) or \( \gamma:\delta \) receptor it will die by apoptosis and in the same way as is observed in B cells, this will be the fate of most T cells (Wiest DL et al 1999).
Figure 8: T cell development

Figure showing a schematic representation of T cell development from the pluripotent stem cell in the bone marrow through to functional T cells in the peripheral blood. Surface markers expressed at each stage are shown.
IL-7, as well as playing a critical role in early thymocyte development (DeLuca D and Clark DR 2002), also plays an important role in TCR gene re-arrangement (Soloff RS et al 1997; Durum SK et al 1998; Berg LJ and Kang J 2001). This has been clearly demonstrated in IL-7 receptor negative murine thymocytes, which do not rearrange their TCR (Durum SK et al 1998).

T cells bearing the α:β receptor are by far the most common T cell found within the immune system and make up 95% of all T cells. It is on these particular T cells that the remaining chapter will focus. Once dedicated to this lineage of receptor, the β-chain locus will, like the genes for immunoglobulin heavy chains in B cells, begin re-arrangement first, with the α segment following second, comparable to the B-cell light chain. After successful rearrangement of the loci and assembly of both receptor protein products, the latter will become associated with CD3 proteins and a ζ chain to become a pre T-cell receptor that will be transported to the cell surface. The emergence of this receptor on the cell surface allows the T cell to communicate with surrounding ligands on dendritic cells and macrophages and provides the important signal for proliferation. T cell expression of the β chain within the pre T cell receptor will now induce expression of the two key markers in T cell development, CD4 and CD8. TECK in particular, has been shown to facilitate the migration of immature thymocytes, at this point CD4- CD8- and known as "double negative", from the outer cortex of the thymus to the inner cortex as they differentiate into CD4+8+ "double positive" cells (Crump AL et al 1993; Norment AM et al 2000)

1.5.1.2 Positive and negative selection

The next crucial stage in T cell development is the positive and negative selection of T cells. This process involves only double positive α:β cells and is based on the ability of the T cells to recognise MHC class I or II on thymic epithelium (Benoist C and Mathis D 1989; Viret C and Janeway CA 1999). Positive selection is the process by which T cells are
selected from the repertoire of developing cells by their ability to recognise peptides presented by another cell bearing a self-MHC molecule (Wong P et al 2000). This process only occurs in about 1-2% of the total T cell population within the cortex of the thymus and the successful process leads to protection from apoptosis and continued maturation (Kisielow P et al 1988; Sha WC et al 1988; Nikolic Zugic J and Bevan MJ 1990; Viret C and Janeway CA 1999). Efficient positive selection of a broad repertoire of T cells depends on the presentation of large numbers of diverse antigen peptides on MHC molecules within the thymus (Marusic-Galesic S et al 1989; Wong P et al 2000). Without a successful binding of the T-cell receptor to an endothelial bound MHC molecule, there will be no positive signal to the cell and it will be eliminated.

Negative T-cell selection also takes place in the thymus and is similar to the process of negative B-cell selection described earlier. Any developing T cell that binds to self-antigen presented by dendritic cells or macrophages will be rendered anergic on arrival in the peripheral blood or undergo apoptosis (Buckland J 2002). It is estimated that half to two-thirds of thymocytes able to undergo positive selection die before full maturation due to negative selection (van Meerwijk JP et al 1997). Whilst positive selection does not permit any unselected cells to leave the thymus, negative selection does.

It is also at this point that the cells function as either a CD4 or a CD8 population is determined (Basson MA and Zamoyska R 2000). After positive selection, cells mature to express either CD4 or CD8. The subject of CD4 and CD8 lineage commitment is controversial. Work has shown that the MHC is critical in determining the T cell lineage as either CD4 or CD8 (van Meerwijk et al 1997; Germain RN 2002). Specific interactions of the T-cell receptor on immature thymocytes with thymic MHC antigens determines the differentiation of double positive CD4/CD8 T cells into either CD4 positive or CD8 positive T cells (Teh HS et al 1988). Double positive T cells that interact with cells expressing MHC class I molecules in the thymus will differentiate to become CD8 T cells.
(Kisielow P et al 1988; van Meerwijk JP and Germain RN 1993). Conversely, double positive T cell interaction with cells expressing MHC class II molecules will differentiate to become CD4 positive T cells (Kruisbeek AM et al 1985; Bikoff EK et al 1993). Recent evidence (Berg LJ and Kang J 2001) also suggests that the quantity of signal through the TCR may determine both positive selection and the commitment of T cells to either CD4 or CD8. Mice with a reduced expression of TCR on their cell surface develop very few CD4 single positive T cells, suggesting that the decrease in TCR signalling results in a failure of positive selection. If the peptide antigen is administered to the mice, CD4 T cells develop normally. At lower levels of antigen, CD8 T cells develop, demonstrating the importance of the TCR in both positive and negative selection and CD4 or CD8 development (Watanabe N et al 2000). Another key contender in lineage specification is Notch signalling, whose role as either committing the T cell to CD4 or CD8 development or in T cell survival is still debated (Berg LJ and Kang J 2001). Signalling through p56lck and Erk pathways may also play key roles in CD4 or CD8 commitment, as blockade of Erk pathways blocks CD4 maturation and promotes CD8 development.

Controversial theories regarding the process of "instruction" versus "selection" for CD4 and CD8 lineage differentiation still exist (Germain N 2002). However, there is, as yet, no clear evidence as to the pathway or pathways responsible for lineage commitment.

1.5.1.3 Antigen presentation within the secondary lymphoid tissues

Once a T cell has successfully entered the peripheral blood, it will circulate into the secondary lymphoid tissues (the spleen, lymph nodes, and mucosal lymphoid tissues), the lymph system and back to the blood. It is within designated areas of the secondary lymphoid tissues that T cells encounter antigen presented by APCs such as dendritic cells in association with MHC class I and II (Pamer EG 1999; Thery C and Amigorena S 2001) and undergo the final stage of their development. T cells that interact successfully with
antigen will divide and become effector T cells. These cells will either stay within the lymph tissues until required, or migrate to sites of infection. Most of the effector T cells generated are relatively short lived and will die as a result of antigen overload or from lack of antigen or cytokines. A proportion of effector T cells will become long-lived memory T cells, able to respond to antigenic stimulation should it be presented to them again following a repeated infection. It is possible that these memory T cells may require repeated antigenic stimulation to remain alive.

1.5.1.4 Differentiation of CD4 and CD8 cells

T-cells of each subset are able to differentiate further, depending on the type of antigen they encounter. The activation of T cells results in the production of a number of soluble factors, known as cytokines. Many cytokines will have profound effects on the same T-cell that secretes it, on bystander T cells and on other key responders in the immune system (Table 10).

**TABLE 10: Key cytokines in T-cell activation**

<table>
<thead>
<tr>
<th>CYTOKINE:</th>
<th>PRODUCED BY:</th>
<th>EFFECT ON T CELLS:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>T cells</td>
<td>Stimulates growth and activation</td>
</tr>
<tr>
<td>IL-4</td>
<td>T cells</td>
<td>Stimulates growth and survival</td>
</tr>
<tr>
<td>IL-6</td>
<td>Activated macrophages and T cells</td>
<td>Stimulates activation</td>
</tr>
<tr>
<td>IL-10</td>
<td>T cells</td>
<td>Inhibits production of T(_{H1}) cells</td>
</tr>
<tr>
<td>IL-12</td>
<td>Dendritic cells and macrophages</td>
<td>Stimulates T(_{H1}) differentiation</td>
</tr>
<tr>
<td>IL-15</td>
<td>Monocytes and epithelial cells</td>
<td>Increases expression of IL-2</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>T cells</td>
<td>Inhibits T cell growth</td>
</tr>
</tbody>
</table>

Table showing the key cytokines involved in T cell activation, from which cell they are normally produced and the effect of the cytokine on the T cell in terms of stimulation and growth.
CD4 T cells can be further sub-divided into two main subsets, Th1 and Th2 (Liew FY 1994; Romagnani S 2000; Raulet DH and Melchers F 2001). Th1 cells are involved with cell-mediated immunity and phagocyte-dependent inflammation and secrete IFN-γ, IL-2 and TNF-γ. The differentiation of T cells into Th1 cells is influenced strongly by signalling through IL-12 and recent evidence suggests that a sustained Th1 immune response may require a continuous supply of IL-12 (Raulet DH and Melchers F 2001). Th2 cells evoke strong antibody responses, IgE production and eosinophil accumulation, but can inhibit phagocyte-independent inflammation. They produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Differentiation into Th2 cells requires IL-4 signalling (Raulet DH and Melchers F 2001). The balance of these two subsets within the immune system can frequently determine the outcome of an immune response.

1.5.2 The T cell in B-CLL

B cell chronic lymphocytic leukaemia is a disease in which there is accumulation of large numbers of B cells within the peripheral blood and bone marrow. These cells are mostly arrested in G0 stage of the cell cycle (fig IV) and are less susceptible to apoptosis (Bannerjit R and Byrd CJ 2000; Caligaris-Cappio F et al 2001). Whilst it is undisputed that this disease is a B cell malignancy, T cells may play an important role in its pathogenesis (Caligaris-Cappio F and Hamblin TJ 1999; Bannerjit R and Byrd CJ 2000; Caligaris-Cappio F et al 2001). The immune system relies on a wide array of specific and critical interactions between many different cell types including B and T cells (Clark EA and Ledbetter JA 1994; Robey E and Allison JP 1995). It is possible that a dysfunction in a cell type other than B cells may play an important contributory role in the development and progression of B-CLL. The T cell undergoes many interactions with the B cell during the course of both B and T cell development and an immune response and it is feasible that a T cell dysfunction could affect normal B cell development and responses.
When compared to the current literature on the B cell, relatively little is known about the T cell in B-CLL. However, B cells are intricately linked to the presence and status of T cells and invariably, one will be unable to respond effectively without the full co-operation of the other. A disease which consists of an uncontrolled B-cell proliferation implies some degree of T cell dysfunction. T cell research within B-CLL has focused on the following five key areas as follows

1. CD4 and CD8 subpopulations and total T cell numbers.
2. T cell response to mitogen
3. T cell colony formation.
4. T cell cytokine expression.
5. Altered T cell phenotype and functional abnormalities.

1.5.2.1 CD4 and CD8 subpopulations and total T-cell numbers

A key finding in T cell studies has been that, whilst B cells numbers are invariably increased, so too are T cell numbers (Kimby E et al 1989; Janssen O et al 1989; Zaknoen SL and Kay NE 1990). Initially it was believed that T cell numbers were reduced, but early work showed that this was in fact a "dilution effect", whereby large numbers of B cells "dilute" the absolute number of T cells, which is actually normal (Catovsky D et al 1974; Totterman H et al 1989; Briggs PG et al 1989). In addition, an imbalance in the normal CD4/CD8 ratio can be seen, with an increase in absolute CD8 numbers (Kay NE et al 1979; Kay NE 1981; Semenzato G et al 1983; Totterman TH et al 1989; Kimby E et al 1989; Janssen O et al 1989; Burger T et al 1990; Goolsby CL et al 2000; Porakishvili N et al 2001). This increase may be linked to the stage of the disease, as the further the disease has progressed, the greater the number of CD8 cells (Herrman F et al 1982). Levels of soluble CD8 have also been shown to be higher in CLL patients compared to normals, and
in those patients with progressive disease, levels were significantly higher than those with more indolent disease (Semenzato G et al 1989; Musolino C et al 1991).

Studies have also shown that there may be differences in the expression of the CD4 and CD8 antigens on the cell surface and that these differences may also be linked to the stage of disease (Dianzani U 1994 et al). CLL patients have been found to have an expansion of the absolute numbers of CD4+ and CD8+ cells but lower levels of CD4 and CD8 expression on each individual T cell (Huang L and Crispe N 1992). These CD4lo and CD8lo cells also both express lower levels of CD3 and TCRαβ antigens on each cell. A correlation could be found between expression of CD4lo and CD8lo with increased expression of HLA-DR and these subsets could be linked to intrinsic features of the tumour. CD4lo HLA-DR+ cell numbers were increased in patients who were in stable remission. In particular, this cell group was expanded in patients with Stage A disease, but not in patients with Stage B or C. These CD4lo CD8lo cells are similar to the double negative T cells which have been identified as expanded populations in both human and murine autoimmune diseases (Huang L and Crispe N 1992). Other similar work has also suggested that these cells may also be specific for self-antigens and may either derive from a distinct pathway of T cell development or belong to a unique T cell lineage that is not dependent on positive or negative selection (von Boehmer H 1992).

1.5.2.2 T-cell response to mitogen

Studies investigating T cell responses to common mitogens such as phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) have shown that there is a reduced proliferation and activation response by CLL T cells (Foa R et al 1981; Foa R et al 1982; Totterman TH et al 1989; Zaknoen SL and Kay NE 1990; Prieto A et al 1993). T cells in CLL also show a reduced ability to co-operate in the production of immunoglobulin (Ig) when cultured with normal B cells, CLL B cells and a transformed B cell line (Callery RT et al 1980). This
would suggest that there is an intrinsic T cell dysfunction which persists after the removal of the malignant B cells and which cannot be corrected by normal B cells. Such CLL T cell abnormalities have been linked to hypogammaglobulinaemia, as earlier studies have also shown that normal B cells are unable to produce immunoglobulin when cultured with CLL T cells (Hersey P et al 1979). Conversely, normal B cells cultured with normal T cells leads to normal immunoglobulin production. Lauria et al (1983) further confirmed these findings by showing that normal B cells were unable to differentiate when cultured with CLL T cells.

In an attempt to distinguish a sub-group of CLL T cells that might be responsible for this reduced B cell support, Sieber et al (1983) showed that the "helper" T cells were functionally quite normal, whilst numbers of suppressor T cells were increased. This increase in suppressor cell numbers was suggested as the cause of the reduced T cell capacity, as opposed to an intrinsic T cell defect. This is supported by the finding that suppressor T cells from CLL patients were as effective as normal suppressor T cells at down-regulating differentiation of normal B cells (Lauria F et al 1983). However, these findings of reduced T cell help for B cells have been challenged, by a study showing that CLL T cells could provide adequate help for normal B cells in the generation of immunoglobulin secretion (Sieber G et al 1981).

A possible cause of the reduced T cell response to mitogens may be a soluble factor present in CLL patient serum. When normal lymphocytes were cultured in either normal AB serum or CLL AB serum and stimulated with PHA or PWM, the lymphocytes cultured in CLL AB serum showed a significantly marked decrease in stimulation as demonstrated by a reduced incorporation of tritiated thymidine (Utsinger PD et al 1975). However, in contrast to this finding, research presented in this thesis has shown that when both normal and CLL purified T cells are cultured in CLL AB serum and stimulated with OKT3, more T cells
express the activation markers CD25 and CD152 compared to those cultured in normal AB serum.

Antica et al (1993) suggest that CLL patients may have three different sub-groups of T cells. The first has normal functional properties, the second has non-responsive properties and the third has greater reactivity than normal control T cell populations.

Overall, the evidence for a dysregulated T cell response to mitogen in CLL is conflicting and there have been no clearly defined, recent studies that unequivocally demonstrate low, normal or increased responses to mitogens.

1.5.2.3 T-cell colony formation

T cells from CLL patients form colonies in vitro less efficiently than those from normal subjects (Jehn U et al 1990; Burger T et al 1990). CLL T cells grown in a double layer T colony-forming assay produced fewer colonies compared with T cells from normal subjects, even after enrichment of the T helper cells. This abnormality correlates with the stage of the disease since more patients in Stage 0 showed colony formation than those at Stages I-IV (Foa R et al 1982). T cell colony formation in one study of untreated B-CLL patients was as good as that obtained from T cells from normal subjects (Femandez LA et al 1983).

Similarly, CLL T cells show a reduced response in a xenogenic graft-versus-host reaction (Stark P et al 1999). T cells were isolated from both normal controls and CLL patients and injected into the skin of rats which had been immunosuppressed with cyclophosphamide. After 4 days, the reaction was measured, calculated by the length, width and thickness of the lesion at the site of injection. T cells from normal controls caused a positive reaction in 97-100% of the tests. In comparison, 37.1% of patients in the stable phase and 13.3% of patients in the progressive phase were able to cause a positive result, implying a dysfunctional T cell status in B-CLL which was worse in advanced disease.
There is clearly an unexplained degree of variability in the capacity to form colonies even at early stages of the disease.

1.5.2.4 T-cell cytokine secretion

Research into the ability of CLL T cells to express appropriate cytokines has shown that this function is also deficient when compared to normals. Significant decreases in production of IFN-γ after PHA and PWM stimulation (Epstein LB and Cline MJ 1974) of IL-4 (Hill SJ et al 1999) and of IL-2 in a Mixed Lymphocyte Reaction (MLR) (Ayanlar-Batuman O et al 1986) have all been shown. However, contrasting results have shown an increase in IL-4 production by CLL T cells (Mu X et al 1996; de-Totero D et al 1999; Kay NE et al 2001) and CLL T cells have also been shown to produce similar quantities of IL-2 when compared to normals (Foa R et al 1985; Janssen O et al 1989; Zaknoen SL and Kay NE 1990). This was reversed when large numbers of autologous leukemic B cells were added to the culture system, suggesting some form of immune control of the CLL T cells by the malignant B cells or of the CLL B cells themselves utilising the IL-2 produced by the T cells (Foa R et al 1985).

CLL B cells have been shown to have high levels of the IL-2 receptor on their cell surface and also to secrete high levels of soluble IL-2 receptor into the serum (Zaknoen SL and Kay NE 1990). It has been shown that these malignant B cells are able to absorb the IL-2 produced by the T cells by both the receptors on the B cell surface and also soluble receptors in the serum. Varying results in the amount of IL-2 are found measured in CLL patients, as the more B cells that are present, the more IL-2 will be absorbed.

A more recent study has shown that CD4 and CD8 T cells from CLL patients produce increased levels of IFN-γ and that this increase in production could contribute to the survival of the CLL B cells as well as to the other various immune abnormalities detected in these patients. CLL B cells have an increased expression of IFN-γ receptors on the cell
surface compared to normals and the increased levels of IFN-γ are believed to inhibit apoptosis and prolong survival of these cells (Zaki M et al 2000).

In a comparison of IL-6 and TNF-α production between patients of different disease stages, levels of both were very high after stimulation in patients with Stage A disease, but little to none in patients with advanced disease (Stage C). Those patients with Stage B disease expressed intermediate amounts (Hulkkonen J et al 1998). Comparable results were observed with TNF-α production, suggesting that as the disease progresses, production of these cytokines decreases, either as a result of diminishing T cell function or increased B cell numbers controlling the T cells or utilising the cytokines themselves. A recent study has also shown an increase in both secreted and intracellular TNF-α and IL-4 in CLL T cells, but a decrease in IL-10 (Mainou-Fowler T et al 2001).

In a study that compared soluble CD4 (sCD4) and soluble CD8 (sCD8) in stable or progressive B-CLL, sCD8 was increased in both groups and sCD4 in the progressive group only (Aguilar-Santelises M et al 1992), suggesting an increased T-suppressor activity in stable B-CLL and a selective increase of helper T cell activity in progressive B-CLL. This suggests that the T cells are affected as the disease progresses. The increase in helper T cell activity might imply that the T cells are trying to respond to the malignant cells or being stimulated to secrete T-helper cell factors by the CLL B cells. An increase in suppressor activity has been reported previously in T cell responses to mitogen and this finding of increased sCD8 would corroborate this finding.

The ability of CLL T cells to respond to cytokines has also been studied (Briggs PG et al 1991). Purified normal and CLL T cells were cultured in the presence of either IL-1 or IL-2 and stimulated with PHA. Normal T cell response was augmented by the addition of both IL-1 and IL-2, whilst CLL T cells showed no response after the addition of IL-1 and only a very small response with IL-2. Such data suggest that CLL T cells have an abnormal
response to cytokines compared to normals and this may contribute to the pathogenesis and to the clinical findings in the disease.

1.5.2.5 T-cell surface antigen expression.

Most studies on the T cell compartment of B-CLL patients have focused on the phenotypic expression of cell surface antigens, in particular those associated with activation and B cell interaction and there is much conflicting evidence. Early reports have shown that significantly more unstimulated CLL T cells express the activation markers CD25 and HLA-DR when resting, compared to normals (Zaknoen SL and Kay NE 1990; Jehn U et al 1990; Garcia-Suarez J et al 1991). In contrast, other work has conversely shown that expression of CD25 and HLA-DR is normal on CLL T cells (Kay NE and Kaplan ME 1986; Briggs PG et al 1989). This PhD thesis has shown that the number of T cells expressing CD25 is significantly reduced and the number of T cells expressing HLA-DR are significantly increased (Scrivener S et al 2001).

A recent report described an assumed acquired deficiency in B-CLL patients of the ability to express the activation marker CD40 ligand (CD154) on anti-CD3 activated T cells (Cantwell M et al 1999). This finding was said to be the result of the uncontrolled proliferation of B cells in B-CLL patients, since stimulated T cells, in the presence of increasing numbers of B cells show rapid down-modulation of the CD154 receptor (Cantwell M et al 1999). Others studies have shown that expression of CD154 on T cells is normal in CLL (Brugnoni D et al 1995 Scrivener S et al 2001). Similarly, levels of expression of CD28 have been reported to be down-regulated (Scrivener S et al 2001) and the zeta (ζ) chain of the CD3 receptor have also been reported to be down regulated in B-CLL patients (Frydecka I et al 1998), although a proportion of these patients had received treatment in the month prior to the study (Rossi E et al 1996).
Expression of the Fas receptor (FasR) was shown to be increased on CD4+ and CD8+ T cells in patients with B-CLL (Tinhofer I et al 1998). Although apoptosis was evident in both subsets, only the CD4 cells died in this manner in response to the Fas antibody CH11. In addition, CD19 cells from B-CLL patients constitutively express functionally active Fas ligand (FasL). This Fas sensitivity correlated with a lower CD4/CD8 ratio than normal and it has been hypothesised that an increase in Fas sensitivity on CD4 cells and the presence of functionally active FasL on B-CLL B cells may be responsible for the inverted CD4/CD8 ratio observed in many patients, as discussed in 1.5.2.1 (Tinhofer I, et al 1998).

There is some controversy surrounding the T-cell phenotype in B-CLL. Early studies (Matutes E et al 1981) described a reduction in patients' T cells which were able to bind to the CD4 monoclonal antibody OKT4 and normal or increased T cell number binding the CD8 monoclonal OKT8. A proportion of the patient T cells were unreactive to all the monoclonal antibodies tested. A later study suggested that there were normal numbers of both treated and un-treated patients' T-cells bound to OKT4 and a statistically significant increase in T-cells binding OKT8, compared to normals (Mills KHG et al 1982). This finding has been contradicted in a more recent study, which suggested that CD4 numbers were increased and these cells had TCRs with oligoclonal patterns (Serrano D et al 1997). Higher numbers of these CD4 T cells with oligoclonal TCRs were observed in patients with more advanced disease, suggesting that there are profound alterations in the T cell compartment of patients with CLL and that there is also evidence of clonality among the CLL T cell population. Other work has also shown evidence of a clonal T cell population in small numbers of patients (Wen T et al 1990). Such results suggest that there may be some degree of host response by the T cells against specific leukaemic antigens and that in early stage disease particularly, the T cells attempt to respond to the leukaemic clone.

Supporting this theory, functional studies have shown that CLL patient CD4 and CD8 T cells express significantly greater levels of CD45RO and decreased absolute numbers of
CD45RA when compared to normals (Frolova EA et al 1995; Porakishvili N et al 2001).
The increase in cells expressing the CD45RO suggests that there is a greater proportion of
T cells in CLL with a "memory" capacity, having previously been exposed to antigenic
challenge or arising as a clonal population from a previously challenged T cell.

Most recently, CLL T cells have been shown to have significantly higher levels of type II
TGFβ receptor when compared to normal T cells (Zaki MH et al 2000). Evidence indicates
that TGFβ can induce growth arrest in mid to late G1 phase of the T cell cycle (Figure 4).
CLL T cells, when stimulated with anti-CD3 and IL-2, show a reduced production of S-
phase fraction, suggesting that they are not completing the full cell cycle of replication.
This could be caused by the increased expression of receptors to TGFβ on the cell surface
preventing passage to further stages of the cell cycle.

CLL T cells, when activated in the presence of CLL B cells, rapidly up-regulate the cell
surface antigen CD30, an affect that is not observed in normal T cells (De Totero D et al
1999; Cerutti A et al 2001). This expression of CD30 on the CLL T cells ultimately
prevents the up-regulation of the CD40 ligand on non-malignant B cells. By preventing
CD40 from binding with CD154 on the T cells, IgA and IgG production by the B cells may
be prevented, providing a possible hypothesis for the increased hypogammaglobulinaemia
in CLL patients. Levels of soluble CD30 are also significantly increased in the supernatant
of activated CLL T cell cultures and in CLL serum samples (De Totero D et al 1999).

CTLA-4 (CD152), the ligand for CD80 and CD86 shows prolonged expression on the
surface of CLL T cells compared to normal T cells after activation (Frydecka IF et al 2001).
Maximum up-regulation of this cell surface antigen was at 72hrs for normals and 24hrs for
CLL patients. After 72hrs, CD152 was undetectable in normals, but expression persisted
on CLL T cells until 120hrs, when expression became undetectable, suggesting a
dysregulation in T cell kinetics and a possible prolonged T cell response.
A recent study of T cell surface markers in CLL has shown that there is a significant reduction in the expression of CD4, CD5 and LFA-1 on unstimulated T cells compared to normals and a significant reduction of CD25, CD28 and CD152 on activated T cells compared to normals (Scrivener S et al 2001). Expression of HLA-DR on stimulated T cells was significantly higher on CLL T cells compared to normals. Removal of the malignant B cells did not correct the defect in surface expression of CD28 and CD152, suggesting a possible intrinsic or long lasting defect in CLL T cells. Permeabilisation of the T cells after activation did not show a difference in intracellular CD25 or CD152 in CLL T cells, but expression of intracellular CD28 was significantly lower in CLL patients. In contrast, resting CLL T cells have significantly higher levels of intracellular CD25 and CD152 compared to normals. This suggests an intrinsic T cell defect that may be affected by external factors, preventing normal surface expression, and altering intracellular expression.

1.6 Summary

The evidence discussed here on the functional capacity of T cells in CLL suggests that there is undoubtedly some degree of T cell dysfunction in CLL. But many studies have produced conflicting evidence, in particular about T cell surface antigen expression and activation status, including the secretion of critical cytokines.

Such variation in results could be a result of the different methods used to stimulate the T cells. Methods that act as general T cell mitogens, such as keyhole limpet haemocyanin (KLH) and OKT3 which acts directly through CD3 and was used in this study, both utilise different methods of T cell activation and may give rise to some of the different responses observed. In addition, early methods of T cell activation involved purifying the T cell population using mouse erythrocyte rosetting, which relies on the CD2 antigen on T cells binding to the sheep red blood cells. By using this method, the T cells can also be
inadvertently activated and resting T cell status is not studied. Methods used today and in this PhD study both employ a procedure of negative selection, where the end T cell population has received no known prior activation signals.

To date, there has been no conclusive study examining an array of key cell surface interaction and activation markers that would shed light on the capabilities of the CLL T cell to "talk" with the malignant B cells.

The aim of this project was to determine the functional status of the T cell compartment in patients with B-CLL. Using cell culture, flow cytometry and ELISA techniques, studies were undertaken to elucidate expression of key activation and interaction markers on both the T cell surface and intracellularly, to measure secretion of key T cell cytokines in both resting and activated T cells and to identify a possible soluble serum factor in CLL patients which may account for the changes described here.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Patients

2.1.1 Patients and control groups

This study was approved by the local research ethics committee. Between September 1997 and July 2001, blood samples were taken from 41 patients with B cell chronic lymphocytic leukaemia (30 males, 11 females median age 70 years, range 48 - 87). Patients were selected from Haematology clinics after having established that they were either not treated or had not received any treatment in the preceding three months. Informed consent was obtained from each patient before samples were taken. Patient details are shown in table 11.

53 Control blood samples were taken from a pool of 22 normal, healthy volunteers (6 males, 16 females, median age 34 years, range 24 - 63).

2.1.2 CLL diagnosis

Patients are diagnosed routinely in the Derriford Laboratory with CLL by a series of haematological tests, as described below.

A Full Blood Count (FBC) is carried out, either as part of a routine check or as part of a clinical investigation. If the FBC reveals an absolute lymphocyte count above $5.0 \times 10^9$/L, a blood film is prepared. The presence of smear cells and/or pleiomorphic lymphocytes indicates the need for an immunophenotyping panel which is done. This analyses expression of CD2, CD5, CD22, CD23, FMC7, kappa and lambda for a more accurate diagnosis. Some patients require no further investigations after this stage. Patients are classed as typical or atypical CLL. Typical CLL may require a bone marrow biopsy to evaluate the degree of cellular infiltration before treatment is decided. Cytogenetic testing is being used with increasing frequency as are molecular tests for gene rearrangement.
Patients are then staged and their subsequent check-ups scheduled according to their disease status.

2.1.3 Patient selection

The patients in this study were either wholly untreated or were free from treatment in the three months prior to blood samples being taken. Three patients had received treatment with purine analogues, but this was a minimum of 2 years before blood samples were taken.
TABLE 11: B-CLL patient details

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>D.O.B</th>
<th>AGE</th>
<th>M/F</th>
<th>WCC</th>
<th>STAGE</th>
<th>PREV. TREATMENT</th>
<th>DURATION</th>
<th>NATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>05.07.22</td>
<td>71</td>
<td>M</td>
<td>30.7</td>
<td>A0</td>
<td>NONE</td>
<td>Sep-96</td>
<td>STABLE</td>
</tr>
<tr>
<td>SL</td>
<td>12.07.50</td>
<td>48</td>
<td>M</td>
<td>32.2</td>
<td>A0</td>
<td>NONE</td>
<td>Jan-97</td>
<td>STABLE</td>
</tr>
<tr>
<td>DT</td>
<td>19.07.19</td>
<td>79</td>
<td>F</td>
<td>111.6</td>
<td>A1</td>
<td>NONE</td>
<td>Nov-89</td>
<td>STABLE</td>
</tr>
<tr>
<td>VB</td>
<td>27.05.17</td>
<td>81</td>
<td>F</td>
<td>115</td>
<td>A0</td>
<td>2X CHLOR SEP '96</td>
<td>Jan-92</td>
<td>STABLE</td>
</tr>
<tr>
<td>BL</td>
<td>08.05.44</td>
<td>54</td>
<td>M</td>
<td>270</td>
<td>B1</td>
<td>7X CHLOR + PRED JUN 97</td>
<td>Sep-92</td>
<td>STABLE</td>
</tr>
<tr>
<td>JB</td>
<td>16.10.43</td>
<td>55</td>
<td>F</td>
<td>60.8</td>
<td>A1</td>
<td>6X CHLOR + PRED DEC 96</td>
<td>Sep-96</td>
<td>STABLE</td>
</tr>
<tr>
<td>MW</td>
<td>05.12.22</td>
<td>76</td>
<td>F</td>
<td>32.7</td>
<td>A0</td>
<td>6X CHLOR OCT 95, CYCLO MAR 98</td>
<td>Jul-90</td>
<td>STABLE</td>
</tr>
<tr>
<td>EM</td>
<td>05.07.22</td>
<td>76</td>
<td>M</td>
<td>46</td>
<td>A0</td>
<td>10X CHLOR FEB 89 6X CHLOR JUNE 96</td>
<td>Oct-88</td>
<td>STABLE</td>
</tr>
<tr>
<td>HS</td>
<td>04.05.13</td>
<td>85</td>
<td>M</td>
<td>85</td>
<td>A0</td>
<td>PRED JAN 94</td>
<td>Jul-93</td>
<td>STABLE</td>
</tr>
<tr>
<td>NP</td>
<td>29.06.29</td>
<td>69</td>
<td>M</td>
<td>170.9</td>
<td>A0</td>
<td>NONE</td>
<td>Jul-95</td>
<td>STABLE</td>
</tr>
<tr>
<td>SS</td>
<td>21.06.42</td>
<td>56</td>
<td>M</td>
<td>11.7</td>
<td>A1</td>
<td>3X CHLOR OCT 93, 7X CHLOR + PRED FEB 94</td>
<td>Mar-90</td>
<td>STABLE</td>
</tr>
<tr>
<td>DH</td>
<td>22.08.26</td>
<td>72</td>
<td>F</td>
<td>8.3</td>
<td>*(4.7)</td>
<td>A0</td>
<td>Jun-90</td>
<td>STABLE</td>
</tr>
<tr>
<td>CG</td>
<td>16.12.18</td>
<td>80</td>
<td>M</td>
<td>18.3</td>
<td>C4</td>
<td>12X CHLOR APR 85 6X CHLOR FEB 93 4X CHLOR JAN 97</td>
<td>Apr-85</td>
<td>STABLE</td>
</tr>
<tr>
<td>PATIENT</td>
<td>D.O.B</td>
<td>AGE</td>
<td>M/F</td>
<td>WCC</td>
<td>STAGE</td>
<td>PREV. TREATMENT</td>
<td>DURATION</td>
<td>NATURE</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
<td>-------</td>
<td>----------------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>SV</td>
<td>31.05.32</td>
<td>66</td>
<td>M</td>
<td>29.6</td>
<td>C3</td>
<td>2X CHLOR FEB 86 1X CHLOR AUG 86 1X CHLOR JUN 88 6X CHLOR + PRED NOV 89 4X CHLOR + PRED AUG 94</td>
<td>Oct-84</td>
<td>STABLE</td>
</tr>
<tr>
<td>RM</td>
<td>24.06.34</td>
<td>64</td>
<td>M</td>
<td>16.9</td>
<td>A0</td>
<td>NONE</td>
<td>Apr-97</td>
<td>STABLE</td>
</tr>
<tr>
<td>GB</td>
<td>07.09.28</td>
<td>71</td>
<td>M</td>
<td>30.3</td>
<td>A0</td>
<td>NONE</td>
<td>Oct-93</td>
<td>STABLE</td>
</tr>
<tr>
<td>PS</td>
<td>18.12.22</td>
<td>76</td>
<td>M</td>
<td>18.5</td>
<td>A0</td>
<td>NONE</td>
<td>Nov-98</td>
<td>STABLE</td>
</tr>
<tr>
<td>RWi</td>
<td>09.07.32</td>
<td>67</td>
<td>M</td>
<td>38.7</td>
<td>A0</td>
<td>NONE</td>
<td>Feb-91</td>
<td>STABLE</td>
</tr>
<tr>
<td>RS</td>
<td>14.01.25</td>
<td>74</td>
<td>M</td>
<td>107</td>
<td>C4</td>
<td>NONE</td>
<td>Aug-98</td>
<td>STABLE</td>
</tr>
<tr>
<td>WR</td>
<td>25.10.19</td>
<td>80</td>
<td>M</td>
<td>20.2</td>
<td>A0</td>
<td>NONE</td>
<td>Apr-97</td>
<td>STABLE</td>
</tr>
<tr>
<td>NR</td>
<td>27.02.34</td>
<td>65</td>
<td>F</td>
<td>40.5</td>
<td>C4</td>
<td>5X CHLOR APR 87 6X CHLOR OCT 91 PRED JAN 92 6X IV FLUD JUN 94 3X CHLOR + PRED JAN 97 5X CHLOR AUG 98</td>
<td>Mar-87</td>
<td>STABLE</td>
</tr>
<tr>
<td>JG</td>
<td>12.05.23</td>
<td>76</td>
<td>M</td>
<td>11.8</td>
<td>A0</td>
<td>NONE</td>
<td>Apr-98</td>
<td>STABLE</td>
</tr>
<tr>
<td>PB</td>
<td>06.03.42</td>
<td>57</td>
<td>F</td>
<td>73.9</td>
<td>A0</td>
<td>NONE</td>
<td>Mar-96</td>
<td>STABLE</td>
</tr>
<tr>
<td>EP</td>
<td>08.12.29</td>
<td>70</td>
<td>M</td>
<td>25.1</td>
<td>A0</td>
<td>NONE</td>
<td>Jul-98</td>
<td>STABLE</td>
</tr>
<tr>
<td>VR</td>
<td>28.03.35</td>
<td>64</td>
<td>M</td>
<td>6.6</td>
<td>A0</td>
<td>6X CHLOR + PRED FEB 99</td>
<td>Jan-99</td>
<td>STABLE</td>
</tr>
<tr>
<td>HC</td>
<td>23.02.21</td>
<td>78</td>
<td>F</td>
<td>45.7</td>
<td>A0</td>
<td>NONE</td>
<td>Apr-96</td>
<td>STABLE</td>
</tr>
<tr>
<td>JC</td>
<td>12.11.23</td>
<td>76</td>
<td>M</td>
<td>27.8</td>
<td>C4</td>
<td>CLAD JUL 98</td>
<td>Jun-98</td>
<td>STABLE</td>
</tr>
<tr>
<td>PATIENT</td>
<td>D.O.B</td>
<td>AGE</td>
<td>M/F</td>
<td>WCC</td>
<td>STAGE</td>
<td>PREV. TREATMENT</td>
<td>DURATION</td>
<td>NATURE</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-------</td>
<td>----------------</td>
<td>----------</td>
<td>--------------</td>
</tr>
<tr>
<td>RB</td>
<td>17.08.37</td>
<td>62</td>
<td>M</td>
<td>19.8</td>
<td>A0</td>
<td>NONE</td>
<td>Dec-99</td>
<td>STABLE</td>
</tr>
<tr>
<td>WHS</td>
<td>04.02.20</td>
<td>79</td>
<td>M</td>
<td>58.9</td>
<td>A0</td>
<td>SPLenic RA OCT 99</td>
<td>Oct-88</td>
<td>STABLE</td>
</tr>
<tr>
<td>RB</td>
<td>01.07.45</td>
<td>54</td>
<td>M</td>
<td>8.9</td>
<td>A0</td>
<td>NONE</td>
<td>May-00</td>
<td>STABLE</td>
</tr>
<tr>
<td>RJ</td>
<td>09.06.33</td>
<td>66</td>
<td>M</td>
<td>46.3</td>
<td>A0</td>
<td>NONE</td>
<td>Sep-96</td>
<td>STABLE</td>
</tr>
<tr>
<td>JC</td>
<td>18.12.26</td>
<td>73</td>
<td>F</td>
<td>29.9</td>
<td>B3</td>
<td>NONE</td>
<td>Dec-00</td>
<td>SLOWLY PROGRESSIVE</td>
</tr>
<tr>
<td>RB</td>
<td>24.07.37</td>
<td>62</td>
<td>M</td>
<td>28.1</td>
<td>A0</td>
<td>NONE</td>
<td>Aug-99</td>
<td>STABLE</td>
</tr>
<tr>
<td>FB</td>
<td>24.07.22</td>
<td>78</td>
<td>F</td>
<td>33.5</td>
<td>A0</td>
<td>NONE</td>
<td>Oct-91</td>
<td>STABLE</td>
</tr>
<tr>
<td>FM</td>
<td>15.05.39</td>
<td>61</td>
<td>M</td>
<td>17.5</td>
<td>A0</td>
<td>2X CHLOR MAR 90 9X CHLOR + PRED MAY 92 6X CHLOR JUL 94 6X CDA + PRED MAY 95</td>
<td>Feb-89</td>
<td>STABLE</td>
</tr>
<tr>
<td>JL</td>
<td>01.02.23</td>
<td>78</td>
<td>M</td>
<td>12.3</td>
<td>A0</td>
<td>NONE</td>
<td>Sep-92</td>
<td>STABLE</td>
</tr>
<tr>
<td>MRM</td>
<td>07.09.14</td>
<td>87</td>
<td>M</td>
<td>83.9</td>
<td>A0</td>
<td>6X CHLOR SEP 91 5X CHLOR APR 99</td>
<td>Sep-91</td>
<td>STABLE</td>
</tr>
<tr>
<td>KM</td>
<td>19.04.31</td>
<td>70</td>
<td>M</td>
<td>60.8</td>
<td>A0</td>
<td>NONE</td>
<td>Oct-00</td>
<td>STABLE</td>
</tr>
<tr>
<td>MS</td>
<td>07.03.40</td>
<td>61</td>
<td>M</td>
<td>14.8</td>
<td>A0</td>
<td>NONE</td>
<td>Nov-00</td>
<td>STABLE</td>
</tr>
<tr>
<td>RWr</td>
<td>22.09.5</td>
<td>65</td>
<td>M</td>
<td>15.2</td>
<td>A0</td>
<td>NONE</td>
<td>Dec-99</td>
<td>STABLE</td>
</tr>
<tr>
<td>HF</td>
<td>17.10.38</td>
<td>63</td>
<td>F</td>
<td>6.2</td>
<td>CR</td>
<td>NONE</td>
<td>Sep-96</td>
<td>STABLE</td>
</tr>
</tbody>
</table>

Table showing all patient details including age, date of birth, sex, white cell count at time of experiment, stage, treatment history, date of diagnosis and nature of disease. * figures in brackets indicate the absolute lymphocyte count, as white cell count below normal range for CLL diagnosis. Increased absolute lymphocyte count indicates CLL. CR = clinical remission, CHLOR = Chlorambucil, PRED = Prednisolone, CDA = Cladribine, FLUD = Fludarabine, RA = radiotherapy, CYCLO = Cyclophosphamide.
2.2 Reagents

2.2.1 Cell culture reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roswell Park Memorial Institute - 1640 (RPMI-1640)</td>
<td>Gibco Life Technologies, Paisley, UK</td>
</tr>
<tr>
<td>Pencillin and Streptomycin antibiotics</td>
<td>Sigma Aldrich, Poole, UK</td>
</tr>
<tr>
<td>Normal human AB serum</td>
<td>National Blood Transfusion Service, Bristol, UK</td>
</tr>
<tr>
<td>Lymphoprep</td>
<td>Nycomed Amersham, Amersham, UK</td>
</tr>
<tr>
<td>Phytohaemagglutinin (PHA)</td>
<td>Murex, Maidenhead, UK</td>
</tr>
<tr>
<td>OKT3</td>
<td>Janssen-Cilag, High Wycombe, UK</td>
</tr>
<tr>
<td>24-well, polystyrene, flat bottom, culture plates</td>
<td>Nunclon, Fisher Scientific UK, Loughborough, Leics, UK</td>
</tr>
<tr>
<td>5 ml, polystyrene, round bottom, Falcon tubes</td>
<td>Marathon Lab Supplies, London, UK</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Lorne Laboratories, Reading, UK</td>
</tr>
<tr>
<td>Interleukin-15 (IL-15)</td>
<td>Cambridge Bioscience, Cambridge, UK</td>
</tr>
</tbody>
</table>
2.2.2 Flow Cytometry reagents

All samples were analysed on a Beckman-Coulter Epics Elite Flow Cytometer (Beckman Coulter, High Wycombe, UK)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow check fluorospheres</td>
<td>Beckman-Coulter, High Wycombe, UK</td>
</tr>
<tr>
<td>CD14/CD45 Leucogate</td>
<td>Serotec, Kidlington, Oxford, UK</td>
</tr>
<tr>
<td>CD2 FITC</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD3 FITC</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD4 PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD5 PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD8 PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD11a (LFA-1) PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD25 (IL-2R) PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD28 PE</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD54 (ICAM-1) PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>CD152 (CTLA-4) PE</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD154 (CD40L) PE</td>
<td>Ancell Corporation, Nottingham, UK</td>
</tr>
<tr>
<td>HLA-DR PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>Mouse IgG1 FITC</td>
<td>Serotec</td>
</tr>
<tr>
<td>Mouse IgG2b FITC</td>
<td>Serotec</td>
</tr>
<tr>
<td>Mouse IgG1 PE</td>
<td>Serotec</td>
</tr>
<tr>
<td>Mouse IgG2a PE</td>
<td>Beckman Coulter</td>
</tr>
</tbody>
</table>

2.2.3 B cell depletion reagents

<table>
<thead>
<tr>
<th>Reagent/Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19 magnetic beads</td>
<td>Miltenyi-Biotech, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma Aldrich, Poole, UK</td>
</tr>
<tr>
<td>LS⁺ separation columns</td>
<td>Miltenyi-Biotech</td>
</tr>
</tbody>
</table>
### 2.2.4 Permeabilisation reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAKO intrastain kit</td>
<td>DAKO, Ely, UK</td>
</tr>
</tbody>
</table>

### 2.2.5 ELISA reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantikine human IL-2 ELISA</td>
<td>R &amp; D systems Europe Ltd., Abingdon, Oxon, UK</td>
</tr>
<tr>
<td>Pelikine Compact Human IFN-γ ELISA</td>
<td>Eurogenetics (UK) Ltd., Hampton, UK</td>
</tr>
<tr>
<td>Carbonate</td>
<td>Sigma Aldrich, Poole, UK</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>Sigma</td>
</tr>
<tr>
<td>PBS: dibasic sodium phosphate, monobasic sodium phosphate, sodium chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Thiomersal</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tetramethylbezidine (TMB)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dimethysulfoxide (DMSO)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>Sigma</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
2.3 Preparation of solutions

2.3.1 Cell culture

*Cell culture medium.* Under sterile class II conditions (MDH Intermed class II cabinet) and at room temperature, 10mls of Penicillin and Streptomycin (Sigma Aldrich, Poole, UK) were added to 500mls RPMI-1640 (Gibco Life Technologies, Paisley, UK) at a final concentration of 500U/ml and 500µg/ml respectively. 5mls of L-glutamine (Sigma) was added to 500mls RPMI at a final concentration of 2mM. Solution was stored at 4°C.

*RPMI + 10% and 50% normal AB serum.* Under sterile class II conditions, 10% or 50% normal AB serum (National Blood Transfusion Service, Bristol, UK) was added to pre-warmed RPMI at 37°C as required.

*RPMI + 50% CLL AB serum.* Blood was taken into sterile plain tubes from an AB Rh (D)⁺ CLL patient and left to stand at room temperature for a minimum of 2 hrs. Once the contents had clotted, the sample was centrifuged and the serum removed under sterile conditions and aliquotted into sterile 1ml tubes (Greiner). Sample was heat inactivated at 57°C for 30 minutes and then frozen for future use. 50% CLL AB serum (5mls) was added to pre-warmed RPMI at 37°C (5mls) when required. The CLL AB serum was analysed for total protein, immunoglobulins and lipids in both fresh and heat inactivated serum.

*Wash buffer.* 400mls 0.01M PBS (Sigma) was supplemented with 1% BSA (4mls) (Lorne Laboratories, Reading, UK).
2.3.2 B-cell depletion solutions

Cell separation (Miltenyi Biotech MACS) buffer. Under sterile class II conditions, 400mls PBS was supplemented with 2mM EDTA (Sigma) and stored at 4°C. Prior to use, 50mls of solution was sterile filtered under class II conditions using a 0.2μ filter (Nalgene, Rochester, New York, USA) and supplemented with 0.5% BSA (250μl) (Lorne Laboratories).

2.3.3 ELISA solutions

All reagents made up at room temperature.

IFN-γ ELISA Coating buffer. Solution A: 0.124g carbonate (Sigma) was added to 10mls distilled water. Solution B: 0.168g bicarbonate (Sigma) was added to 20mls distilled water. Solution A was added to solution B until the pH was 9.6. Approximately 7mls of solution A and 17.5mls solution B were needed. 120μl of the supplied coating antibody (R & D systems Europe Ltd, Abingdon, Oxon, UK) was added to 12mls of this coating buffer.

PBS stock solution. 16g dibasic sodium phosphate (Sigma), 3g monobasic sodium phosphate (Sigma) and 82g sodium chloride (Sigma) were added to 450mls distilled water, the pH adjusted to 6.8-6.9. 10mg thiomersal (Sigma) was added as a preservative.

Working strength PBS. PBS stock solution was diluted 1:20 in distilled water

Blocking buffer. 500μl of supplied blocking buffer (R&D systems) was added to 25mls of working strength PBS.

Washing buffer. 50μl of tween 20 (Sigma) was added to 1L of working strength PBS.

Biotinylated IFN-γ. 120μl of supplied biotinylated IFN-γ (R&D systems) was added to 12mls of working strength dilution buffer
Substrate buffer. 1.5g of sodium acetate (Sigma) was dissolved in 80mls distilled water. pH was adjusted to 5.5 with glacial acetic acid (Sigma) and distilled water was added to make up 1L.

TMB stock solution. 30mg tetramethylbezidine (TMB) (Sigma) was dissolved in 5mls dimethylsulfoxide (DMSO) (Sigma).

Hydrogen peroxide solution. 3% (1ml of a 30% stock solution) of hydrogen peroxide (H₂O₂) (Sigma) was added to 10mls of distilled water.

Substrate solution. 12mls of substrate buffer was added to 200μl of TMB stock solution and 12μl of H₂O₂ stock solution.

Stop solution. H₂SO₄ (Sigma) was added to distilled water at a final concentration of 1.8M.
2.4 METHODS

2.4.1 Flow cytometry on the Beckman Coulter Epics Elite

All cells were analysed on a Beckman Coulter Epics Elite.

Before analysing patient and control samples, the flow cytometer was aligned using Beckman Coulter Flow Check beads. This was carried out at the start of every experiment to ensure that the laser was correctly aligned with the photomultiplier tubes (PMTs). This was established by ensuring both visually that the beads were aligned inside the central box in histogram 1 and that the peaks which fell within the set limits of PMT1, PMT2, PMT3, PMT4 and PMT5 were narrow and high representing the particles passing the centre of the laser beam. Correct alignment was also verified numerically. The half coefficient variant (cv) value for each of the PMTs should be below 2.0. If either of these parameters fell out of their normal ranges, a full or partial re-alignment was carried out, as explained in the Beckman Coulter Epics Elite trouble shooting guide.

After correct alignment was established, each set of samples was analysed in the same order, to ensure correct negative and positive gating and correct compensation. The negative control sample was always analysed first, to set the gates around the negative cells and to ensure that there were no cells that stained positive for the relevant mouse isotypic controls (fig 9).

The cells stained with either CD2 FITC or CD3 FITC were analysed second to check that compensation between FITC and PE was correct. Different cell types and cells in different stages of activation could require compensation to prevent FITC fluorescence being picked up by PMT3 instead of PMT2. If this occurred, the voltage for PMT2 was increased and the cells re-aligned in the correct upper right quadrant of the dot plot. CD2 or CD3 were also analysed individually to obtain a total T cell number from which percentages could be calculated.
A sample containing the monoclonals CD45 FITC/CD14 PE (Leucogate) was always
analysed. This allowed the gated cells to be identified as lymphocytes and not monocytes
(fig. 10).

The relevant samples were then analysed. Once the lymphocyte gate had been set, each
sample was counted for a total of 5000 cells (lymphocytes).
Figure 9: Flow cytometry dot plots showing cell populations after staining with negative isotypes.

Figures showing two dot plots after staining with negative isotypes. The top figure shows the gating of the lymphocytes (within gate A) and the second figure shows no positivity ie all cells are negative for the isotypic controls and any subsequent binding is not due to inappropriate antibody recognition of the immunoglobulin class.
Figure 10: Flow cytometry dot plots showing cell populations after staining with Leucogate.

Figures showing two dot plots after staining with Leucogate. The top figure shows gating of the lymphocytes (within gate A). The bottom figure shows positivity within region B4 only (FITC), indicating that all cells within gate A are positive for CD45 and are therefore all lymphocytes and not monocytes.
2.4.2 PBMC isolation

Blood was drawn into 10ml sodium heparin anticoagulated tubes and under sterile class II conditions, mixed in equal volumes with pre-warmed RPMI-1640 medium (Gibco Life Technologies, Paisley, UK) at 37°C containing penicillin, streptomycin and L-glutamine (Sigma Aldrich, Poole, UK) and layered in equal volumes on to the density gradient separation medium Lymphoprep (Nycomed Amersham, Amersham, UK). Cells were centrifuged for 20 minutes at 450g with the brake off (Labofuge 400R, Heraeus, Kendro Laboratory Products Ltd, Bishops Stortford, Hertfordshire, UK). PBMCs were removed from the interface, transferred to clean, sterile 12ml tubes (Greiner Bio-One, Stonehouse, Gloucester, UK) and washed twice in excess RPMI + 10% AB serum (National Blood Transfusion Service (NBTS), Bristol, UK) as described previously. Cells were resuspended in RPMI (Gibco) + 10% AB serum and counted by a haemocytometer (Sigma Aldrich, Poole, UK).

2.4.3 Cell culture methods for cell surface expression of antigens CD25, CD28, CD152, CD154 and HLA-DR

PBMCs were cultured under conditions described below to evaluate expression of the cell surface markers CD25, CD28, CD152, CD154 and HLA-DR on T cells after activation. For expression of CD28, CD154 and HLA-DR, the monoclonal antibody OKT3 (Janssen-Cilag, High Wycombe, UK) was added to PBS at a final concentration of 1mg/ml and 500μl was plated on 6 wells of a 24 well, flat bottom, polystyrene, culture plate (Nunclon, Fisher Scientific UK, Leics, UK) for 2 hours at room temperature under class II sterile conditions. 6 wells on a separate 24 well plate were set up as controls containing 500μl PBS alone in addition to the OKT3 wells. PBMCs were isolated as described in 2.4.2. The 24-well plate containing the OKT3 wells and the control 24 well plate containing the PBS wells were washed 3 times in 500μl of PBS. Cells for CD28, CD154 and HLA-DR
analysis were plated at a final concentration of $1 \times 10^6$ in 1 ml RPMI (Gibco) + 10% AB serum (NBTS) per well in each plate.

Cells for CD25 and CD152 analysis were plated on 4 wells in a 24-well plate at a final concentration of $1 \times 10^6$ /ml in RPMI + 10% AB serum per well. Cells for CD25 and CD152 were not cultured with OKT3 in these initial experiments. Instead, 5μl of PHA (Murex, Maidenhead, UK) was added to each well of PBMCs at a final concentration of 5μg/ml. Corresponding controls were set up in a separate 24 well plate containing $1 \times 10^6$ PBMCs in 1 ml RPMI + 10% AB serum in each well with no PHA.

After cells for CD25, CD152 and HLA-DR analysis had been plated on the 24-well plates containing both the OKT3 and the control PBS only, they were cultured for 4 hours in the incubator (Sanyo IR Sensor, Jencons Ptc) at 37°C and in 5% CO₂. After culture, the cells from each well were transferred to separate 5ml, polystyrene, round bottom tubes (Falcon, Marathon Lab Supplies, London, UK) centrifuged for 5 minutes at 1400rpm and washed twice in 1ml wash buffer.

After cells for CD28 and CD154 analysis had been plated on the 24-well plates containing both the PHA and the control wells, they were cultured in the incubator for 48 hours at 37°C and in 5% CO₂. After culture, the cells from each well were transferred to separate 5ml tubes (Falcon), centrifuged for 5 minutes at 220g and washed twice in 1ml wash buffer.

10μl of antibody for negative controls (equivalent mouse isotypic controls), Leucogate (lymphocyte gating), CD2 (T cell confirmation), CD3 (T cell confirmation), CD3/CD25 (test), CD2/CD28 (test), CD3/CD152 (test), CD2/CD154 (test) and CD2/HLA-DR (test) (as described in 2.2.2 and 2.4.1) were added to each 5ml tube per $10^6$ cells and incubated for 15 minutes in the dark at room temperature in air.

PBMCs were washed twice in wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed using flow cytometry.
2.4.4 Cell preparation methods for surface expression of CD4, CD5, CD8, CD11a, CD54 and TCRαβ

PBMCs were isolated as described in 2.4.2. 10μl of antibody for negative control (equivalent mouse isotypic controls), Leucogate (lymphocyte gating), CD3 (T cell confirmation), CD3/CD4 (test), CD3/CD5 (test), CD3/CD8 (test), CD3/CD11a (test), CD3/CD54 (test) and CD3/TCRαβ (test) (as described in 2.2.2 and 2.4.1) were added to each tube per 1 x 10^6 cells and incubated for 15 minutes in the dark at room temperature. PBMCs were washed twice in wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed using flow cytometry.

2.4.5 T cell enrichment

10mls of magnesium and calcium free PBS, kept on ice to prevent antibody capping during staining, was added to each sample of 10mls of sodium heparin anticoagulated blood (no more than 8 hours old) and PBMCs were isolated as described in 2.4.2, with the exception that all wash stages in the centrifuge were carried out at 18°C. After final wash stage, 1ml of cell separation buffer (described in 2.3.2) was added to the cells and they were immediately counted by a haemocytometer (Sigma Aldrich, Poole, UK). All buffers and reagents were kept on ice throughout.

After the cells had been counted, they were diluted with 80μl of cell separation buffer and 20μl of CD19 microbeads (Miltenyi-Biotech, Bergisch Gladbach, Germany) for every 10^7 total cells, vortexed (Clifton Cyclone, Jencons Plc) and incubated at 4°C for 15 minutes in the dark. Cells were then washed in excess cell separation buffer at 220g for 10 minutes at 18°C.

The cell separation MACS LS+ column (Miltenyi Biotech, Bergisch Gladbach, Germany) was washed 3 times with 1ml of cell separation buffer, avoiding air bubbles. The cells to be
separated were resuspended in 500μl of cell separation buffer per 10^8 cells and run through the column in 500μl aliquots and the supernatant collected. The column was washed through with 3mls of cell separation buffer and the supernatant collected in 12ml tubes (Greiner Bio-One, Stonehouse, Gloucester, UK). Cells that were collected in the supernatant were washed at 220g with excess cell separation buffer, resuspended in 1ml of RPMI (Gibco Life Technologies) + 10% AB serum (NBTS) and counted by a haemocytometer (Sigma).

White cell numbers were often high in CLL patients samples which can have much greater numbers of B cells than control samples, so it was necessary to titrate the amount of CD19 MACs (Miltenyi) beads required. In an attempt to achieve maximum purification of the isolated T cells, the above experiment was repeated, titrating the levels of CD19 beads at concentrations ranging from 30μl to 70μl per 10^7 total cells and staining with CD20 (Sigma) to evaluate B cells remaining after depletion. Total CD20 numbers were analysed using flow cytometry.

2.4.6 Permeabilisation methods

To evaluate the expression of intracellular CD25, CD28 and CD152, T cells were enriched as described in 2.4.5 and cultured as described in 2.4.3, with the addition that after time course and optimisation studies (described in 2.5.8), all cells were cultured for 48 hours and activated using OKT3. Intrastain reagents fix cell surface monoclonal antibodies and then permeabilise the cell membrane to allow for intracellular staining. After culture, cells were harvested from the 24-well, polystyrene, round bottom plates (Nunclon, Fisher Scientific) and 1 x 10^6 cells transferred to each of 6 clean, 5ml polystyrene tubes (Falcon) and washed twice in 1ml of PBS at room temperature. Cells were then stained with 10μl of antibody for negative control (equivalent mouse isotypic controls), Leucogate (lymphocyte
gating) and CD2 (T cell confirmation) as described in 2.2.2 and 2.4.1 for 15 minutes in the dark. 100µl of DAKO intrastain reagent A (DAKO, Ely, UK) was added to each tube and incubated for 15 minutes in the dark at room temperature. Cells were then washed twice in 2mls of PBS at 320g for 5 minutes.

100µl of DAKO intrastain reagent B (DAKO) was added to each tube and vortexed (Clifton Cyclone, Jencons Plc). Monoclonal antibodies for intracellular staining were added to the cells (CD25, CD28 and CD152, as described in 2.2.2) and left for 15 minutes in the dark at room temperature. Cells were then washed twice in 1ml PBS at 320g for 5 minutes, resuspended in 1ml PBS and analysed on the flow cytometer.

2.4.7 Expression of CD25, CD28 and CD152 in normal controls and CLL patients after B-cell depletion and culture with OKT3 and 50% CLL AB serum

T cells were enriched as described in 2.4.5 and prepared for culture with OKT3 (Janssen-Cilag) as described in 2.4.3. Cells were resuspended in RPMI (Gibco) containing either 50% CLL AB serum or 50% normal AB serum (NBTS) as described in 2.3.1 and cultured as described in 2.4.3 with the addition that after time course and optimisation studies (described in 2.5.8), all cells were cultured for 48 hours and activated using OKT3 (Janssen-Cilag). Cells were harvested at 48 hours and transferred from the 24-well, polystyrene plates (Nunclon, Fisher Scientific) into each of 6 clean 5ml polystyrene tubes (Falcon, Marathon Lab Supplies). 10µl of antibody for negative controls (equivalent mouse isotypic controls), Leucogate (lymphocyte gating), CD2 (T cell confirmation), CD2/CD25 (test), CD2/CD28 (test) and CD2/CD152 (test) (as described in 2.2.2 and 2.4.1) were added per 1 x 10^6 cells in 1ml and incubated for 15 minutes in the dark at room temperature. Cells were washed twice in 1ml of wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed in the flow cytometer.
A direct comparison was made between expression of CD25, CD28 and CD152 after culture in CLL AB serum or in normal AB serum.

2.4.8 Mixed lymphocyte reaction (MLR)

For responder cells, PBMCs were isolated and B cells depleted as described previously in T cell enrichment (2.4.5). For stimulator cells, PBMCs were isolated as described previously (2.4.2). Stimulator PBMCs were irradiated at 25Gy (Gammacell 1000 Cs-137 irradiator, Schering Health Care Ltd., Burgess Hill, West Sussex, UK) for 10.1 minutes, counted in a haemocytometer (Sigma) and resuspended in RPMI (Gibco) + 10% normal AB serum (NBTS). Responder cells were counted in a haemocytometer and resuspended in RPMI + 10% AB serum. 10^6 stimulator cells in 1ml were plated with 10^6 responder cells in 1ml in 24 well, polystyrene, flat bottom plates (Nunclon, Fisher Scientific) for the MLR. Control wells were set up containing responder cells only. Cells were cultured for 72 hours for IL-2 secretion and 96 hours for IFN-γ secretion for optimum cytokine expression. At the appropriate time points, the medium was harvested under sterile class II conditions and centrifuged for 5 minutes at 400g. Leaving the cells in the tubes, the supernatant was removed under sterile conditions and transferred to sterile 1.5ml tubes (Nalgene Cryovials, Nalgene) and stored at -20°C until required.

2.4.9 IL-2 ELISA

Supernatants were thawed and all reagents brought to room temperature before use. The wash buffer (R&D systems) was reconstituted by adding 20ml of concentrate to 480mls of deionised water. 5mls of the calibrator RD5 (R&D), for use with the cell supernatant, was added to the supplied IL-2 standard stock solution (R&D) to make a final concentration of 2000pg/ml of IL-2. This was left for a minimum of 15 minutes at room temperature with gentle agitation. 500μl of calibrator RD5 was added to each of 6, 5ml,
polystyrene tubes (Falcon, Marathon Lab Supplies) and to the first tube, 500µl of IL-2 standard was added. A dilution series was carried out, with 500µl of the solution from the preceding tube being added to the next tube, to produce 6 tubes containing 1000, 500, 250, 125, 62.5 and 31.2 pg/ml of IL-2 standard. The 120-well microplate (R&D) was removed from its foil pouch and 100µl of supplied assay diluent RDIA (R&D) was added to each well. 100µl of each of the IL-2 dilution series of standards was added in duplicate with 2 wells left for blanks.

All patient and control samples were set up in duplicate at a 1:2 and a 1:5 dilution. For the 1:2 dilutions, each well contained 100µl of supplied assay diluent (R&D) and 100µl of patient or control supernatant from the MLR. For the 1:5 dilutions, each well contained 160µl of assay diluent and 40µl of patient or control supernatant from the MLR. The whole plate was covered with an adhesive strip, incubated in air for 2 hours at room temperature and then washed 3 times in wash buffer in the autowasher (Kodak Amerlite, Kodak Clinical Diagnostics Ltd, Amersham, UK). The autowasher filled each well with 200µl of appropriate buffer and then subsequently removed it on each of the 3 cycles. The wells were left empty at the end of the three cycles.

After washing, 200µl of the IL-2 conjugate (R&D) was added to each well. The whole microplate was covered with a new adhesive strip and incubated for a further 2 hours at room temperature and then washed 3 times in wash buffer in the autowasher (Kodak Amerlite), as described previously. The colour reagents A and B (R&D) were added in equal concentrations to make the substrate solution to be used within 15 minutes of mixing. After washing the microplate, 200µl of this substrate solution was added to each well of the microplate and incubated for 20 minutes at room temperature in air, protected from light. 50µl of stop solution (R&D) was then added to each well and a uniform colour change obtained by gently tapping the plate. The optical density of each well was measured using a microplate reader (DIAS, Dynatech Laboratories, West Sussex, UK). The test
wavelength was set to 450nm and the correction wavelength to 540nm. Using this dual wavelength reading mode to measure the absorbance of the sample, reading errors caused by dirt, scratches or moisture on the base of the plate were eliminated.

Both normal and CLL supernatants were analysed on the same ELISA plates simultaneously to avoid any intra assay variation.

2.4.10 IFN-γ ELISA

The 120-well IFN-γ microplate (Eurogenetics UK Ltd) was removed from its foil pouch. 120μl of IFN-γ coating antibody (Eurogenetics) was added to 12mls of IFN-γ coating buffer (described in 2.3.3). 100μl of coating buffer plus coating antibody was then added to each well and incubated overnight at room temperature in air. The microplate was then washed 3 times in the autowasher (Kodak Amerlite), as described previously in 2.4.9, with wash buffer (described in 2.3.3). 500μl of blocking buffer (described in 2.3.3) was added to 25mls of working strength PBS and 200μl of the resulting solution was added to all wells, which were mixed by tapping the side of the microplate. The whole plate was then covered with an adhesive seal and incubated for 1 hour at room temperature in air.

500μl of distilled water was added to 5300pg/ml of IFN-γ standard lyophilised powder (Eurogenetics) and incubated for 10 minutes at room temperature in air, with gentle mixing. Another diluting series of standards was set up with 7, 5ml polystyrene tubes (Falcon, Marathon Lab Supplies), labelled 500, 200, 80, 32, 12.8, 5.1 and 2.0 pg/ml. The concentrated dilution buffer (Eurogenetics) was diluted 5 times in distilled water to produce working strength dilution buffer. 480μl of this working strength dilution buffer was added to the first tube labelled 500pg/ml and 300μl to each of the other 6 tubes. 50μl of the IFN-γ standard was added to the first tube labelled 500 pg/ml and 200μl of this solution was then transferred to the 200pg/ml (second) tube. The series was then completed by transferring 200μl from each preceding tube to the next.
The microplate was washed 3 times in wash buffer using the autowasher (Kodak Amerlite), as described previously. 100μl of each standard from the 7 tubes was added to the microplate in duplicate, leaving the blank wells empty. Samples from the MLR supernatant were added in duplicate at a 1:2 dilution and at a 1:5 dilution. For the 1:2 dilution, 200μl of the samples were pre-diluted with 200μl of working strength dilution buffer and for the 1:5 dilution, 80μl of the samples were pre-diluted in 320μl of working strength dilution buffer. 100μl of sample was added to each well. The whole microplate was covered with a new adhesive strip and incubated for 1 hour at room temperature in air. After 1 hour, the microplate was washed 3 times in wash buffer using the autowasher, as described previously. 120μl of biotinylated IFN-γ (Eurogenetics) was added to 12mls of working strength dilution buffer just before use and 100μl was added to each well, leaving the blank wells empty. The whole microplate was covered with a new adhesive strip and incubated for 1 hour at room temperature in air and then washed 3 times in the autowasher, as described previously.

Just prior to use, the concentrated streptavidin-HRP conjugate (Eurogentics) was removed from the -20°C freezers and 3μl was added to 30mls of working strength dilution buffer. 100μl of this solution was added to each well, leaving the blank wells empty. The whole microplate was covered with a new adhesive strip and incubated for 30 minutes at room temperature in air and then washed 3 times in the autowasher, as described previously.

The substrate solution (described in 2.3.3) was prepared within 10 minutes of use. 100μl was added to each well, including the blank wells. The plate was covered with a lid, gently tapped to mix and incubated for 30 minutes at room temperature in the dark.

100μl of stop solution was added to all wells and the plate read in the plate reader (DIAS, Dynatech Laboratories, West Sussex, UK). The test wavelength was set to 450nm and the correction wavelength to 540nm. Using this dual wavelength reading mode to measure the
absorbance of the sample, reading errors caused by dirt, scratches or moisture on the base of the plate were eliminated.

Both normal and CLL supernatants were analysed on the same ELISA plates simultaneously to avoid any intra assay variation.

2.4.11 MLR with IL-15

For responder cells, PBMCs were isolated and B cells depleted as described previously in 2.4.5. For stimulator cells, PBMCs were isolated as described in 2.4.2. Stimulator PBMCs were re-suspended in PBS, counted using a haemocytometer (Sigma) and irradiated at 25Gy (Gammacell 1000 Cs-137, CIS Radiopharmaceuticals, Schering Health Care Ltd, West Sussex, UK). After irradiation the stimulator cells were spun for 5 minutes at 320g and resuspended in RPMI (Gibco) plus 10% normal AB serum (NBTS). After T cell enrichment, responder cells were re-suspended in RPMI plus 10% AB serum and counted using a haemocytometer. $10^6$ stimulator cells in 1ml RPMI plus 10% AB serum were plated with $10^6$ responder cells in 1ml RPMI plus 10% AB serum in 3 wells of a 24 well polystyrene plate (Nunclon, Fisher Scientific UK) for the MLR. Corresponding controls were set up in 3 wells of a separate 24-well plate containing $10^6$ responder cells in 1ml RPMI plus 10% AB serum only. Interleukin-15 (IL-15) (Cambridge Bioscience, Cambridge, UK) was added to the first MLR and control wells at concentration of 10ng/ml, to the second MLR and control well at 1ng/ml and the final MLR and control wells were left with no addition of IL-15. For IL-2 secretion, cells were cultured for 72 hours in an incubator at 37°C and 5% CO$_2$. After 72h, the medium was harvested, transferred to 6 separate 5ml, polystyrene tubes (Falcon) and centrifuged for 5 minutes at 400g. Leaving the cells in the tubes, the supernatant was removed and transferred to 1.5ml tubes (Greiner Bio-One, Stonehouse, Gloucester, UK) and stored at -20°C until required.
Supernatants were thawed when required and evaluated for IL-2 secretion, as described previously in 2.4.9. Supernatants were only frozen and thawed once.

2.4.12 Expression of CD25, CD28 and CD152 after OKT3 stimulation, IL-15 and permeabilisation.

T cells were enriched as described in 2.4.5 and cell cultures with OKT3 (Janssen-Cilag) prepared as described in 2.4.3. 6 wells of a 24 well, polystyrene plate (Nunclon, Fisher Scientific UK) were set up with OKT3. 6 wells were set up as controls with no OKT3. After T cell enrichment, cells were re-suspended in RPMI (Gibco) plus 10% normal AB serum (NBTS) and plated on 12 wells of a 24 well plate at $10^6$ per well in 1ml. IL-15 (Cambridge Bioscience) was added to the first four wells of the 24 well plate at a concentration of 10ng/ml, to the second four wells at a concentration of 1ng/ml and the last four wells were left without IL-15. Cells were cultured as described in 2.4.3 with the addition that, after time course and optimisation studies (described in 2.5.8), all cells were cultured for 48 hours and activated using OKT3. To half the cells were added 10µl of antibody for negative control (equivalent mouse isotypic controls), Leucogate (lymphocyte gating), CD2 (T cell confirmation), CD2/CD25 (test), CD2/CD28 (test) and CD2/CD152 (test) (as described in 2.2.2 and 2.4.1) per $10^6$ cells, incubated for 15 minutes in the dark in air and at room temperature, washed twice in 1ml of wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed in the flow cytometer.

The remaining cells were permeabilised and stained for intracellular antigens as described previously in 2.4.6.
2.5 OPTIMISATION METHODS

A series of experiments were carried out to establish optimum time points for each of the cell surface markers studies and for culture with OKT3. Although the majority of these data have been previously demonstrated, as indicated by references in each subsection, it was important to verify them to ensure accurate comparisons between normal and CLL samples. Direct comparisons were also made between the different methods for cell separation and cell permeabilisation. CLL cells are known to be more fragile and it was therefore important to establish a suitable protocol for both normal and CLL cells.

Where appropriate, data was analysed using means plus coefficient of variation (cv). Cv is expressed as a percentage and has been used to assess sampling methods and the spread of data within each test.

2.5.1 Time course for OKT3 adherence to culture plates

Under sterile class II conditions, OKT3 (Janssen-Cillag) was added to PBS to a final concentration of 1mg/ml. 500μl was plated on 1 well in each of 6 separate 24 well, polystyrene culture plates (Nunclon, Fisher Scientific UK). Half of these were left at room temperature and half at 4°C, so that each time point was studied at two different temperatures. 500μl of PBS only was also plated into a separate well of each of these six 24 well plates as controls. OKT3 was left to adhere for either 2, 16 or 24 hours. After each relevant time point, the appropriate well was washed 3 times in 1ml of PBS. PBMCs were isolated as described previously in 2.4.2 and were added to both the OKT3 and control wells in each of the 24 well plates at a concentration of $10^6$ in RPMI (Gibco) plus 10% AB serum (NBTS) and cultured for 4 hours in an incubator at 37°C in 5% CO₂ in air. After culture, medium and cells were harvested, transferred to clean 5ml, polystyrene tubes (Falcon, Marathon Lab Supplies) then centrifuged at 220g for 5 minutes and the supernatant removed. 1ml wash buffer was added to each tube and cells were washed for 5
minutes at 220g. 10μl of antibody for negative controls (equivalent mouse isotypic controls), Leucogate (lymphocyte gating), CD2 (T cell confirmation) and CD2/CD154 (test) (as described in 2.2.2 and 2.4.1) was added to 10^6 cells and incubated for 15 minutes in the dark at room temperature in air. Only normals' cells were used. Cells were then washed twice in 1ml wash buffer at 220g for 5 minutes, resuspended in 1ml PBS and analysed using flow cytometry. Results for CD2/CD154 only are shown in the results section (table 12). Other control data not shown in the results sections are available outside the thesis.

The optimum time scale for leaving OKT3 binding to culture plates could be determined by the expression of the activation marker, CD154, after culture with each OKT3 plate, as described in section 3.1. CD154 was selected as the activation marker as it is up-regulated in a relatively short period of time and also because it was a marker to be studied.

2.5.2 The use of CD2 or CD3 for T cell isolation following OKT3 activation

As previous studies show that OKT3 binds to the CD3 receptor (Bonnefoy-Berard N and Revillard JP 1996; Broders N et al 1998; Brusa P et al 1998), it was important to determine whether CD2 might detect a significantly different number of T cells compared to CD3 after OKT3 activation.

For this optimisation study, 4 normal subjects were used. Under sterile class II conditions, OKT3 (Janssen-Cilag) was added to PBS at a final concentration of 1mg/ml. 500μl was plated into four wells of a 24 well, polystyrene plate (Nunclon, Fisher Scientific UK) for each normal subject analysed. In addition, another four wells containing 500μl of PBS only were set up as controls on the same plate for each individual analysed. Each time a subject or subjects were studied, a single plate was left for 2 hours in air at room temperature for the OKT3 to adhere to the wells. PBMCs were isolated from normals only as described
previously in 2.4.2, re-suspended in RPMI (Gibco) plus 10% AB serum (NBTS) and plated into both the OKT3 and the control wells at a final concentration of $10^6$ cells in 1ml. The plate was then cultured in the incubator for 4 hours at 37°C in 5% CO$_2$ in air. After culture, cells and medium were harvested and transferred to clean 5ml, polystyrene tubes (Falcon, Marathon Lab Supplies), centrifuged at 220g for 5 minutes and the supernatant removed. 1ml wash buffer was added to each tube and cells were washed for 5 minutes at 220g. A direct comparison was made between the binding of CD2 and CD3 after culture with OKT3. 10µl of appropriate monoclonal antibodies, consisting of negative controls (equivalent mouse isotypic antibodies), Leucogate (lymphocyte gating) and CD2 (T cell numbers) (as described in 2.2.2 and 2.4.1) were added to $10^6$ cells in the first experiment. 10µl of appropriate monoclonal antibodies, consisting of negative control, Leucogate and CD3, were added to $10^6$ cells in the second experiment. All cells were incubated for 15 minutes in the dark at room temperature in air. Cells were then washed twice in 1ml wash buffer at 220g for 5 minutes, resuspended in 1ml PBS and analysed using flow cytometry. Results for CD2 and CD3 only are shown in the results section (table 13). Other control data not shown in the results section are available outside the thesis.

A comparison could be made between monoclonal antibody binding of CD2 to the CD2 receptor and monoclonal antibody binding of CD3 to the CD3 receptor after 4h incubation with OKT3 (anti-CD3), as described in more detail in section 3.2.

2.5.3 Time course for expression of CD154

These experiments were performed to confirm previous findings (Foy TM et al 1994; Clark LB et al 1996; Cantwell M et al 1997) for expression of CD154 under these experimental conditions.
Two normal controls were analysed for this optimisation study. Under sterile class II conditions, OKT3 (Janssen-Cilag) was added to PBS at a final concentration of 1mg/ml. For each normal control studied, 500μl of OKT3 was plated into six wells of a 24 well, polystyrene plate (Nunclon, Fisher Scientific UK). Six wells containing 500μl of PBS only were set up as controls on the same plate. The plate was left for 2 hours for the OKT3 to adhere to the wells. From each normal, PBMCs were isolated as described previously in 2.4.2, re-suspended in RPMI (Gibco) plus 10% AB serum (NBTS) and then plated into six of both the OKT3 and the control wells at a final concentration of 10^6 cells in 1ml. The plate was cultured in the incubator for 2, 4 and 6 hours at 37°C in 5% CO₂ in air. Two wells of OKT3 (activated) and 2 wells of control (PBS) cells were harvested at each time point, transferred to clean 5ml, polystyrene tubes (Falcon) and washed twice in 1ml wash buffer for 5 minutes at 220g. Cells from both OKT3 and control tubes were then divided into four tubes and monoclonal antibodies to stain for negative controls (equivalent mouse isotypic antibodies), Leucogate (lymphocyte gating), CD2 (T cell confirmation) and CD2/CD154 (test) (as described in 2.2.2 and 2.4.1) were added at 10μl per 10^6 cells and incubated for 15 minutes in the dark at room temperature in air. PBMCs were then washed twice in wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed using flow cytometry. Only the CD2/CD154 data are shown in the results (table 14). Control data not shown in the results section are available outside the thesis.

The optimum time point for expression of CD154 was determined, as described in section 3.3.

2.5.4 Time course for expression of CD25, CD28 and CD152

These experiments were performed to confirm previous findings (Waldmann TA 1986; Waldmann TA 1991; Lindsten T et al 1993; Walunas TL et al 1994; Krummel MF and
Allison P 1995; Alegre ML et al 1996) for expression of CD25, CD28 and CD154 under these experimental conditions.

Two normal controls were analysed. Under sterile class II conditions, OKT3 (Janssen-Cilag) was added to PBS at a final concentration of 1mg/ml. 500µl was plated into 6 wells of 3 separate 24 well, polystyrene plates (Nunclon, Fisher Scientific UK). 6 wells containing 500µl of PBS only were set up as controls on each of the three 24 well plates. All 3 plates were left for 2 hours at room temperature in air for the OKT3 to adhere to the wells. PBMCs were isolated as described previously in 2.4.2, re-suspended in RPMI (Gibco) plus 10% AB serum (NBTS) and plated into both the OKT3 plates and the control plates at a final concentration of 10^6 cells in 1ml in each well. One 24 well plate was cultured in the incubator for each of the time intervals, 24, 48 and 72 hours, at 37°C in 5% CO₂ in air. Cells were harvested from the 24 well plates at each time point, transferred to clean 5ml, polystyrene tubes (Falcon, Marathon Lab Supplies) and washed twice in 1ml wash buffer for 5 minutes at 220g. Monoclonal antibodies for negative controls (equivalent mouse isotypic controls), Leucogate (lymphocyte gating) CD2 (T cell confirmation), CD2/CD25 (test), CD2/CD28 (test) and CD2/CD152 (test) (as described in 2.2.2 and 2.4.1) were added at 10µl per 10^6 cells and incubated for 15 minutes in the dark at room temperature in air. PBMCs were then washed twice in wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed using flow cytometry. Results for CD2/CD25, CD2/CD28 and CD2/CD152 only are shown in the results section (table 15). Other control data not shown in the results section are available outside the thesis.

The time points for optimum expression of surface CD25, CD28 and CD152 were determined, as described in section 3.4.
2.5.5 Time course for expression of HLA-DR

These experiments were performed to confirm expression of HLA-DR under these experimental conditions.

One normal control was analysed. Under sterile class II conditions, OKT3 (Janssen-Cilag) was added to PBS at a final concentration of 1mg/ml. 500μl was plated on 6 wells of a 24 well, polystyrene plate (Nunclon, Fisher Scientific UK). Six wells containing 500μl of PBS only were set up as controls. The plate was left for 2 hours for the OKT3 to adhere to the wells. PBMCs were isolated as described previously in 2.4.2, re-suspended in RPMI (Gibco) plus 10% AB serum (NBTS) and plated into both the OKT3 and the control wells at a final concentration of $10^6$ cells in 1ml. The plate was cultured in the incubator at 37°C in 5% CO₂ in air. Under sterile conditions, cells from two wells of the OKT3 and control samples were harvested from the 24 well plate at each time point (24, 48 and 72 hours) transferred to clean 5ml, polystyrene tubes (Falcon, Marathon Lab Supplies) and washed twice in 1ml wash buffer for 5 minutes at 220g. Monoclonal antibodies for negative controls (equivalent mouse isotypic controls), Leucogate (lymphocyte gating), CD3 (T cell confirmation) and CD3/HLA-DR (test) (as described in 2.2.2 and 2.4.1) were added at 10μl per $10^6$ cells and incubated for 15 minutes in the dark at room temperature in air. PBMCs were then washed twice in wash buffer for 5 minutes at 220g, resuspended in 1ml PBS and analysed using flow cytometry. Results for CD2/HLA-DR only are shown in the results section (table 16). Other control data not shown in the results section are available outside the thesis.

The time point for optimum expression of HLA-DR was determined, as described in section 3.5.
2.5.6 Comparison of B-cell depletion methods

Two different methods of B and T cell separation were evaluated. A comparison was made between these two different methods of T cell purification for the most effective way of removing the large numbers of B cells from CLL patients' blood samples and obtaining a pure T cell population.

In addition to the MACs method of T cell purification, described in 2.4.5, Dynabeads were also evaluated, as described below:

Dynabeads

The required amount of Dynabeads (Dynal Biotech Ltd, Bromborough, Wirral, UK.) for isolating $10^6$ cells was

- 320μl / 4mls whole washed blood for CD4 cells and
- 200μl / 4mls whole washed blood for CD8 cells

Dynabead protocol

Under sterile class II conditions, the required amounts of CD4 and CD8 beads were placed in 12ml tubes (Greiner Bio-One) and put into the magnetic particle concentrator (MPC) for 60 seconds. The fluid was pipetted off and discarded. The 12ml tube was removed from the MPC and washed in excess PBS + 0.3% BSA (Lorne Laboratories, Reading, UK).

The washing procedure was then repeated. The washed Dynabeads were resuspended in a volume of PBS/BSA equal to that which was originally pipetted from the 12ml tube. Blood was drawn into sodium heparin tubes, transferred to 20ml sterile tubes (Greiner Bio-One) and washed in an equal volume of PBS. The washed Dynabeads were added to 4 mls of the sodium-heparin anticoagulated blood pre-washed in PBS, ensuring that the ratio of beads to target cells was now at 10:1. All samples and buffers were kept cold during the entire procedure by keeping on ice. Blood was incubated for 60 minutes at 4°C, tilting and
rotating continuously. The tube containing the blood and Dynabeads together was placed in the MPC for 3 minutes, drawing the rosetted cells to the side of the tube. While the tube remained on the MPC, the supernatant was pipetted off and discarded. The tube was removed from the MPC. The CD4 and CD8 cells rosetted around the Dynabeads were then resuspended in PBS/BSA.

**Detachabead protocol**

20μl of Detachabeads (Dynal Biotech Ltd) were added to the rosetted cells/Dynabeads in the same 12ml tube (Greiner-Bio-One) and incubated for 45 minutes at room temperature in air, keeping the sample tilting and rotating. The 12ml tube was then placed in the MPC for 3 minutes, drawing the Dynabeads and Detachabeads only to the side of the 12ml tube and leaving the CD4 and CD8 cells in the suspension. The supernatant containing these CD4 and CD8 cells was then pipetted out of the tube and placed in a fresh sterile 12ml container (Greiner Bio-One). The tube containing the Detachabeads and Dynabeads was then removed from the MPC and washed 2-3 times in PBS + 0.3% BSA (Lorne Laboratories). This released any remaining CD4 and CD8 cells from the Dynabeads. The tube was placed back on the MPC and again, the suspension containing the CD4 and CD8 cells was pipetted off and retained. The cell suspensions containing the CD4 and CD8 T cells were combined and washed 2-3 times to remove any remaining Detachabeads. The cells were resuspended in a volume of RPMI (Gibco) plus 10% AB serum (NBTS) to equal 10^6 cells/ml.
2.5.7 Comparison of cell permeabilisation methods - paraformaldehyde and saponin vs DAKO intrastain kit

In addition to the DAKO intrastain method of cell permeabilisation (as described in 2.4.6), a second method was also evaluated as described below.

Paraformaldehyde and Saponin

After culture as described in 2.4.3 for 48 hrs, cells were harvested from the 24 well, polystyrene plates (Nunclon, Fisher Scientific UK), transferred to clean 5ml, polystyrene tubes (Falcon, Marathon Lab Supplies) and resuspended in 100μl of 4% fixation buffer (PBS + 4% paraformaldehyde, (Sigma)) for 15 minutes at room temperature in air. Cells were stained with monoclonal antibodies for negative controls (equivalent mouse isotypic controls), Leucogate (lymphocyte gating) and CD2 (test) (as described in 2.2.2 and 2.4.1) and incubated for 15 minutes in the dark at room temperature.

Cells were then washed twice in 1ml permeabilisation buffer (PBS + 10% AB serum + 0.1% saponin (Sigma)) for 5 minutes at 320g. PBMCs were resuspended in 100μl of permeabilisation buffer and incubated for 1 hour in the dark at room temperature in air. Appropriate antibodies for intracellular analysis (CD25) were added at 10μl per 10⁶ total cells and incubated for 15 minutes in the dark at room temperature in air. Cells were washed twice in 1ml staining buffer for 5 minutes at 160g, resuspended in 1ml PBS and analysed using flow cytometry.

A comparison was made between the two methods of permeabilisation based upon cell structure, intact cell membranes and expression of intracellular and surface antigens using the flow cytometer, as described in section 3.7.
2.5.8 Time course for expression of CD25, CD28 and CD152 after B-cell depletion and permeabilisation

As no data were found in the literature on the time course of expression of these markers intracellularly, the following optimisation experiments were conducted.

Under sterile class II conditions, OKT3 (Janssen-Cilag) was added to PBS at a final concentration of 1 mg/ml. 500µl was plated on 6 wells on three 24 well, polystyrene plates (Nunclon, Fisher Scientific UK). 6 wells containing 500µl of PBS only were set up as controls on each of the 24 well plates. Each plate was left for 2 hours for the OKT3 to adhere to the wells. PBMCs were isolated as described previously in 2.4.2, re-suspended in RPMI (Gibco) plus 10% AB serum (NBTS) and plated on both the OKT3 plates and the control plates at a final concentration of $10^6$ cells in 1ml. 24 well plates were cultured in the incubator for 24, 48 and 72 hours at 37°C in 5% CO₂. Cells were harvested from the 24 well plate at each time point, transferred to clean 5ml, polystyrene tubes (Falcon, Marathon Lab Supplies) and washed twice in 1ml wash buffer for 5 minutes at 220g. At each time point, cells were fixed, stained and permeabilised, resuspended in 1ml PBS and analysed using flow cytometry as described in 2.4.1. Only results for CD2/CD25, CD2/CD28 and CD2/CD152 are shown in the results section (table 17). Other control data not shown in the results section are available outside the thesis.

The optimum times for intracellular expression of CD25, CD28 and CD152 were determined, as described in section 3.8.
CHAPTER THREE: RESULTS OPTIMISATION

3.0 Results of optimisation studies

3.1 Time course for OKT3 adherence to culture plates

A time course study was carried out to establish the most effective time and temperature to achieve maximum adherence of OKT3 on 24 well culture plates, measured by expression of CD154 on T cells after each time point. OKT3 was plated for 2 hours, 16 hours and 24 hours, left at room temperature or at 4°C, washed and cells cultured for 4 hours for expression of CD154 as described in 2.5.1 (Table 12). As the time for OKT3 adherence increased from 2 hours through to 24 hours, total CD154 expression decreased (2 hours = 26.1%, 16 hours = 20.3% and 24 hours = 15.9%). Statistical analysis was not carried out on these figures as data were required from one individual experiment only to verify previous routine laboratory protocols. Plates left to adhere at 4°C produced less CD154 expression compared to plates left at room temperature. The optimum plating time for maximum OKT3 adherence was 2 hours at room temperature, as this produced the highest proportion of CD154 expressing cells. This suggests that if OKT3 is left on the plates for longer than 2 hours, the antibody will start to disassociate from the plate and will therefore be unable to stimulate as many T cells.

Table 12: Optimal coating conditions for T cell stimulation as determined by expression of CD154 over a time period and at different temperatures

<table>
<thead>
<tr>
<th>Time point for plating OKT3</th>
<th>OKT3 room temperature</th>
<th>OKT3 4°C</th>
<th>Control room temperature</th>
<th>Control 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hours</td>
<td>26.1%</td>
<td>17.8%</td>
<td>0.9%</td>
<td>1.2%</td>
</tr>
<tr>
<td>16 hours</td>
<td>20.3%</td>
<td>17.5%</td>
<td>2.6%</td>
<td>3.6%</td>
</tr>
<tr>
<td>24 hours</td>
<td>15.9%</td>
<td>12.6%</td>
<td>0.8%</td>
<td>1.0%</td>
</tr>
</tbody>
</table>

Table showing expression of CD154 after adherence of OKT3 to 24-well plates over a 2 to 16 hour time period and at room temperature or 4°C. Corresponding controls of PBS only were set up on the same plates to ensure CD154 expression was a result of OKT3 stimulation.
3.2 CD2/CD3 for identification of T cell populations after OKT3 stimulation

The monoclonal antibodies directed against the T cell markers CD2 and CD3 were studied on the flow cytometer of PBMCs from 4 normal subjects to determine whether they detected similar numbers of T cells following activation with the anti-CD3 monoclonal OKT3 as described in 2.5.2. As previous studies show that OKT3 binds to the CD3 receptor (Bonnefoy-Berard N and Revillard JP 1996; Broders N et al 1998; Brusa P et al 1998), it was important to determine whether CD2 might detect a significantly different number of T cells compared to CD3 after OKT3 activation. The total number of cells studied was the same for CD2 and CD3. CD3 counts were always lower than CD2 counts in each normal subject (table 13) and variation about the mean was low. Following this finding, CD2 was used in all subsequent experiments as the pan T cell marker where OKT3 was used as the mitogen.

However, it should be noted that another reason why CD2 numbers may be consistently higher than CD3 numbers is the presence of Natural Killer (NK) cells. NK cells also express CD2 and small numbers of them may have been included within the CD2 counts. Although CD3 binding appears to be affected by pre-incubation with OKT3, T cell numbers may not be accurate following CD3 binding as the inclusion of NK cells within the CD2 population cannot be excluded. This could potentially have been ruled out using two different methods. A comparison could have been made between CD3^{+ve} T cells in OKT3 stimulated and control populations vs CD2^{+ve}/CD56^{+ve} in OKT3 and control populations, to exclude the presence of NK cells. Additionally, measuring relative intensity of CD3 vs CD2 expression pre and post OKT3 stimulation would allow occupation of CD3 sites by OKT3 to be identified. However, at the time of testing, measurement of relative intensity was not a routine procedure and was not carried out.

Although the possibility of NK cell inclusion is possible, the majority of key markers studied in this thesis are not known to be expressed on the surface of NK cells.
Table 13: Surface staining of CD2 and CD3 after 4 hours activation with OKT3

<table>
<thead>
<tr>
<th>T cell marker</th>
<th>4hrs incubation with OKT3 (mean) (n=4)</th>
<th>cv</th>
<th>4hrs incubation with PBS control (mean) (n=4)</th>
<th>cv</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>83.1%</td>
<td>6%</td>
<td>85.8%</td>
<td>10%</td>
</tr>
<tr>
<td>CD3</td>
<td>71.3%</td>
<td>10%</td>
<td>77.5%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Table showing the difference in expression of the monoclonal antibodies CD2 and CD3 after 4 hours incubation with OKT3 or 4 hours incubation with PBS alone as control (described in 2.5.1).

cv = coefficient of variation
3.3 Time course for optimal expression of CD154

A time course study was done to determine the time of maximum expression of CD154 over a six hour period as described in 2.5.3. CD154 expression was determined at shown intervals by flow cytometry of OKT3 stimulated PBMCs from two normal subjects. In both, maximum expression (mean = 19.3%) was obtained between 4 and 6 hours (table 14). In unstimulated PBMCs, there was consistently very low CD154 expression at all times. Statistical analysis (cv) shows method performance to be good for the stimulated T cell populations, as variation around the mean is low. Previous findings also showed maximum expression of CD154 after 4-6 hours of activation (Foy TM et al 1994; Clark LB et al 1996; Cantwell M et al 1997).

Table 14: Time course for optimal expression of CD154

<table>
<thead>
<tr>
<th>Time point</th>
<th>OKT3 activation (mean) (n=2)</th>
<th>cv</th>
<th>Control (mean) (n=2)</th>
<th>cv</th>
</tr>
</thead>
<tbody>
<tr>
<td>2hrs</td>
<td>9.4%</td>
<td>10%</td>
<td>0.25%</td>
<td>20%</td>
</tr>
<tr>
<td>4hrs</td>
<td>19.3%</td>
<td>10%</td>
<td>0.25%</td>
<td>20%</td>
</tr>
<tr>
<td>6hrs</td>
<td>18.5%</td>
<td>10%</td>
<td>0.1%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table showing expression of CD154 over a 6 hour time period with OKT3 activation. Corresponding controls of PBS only were set up on the same plates to ensure CD154 expression was a result of OKT3 stimulation.

cv = coefficient of variation
3.4 Time course for optimal expression of surface CD25, CD28 and CD152

A time course study was set up to determine the maximum expression of surface CD25, CD28 and CD152 over a 72 hour period at intervals of 24 hours as described in 2.5.4 (table 15). In 2 normal controls, maximum expression of CD25 occurred at 48 hours, CD28 was equally highly expressed at each time point and CD152 was expressed maximally at 48 hours, but CD152 expression was consistently much lower than either CD25 or CD28. Statistical analysis (mean + cv) shows a variable cv, suggesting a variation of expression within normal controls, although number of controls used is low. Previous findings have shown surface expression of CD25, CD28 and CD152 at the same time points (Waldmann TA 1986; Waldmann TA 1991; Lindsten T et al 1993; Walunas TL et al 1994; Krummel MF and Allison P 1995; Alegre ML et al 1996).

**Table 15: Time course for optimal expression of surface CD25, CD28 and CD152**

<table>
<thead>
<tr>
<th>Time point</th>
<th>CD25 (mean) (n=2)</th>
<th>cv</th>
<th>CD28 (mean) (n=2)</th>
<th>cv</th>
<th>CD152 (mean) (n=2)</th>
<th>cv</th>
</tr>
</thead>
<tbody>
<tr>
<td>24hrs</td>
<td>48.9%</td>
<td>40%</td>
<td>88.8%</td>
<td>8%</td>
<td>7.3%</td>
<td>30%</td>
</tr>
<tr>
<td>48hrs</td>
<td>80.5%</td>
<td>10%</td>
<td>90.2%</td>
<td>30%</td>
<td>16.7%</td>
<td>10%</td>
</tr>
<tr>
<td>72hrs</td>
<td>61.6%</td>
<td>30%</td>
<td>93.5%</td>
<td>6%</td>
<td>6.5%</td>
<td>30%</td>
</tr>
</tbody>
</table>

Table showing surface expression of CD25, CD28 and CD152 after OKT3 activation for 24, 48 and 72 hours in two normal controls.

cv = coefficient of variation
3.5 Time course for optimal expression of HLA-DR

A time course study was carried out to identify the time point for optimal expression of HLA-DR as described in 2.5.5. Results showed that over a 72 hour period of activation with OKT3, maximum HLA-DR expression occurred at 48 hours, but did not exceed 20% (table 16). Statistical analysis was not carried out, as this data was verification of previous findings that have shown surface expression of HLA-DR at the same time point (Griffin JD et al 1985; Griffiths CE et al 1989; Fratazzi C and Carini C 1997).

Table 16: Time course for optimal expression of HLA-DR

<table>
<thead>
<tr>
<th>Time point</th>
<th>HLA-DR expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hrs</td>
<td>10.0%</td>
</tr>
<tr>
<td>48 hrs</td>
<td>18.9%</td>
</tr>
<tr>
<td>72 hrs</td>
<td>4.5%</td>
</tr>
</tbody>
</table>

Table showing surface expression of HLA-DR after OKT3 activation for 24, 48 and 72 hours in one normal control.
3.6 Comparison of B-cell depletion methods

A study was carried out to determine the most efficient method of depleting the large number of B cells from CLL patients as described in 2.5.6. Dynabeads and MACS beads were both initially evaluated for T cell enrichment using flow cytometry to analyse the cell population remaining after B cell depletion. Studies with Dynabeads gave an unsatisfactory result, with the presence of a large number of debris cells. Results with MACS beads gave a cleaner population of cells in the flow cytometer (Figure 12) compared to Dynabeads (Figure 13). Mean CD20 count after B cell depletion with MACS was 3.2% for CLL patients (n=4) and 2.4% for normal controls (n=4) after titration experiments for the optimum amount of beads required to deplete the B cells. CD20 was used to evaluate remaining B cells as CD19 always gave a negative result. This was due to the CD19 beads used for depletion occupying the CD19 receptor site on the B cell surface preventing CD19 monoclonal antibody binding. The MACS method was used for all subsequent T cell enrichment experiments.

However, it must be pointed out that the two methods of cell separation, whilst both removing B cells and leaving an untouched T cell population, have important differences in their methods that may explain why the flow cytometry results are different. Dynabead cell separation starts with whole blood, whilst MACs cell separation begins with a sample that has already had red blood cells and other possible contaminating cells removed during the lymphoprepping procedure. As a result of this difference, there will be a proportionately greater number of contaminating cells and debris (ie red blood cells and neutrophils) within the Dynabead separated cell populations compared to the MACs separated cell populations and this fact must not be ignored when selecting a suitable method for B cell removal and T cell enrichment.
Figure 11: Flow cytometry dot plot showing cell population after B cell depletion using CD19 MACS beads

Figure showing a forward scatter/side scatter dot plot of PBMCs after depletion of CD19+ve cells with MACS beads. Note the clean and evenly distributed cell populations with very little debris.
Figure 12: Flow cytometry dot plot showing cell population after B cell depletion using CD19 Dynabeads.

Figure showing a forward scatter/side scatter dot plot of PBMCs after depletion of CD19+ve cells with Dynabeads. Note the large amount of cell debris in the bottom left hand corner.
3.7 Comparison of cell permeabilisation methods

A study was carried out to determine the most effective way of fixing cell surface markers and permeabilising the cell to analyse intracellular CD25, CD28 and CD152 expression as described in 2.5.7. The first method using paraformaldehyde and saponin as described in 3.6 did not give a satisfactory result when analysed on the flow cytometer for cell structure and the expression of both intracellular and surface antigens. In comparison, the DAKO intrastain method described in 2.4.6 gave a good result on the flow cytometer with an intact cell structure and expression of both intracellular and surface antigens. The DAKO intrastain method was used for all subsequent cell permeabilisation experiments.
3.8 Time course for optimal expression of CD25, CD28 and CD152 on stimulated T cells after B cell depletion and permeabilisation

A time course study was carried out on one normal control to identify the time point for maximum expression of CD25, CD28 and CD152 after B cell depletion, OKT3 stimulation and permeabilisation over a 48 hour time period at 24 hour intervals as described in 2.5.8 (table 17). Expression of intracellular CD25 and CD152 was highest at 48 hours (CD25 = 64.2%, CD152 = 71.3%). Levels of intracellular CD152 were much higher than expression on the cell surface at 48 hours and rose steadily over the time course (CD152 surface expression = 8.6%, CD152 intracellular expression = 71.3%). Levels of intracellular CD28 did not change significantly over the 48 hour time period (0hrs = 41.3%, 24hrs = 31.9%, 48hrs = 54.1%). All subsequent cell permeabilisation experiments for analysis of CD25, CD28 and for CD152 were cultured for 48 hours in OKT3 before permeabilisation.

Table 17: Time course for expression of CD25, CD28 and CD152 on stimulated T cells permeabilised and non-permeabilised.

<table>
<thead>
<tr>
<th>Time point</th>
<th>CD25</th>
<th>CD28</th>
<th>CD152</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hrs permeabilised</td>
<td>4.9%</td>
<td>41.3%</td>
<td>7.0%</td>
</tr>
<tr>
<td>0 hrs non-permeabilised</td>
<td>4.5%</td>
<td>52.9%</td>
<td>0.9%</td>
</tr>
<tr>
<td>24 hrs permeabilised</td>
<td>25.4%</td>
<td>31.9%</td>
<td>27.9%</td>
</tr>
<tr>
<td>24 hrs non-permeabilised</td>
<td>47.8%</td>
<td>62.1%</td>
<td>3.3%</td>
</tr>
<tr>
<td>48 hrs permeabilised</td>
<td>64.2%</td>
<td>54.1%</td>
<td>71.3%</td>
</tr>
<tr>
<td>48 hrs non-permeabilised</td>
<td>73.5%</td>
<td>58.5%</td>
<td>8.6%</td>
</tr>
</tbody>
</table>

Table showing surface and intracellular expression of CD25, CD28 and CD152 after OKT3 activation for 0, 24 and 48 hours in one normal control. A comparison is made at each time point between the permeabilised (intracellular) and non-permeabilised (surface) expression of each antigen.

149
Optimisation - summary of results

The results from these experiments enabled parameters to be set for the culture and activation of T cells and the subsequent B cell depletion and T cell permeabilisation for intracellular studies. The results showed that the optimum time point for plating OKT3 prior to PBMC or T cell culture was two hours and that after OKT3 culture, CD2 was the most efficient monoclonal for correctly identifying total T cell numbers. Time course studies showed that CD154 was optimally expressed at 4 hours, whilst CD25, CD152 and HLA-DR had optimum expression at 48 hours. CD28 was generally expressed at similar levels at all time points and could therefore be investigated at any time point.

B cell depletion experiments showed that for this study involving the depletion of large numbers of B cells, leaving the T cells untouched, the MACS system was most effective compared to Dynabeads. This does not imply that the Dynabead system is ineffective, simply that for the purpose of this research, MACS was more appropriate and gave a cleaner and better defined untouched T cell population. In comparison, the DAKO system for cellular permeabilisation also gave a better result when compared to using saponin and paraformaldehyde. Cell integrity was retained with the DAKO method, as shown by direct comparisons between permeabilised and non-permeabilised cells during forward scatter and side scatter analysis on the flow cytometer.

Time course experiments for intracellular expression of CD25, CD28 and CD152 showed that, in keeping with earlier experiments, CD25 and CD152 were optimally expressed at 48 hours and CD28 was continually expressed at all time points.

From the results obtained in these studies, it was then possible to begin experiments studying expression of cell surface and intracellular markers in CLL and normal T cells.
CHAPTER FOUR: RESULTS - SURFACE MARKERS

All statistical analysis was carried out using the Mann-Whitney (Wilcoxon) comparison of medians. The number of patients used in each group was too small to assess normality and as a result, the non-parametric Mann-Whitney test has been used throughout.

4.0 Expression of activation and interaction markers in CLL patients and normals

4.1 Expression of CD25, CD28, CD152, TCRαβ, CD4, CD5, CD8 and HLA-DR on T cells from B-CLL patients and normals

Significantly fewer T cells from B-CLL patients (n=10) compared to normals (n=7) expressed the markers CD25 (IL-2R) (23.1% v 80.8%: p= 0.007), CD28 (52.8% v 84.5%: p= 0.01) and CD152 (CTLA-4) (0.0% v 16.5%: p=0.001) after stimulation described in 2.4.3. Fifty percent of B-CLL patients failed to express any surface CD152 following OKT3 activation (range 0% - 7.5%), whilst the percentages of CD2+ve T-cells expressing CD25 after activation varied greatly between patients (range 1.8% - 91.2%) (Table 18) (Figure 13).

In unstimulated T cells from patients with B-CLL (n=7) compared to normals (n=5) described in 2.4.4, there was a significant reduction of the percentages expressing the cell surface markers CD4 (54.8% v 70.2%: p=0.03) and CD5 (83.3% v 98.5%: p=0.05), with a wide range of expression between patients (Table 18).

Percentages of unstimulated CD2+ve cells expressing CD8 and TCRαβ, as described in 2.4.4, were reduced, but not significantly (Table 19).

Greater numbers of stimulated CD2+ve cells from CLL patients (n=4), as described in 2.4.3, expressed HLA-DR compared to normals. Although this was not significant, the range of expression between patients and normals was striking (n=4) (100% v 63.9%: p=0.05) (Table 19).
Table 18: Expression of cell surface markers that were significantly different between CLL patients and normal controls

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>B-CLL (median)</th>
<th>Normal (median)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>CD4</td>
<td>54.8% (16.6 - 100%)</td>
<td>70.2% (58.7 - 71.3%)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=7)</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD5</td>
<td>83.3% (74.5 - 100%)</td>
<td>98.5% (99.6 - 100%)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=5)</td>
<td>(n=4)</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD25</td>
<td>23.1% (1.8 - 91.2%)</td>
<td>80.8% (43.0 - 95.1%)</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=10)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>52.8% (19.5 - 100%)</td>
<td>84.5% (56.4 - 92.4%)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=10)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>0.0% (0.0 - 7.5%)</td>
<td>16.5% (6.4 - 24.4%)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=10)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>CD11a</td>
<td>89.4% (43.6 - 100%)</td>
<td>100% (99.8 - 100%)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=6)</td>
<td>(n=6)</td>
<td></td>
</tr>
</tbody>
</table>

* = range

Table showing expression of T cell surface markers on both CLL patients and normal controls. T cells were stimulated for expression of CD25, CD28 and CD152. T cells were unstimulated for expression of CD4, CD5 and CD11a. Expression of all surface antigens was significantly lower in CLL patients compared to normals.
Table 19: Expression of cell surface markers that showed no significant difference between patients and normals

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>B-CLL (median)</th>
<th>Normal (median)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>TCRαβ</td>
<td>87.9% (38.1 - 100%)* (n=7)</td>
<td>95.2% (93.6 - 98.6%)* (n=5)</td>
<td>ns</td>
</tr>
<tr>
<td>CD3</td>
<td>CD8</td>
<td>37.3% (20.3 - 43.6%)* (n=5)</td>
<td>37.1% (32.6 - 42.8%)* (n=4)</td>
<td>ns</td>
</tr>
<tr>
<td>CD3</td>
<td>CD54</td>
<td>40.4% (23.4 - 65.1%)* (n=6)</td>
<td>44.3% (19.9 - 54.6%)* (n=6)</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD154</td>
<td>10.5% (3.6 - 51.4%)* (n=10)</td>
<td>10.4% (5.5 - 18.5%)* (n=7)</td>
<td>ns</td>
</tr>
<tr>
<td>CD3</td>
<td>HLA-DR</td>
<td>100% (63.1 - 100%)* (n=4)</td>
<td>63.9% (6.8 - 89.2%)* (n=4)</td>
<td>ns</td>
</tr>
</tbody>
</table>

* = range

Table showing expression of T cell surface markers on both CLL patients and normal controls. T cells were stimulated for expression of CD154 and HLA-DR. T cells were not stimulated for expression of CD8, CD54 and TCR αβ. Expression of CD154 is higher in CLL patients but this difference is not significant.

ns: not significant
4.2 Expression of CD154 on T cells from B-CLL patients and normals

A greater number of CD2+ve T-cells from patients with B-CLL (n=10) expressed the CD40 ligand, CD154, described in 2.4.3, compared to normals (n=7) after stimulation (16.6% v 11.1%; p = 0.5) although this difference was not significant (Table 19).

4.3 Expression of the adhesion molecules LFA-1 (CD11a) and ICAM-1 (CD54) on the T cells from B-CLL patients and normals

Significantly fewer T cells of patients with B-CLL (n=6) expressed the adhesion molecule LFA-1 (CD11a) compared to normals (n=6), described in 2.4.4 (77.9% v 99.9%; p=0.01) (Table 18) (Figure 13) in unstimulated cells. Expression of ICAM-1 (CD54) was not significantly different between patients and normals (Table 19).
**Cell surface markers - summary of results**

The results in this section show that significantly less T cells from CLL patients express the cell surface markers CD25, CD28 and CD152 when activated in a PBMC population. In an unactivated PBMC cell population significantly less T cells from CLL patients express the cell surface markers CD4, CD5 and CD11a (LFA-1). HLA-DR was unchanged, although range of expression in normals was much larger compared to patients, and expression of CD154, CD8 and CD54 (ICAM-1) was unchanged between CLL and normal T cells. These results suggest a profound dysregulation in the up-regulation of key activation markers, in addition to a dysregulation in expression of markers normally present on resting T cells.

Results obtained from this section were carried out by analysis of T cells from a PBMC population with the malignant B cells present. In order to determine whether the B cells may be in some way responsible for this effect seen in the T cells, it was necessary to remove the B cells and repeat some of the above experiments on two key markers - CD152, expressed after activation with OKT3 and CD28, expressed continually.
4.4 Expression of CD28 and CD152 on stimulated T cells after B cell depletion

Fewer of the CLL patients' T cells (n=4) expressed the two surface antigens CD28 (49.9% v 92.2%; p = 0.01) and CD152 (4.9% v 17.3%; p = 0.05) compared to normals (n=3) after depletion of the malignant CD19+ve B cell population, as described in 2.4.5, and stimulation of the remaining cell population with OKT3 (Table 20). CD19 cells were depleted from PBMCs and any remaining B cells counted using CD20. Mean CD20 count after B cell depletion was 3.2% for CLL patients and 2.3% for normal controls.

Table 20: Expression of CD28 and CD152 on stimulated T cells after B cell depletion

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>B-CLL (n=4) (median)</th>
<th>Normal (n=3) (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>49.9% (10.3 - 61.5%)*</td>
<td>92.2% (88.2 - 96.3%)*</td>
<td>0.01</td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>4.9% (3.1 - 14.3%)*</td>
<td>17.3% (10.7 - 26.3%)*</td>
<td>ns</td>
</tr>
</tbody>
</table>

* = range

Table showing expression of CD28 and CD152 on the surface of T cells from CLL patients and normal controls after magnetic cell depletion of the CD19+ve B cells and stimulation with OKT3.

ns: not significant
B cell depletion - summary of results

Using magnetic CD19 B cell depletion methods, B cells were removed from both normal and CLL PBMC populations, with maximum B cells remaining after depletion no greater than 3.2% for CLL patients and 2.4% for normal controls as measured using CD20. After removal of the B cells, T cells were cultured for 48 hours to allow for any immediate effect the B cells may have had on T cell status. Despite this, significantly less T cells from CLL patients expressed the cell surface markers CD28 and CD152 after culture with OKT3.

As removal of the B cells could not be seen to have a noticeable effect on cell surface up-regulation, expression of the two same antigens CD28 and CD152 and also CD25, were investigated intracellularly to look for any possible dysfunction in production of these markers and their expression on the cell surface.
4.5 Expression of intracellular CD25, CD28 and CD152 on stimulated and unstimulated T cells after B cell depletion

After permeabilisation of stimulated CD2+ve cells, as described in 2.4.6, intracellular CD28 was expressed in significantly fewer cells in B-CLL patients (n=7) compared to normals (n=7) (44.2% v 67.5%: p = 0.02) (Table 21) (figure 14). CD25 and CD152 positivity were unchanged between patients and normals. In contrast, significantly more unstimulated CD2+ve cells from CLL patients expressed intracellular CD25 (5.8% v 2.5%: p = 0.01) and intracellular CD152 (8.7% v 4.5%: p = 0.03) compared to normals (table 22) (figure 15). Intracellular CD28 positivity was unchanged in CLL patients and normals.

When levels of intracellular CD25, CD28 and CD152 were compared in activated and unactivated cells in normal and patients, there was a significant difference seen in expression of CD25 and CD152 after activation. In contrast, levels of CD28 were not significantly different in either patients or controls after activation.
### Table 21: Expression of intracellular CD25, CD28 and CD152 on stimulated T cells following B cell depletion and permeabilisation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>B-CLL (n=7) (median)</th>
<th>Normal (n=7) (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>CD25</td>
<td>44.2% (32.1 - 61.2%)*</td>
<td>52.7% (28.7 - 86.5%)*</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>44.2% (21.5 - 70%)*</td>
<td>67.5% (54.1 - 74.7%)*</td>
<td>0.02</td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>53.9% (39.9 - 80.3%)*</td>
<td>69.4% (57.4 - 75.3%)*</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* = range
Table showing expression of intracellular CD25, CD28 and CD152 in CLL and normal control T cells after magnetic depletion of CD19+ve B cells and stimulation with OKT3 for 48 hours. There was a significantly lower expression of intracellular CD28 in CLL patients T cells compared to normals. There was no significant difference in expression of CD25 or CD152.

ns: not significant

### Table 22: Expression of intracellular CD25, CD28 and CD152 on unstimulated T cells following B cell depletion and permeabilisation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>B-CLL (n=7) (median)</th>
<th>Normal (n=7) (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>CD25</td>
<td>5.8% (3.7 - 11.8%)*</td>
<td>2.5% (1.8 - 8.0%)*</td>
<td>0.01</td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>40.9% (18.1 - 64.0%)*</td>
<td>55.6% (24.9 - 80.9%)*</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>8.7% (2.7 - 13.2%)*</td>
<td>4.5% (2.5 - 10.0%)*</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* = range
Table showing expression of intracellular CD25, CD28 and CD152 in CLL and normal control T cells after magnetic depletion of CD19+ve B cells. The T cells were not activated and were cultured for 48 hours with no mitogen. There was a significantly higher expression of intracellular CD25 and CD152 in CLL patients T cells compared to normals. There was no significant difference in expression of CD28.

ns: not significant
**Permeabilisation - summary of results**

B cell depleted PBMCs were cultured for 48 hours using OKT3. A control population of unstimulated T cells was also studied. CLL T cells cultured with OKT3 and permeabilised expressed significantly less CD28 compared to normal T cells. There was no difference in intracellular expression of CD25 and CD152 in activated CLL and normal T cells. However, in the resting (control) T cell populations, CLL T cells expressed significantly less intracellular CD25 and CD152 compared to normal T cells, whilst CD28 levels were unchanged between the two.

Under normal conditions, CD28 is expressed continually on the T cell surface during T cell activation, whilst CD25 and CD152 are up-regulated approximately 48 hours post activation. The fact that less CLL T cells express intracellular CD28 and less surface CD28 after activation suggests a profound dysregulation in the ability of T cell to both up-regulate and express this key second marker. CD28 interacts with CD80 and CD86 on B cells (and other APC) (Holdorf AD et al 2000) during activation to provide the critical second signal required for complete T cell activation. Without this second signal, the T cell would be unable to activate efficiently and would not up-regulate key markers such as CD25 (Jenkins MK et al 1991; Powell JD et al 1998; Boulougouris G et al 1999; Boonen GJJJC et al 1999; Holdorf AD et al 2000). IL-2 production would be compromised and some evidence suggests that anergy may result (Lenschow DJ et al 1996; Chambers CA and Allison P 1999; Boulougouris G et al 1999). The fact that CD28 levels are normal in resting CLL T cells implies there may be a problem with T cell activation and its response to the activation signals.

In comparison, intracellular CD25 and CD152 were unchanged between CLL and normal T cells after activation, but significantly less CLL T cells expressed intracellular CD25 and CD152 in resting (control) populations. The finding that levels of intracellular CD25 and
CD152 were unchanged between normal and CLL T cells after activation, whilst surface expression was down-regulated, suggested that there may be a soluble factor produced by the CLL B cells that persists even after B cell removal and that may have an effect on T cell activation and up-regulation of these key markers. In order to study this further, normal and CLL T cells were both cultured in the presence of CLL AB serum, to determine if this would effect T cell surface marker expression. A patient who was AB Rh (D) + gave blood samples into plain sterile tubes, which were left to stand at room temperature for a minimum of 2 hrs. Once the contents had clotted, the sample was centrifuged and the serum removed under sterile conditions, heat inactivated for 30 minutes at 57°C and frozen for future use. The samples were analysed for total protein, immunoglobulins and lipids to ensure that there were no obvious factors that may cause T cell activation. Normal and CLL T cells were cultured with this serum.
4.6 Expression of CD25, CD28 and CD152 on normal and CLL T cells after B cell depletion and OKT3 stimulation in 50% CLL AB serum and 50% normal AB serum.

When normal T cells are cultured in RPMI containing 50% CLL AB serum, as described in 2.4.7, the proportion of cells expressing CD25 and CD152 significantly increased (n=6) when compared to culture in 50% normal human AB serum (n=6) (CD25 73.5% v 19.8%: p = 0.002) (CD152 13.6% v 4.6%: p = 0.01) (Table 23) (figure 16). Significantly more CLL T cells, when cultured in 50% CLL AB serum (n=4), expressed CD25 compared to culture in normal AB serum (n=4) (57.6% v 16.6%: p = 0.04) (Table 24) (fig 17). Levels of T cells expressing CD28 were unchanged in CLL AB when compared to normal AB serum in both CLL and normal controls. The proportion of CLL T cells expressing CD152 was unchanged after culture in CLL AB serum (table 24) (figure 17). In one patient who failed to express any surface CD152 after culture in normal AB serum, 1.2% of T cells expressed CD152 after culture in CLL AB serum (table 24).
Table 23: Expression of surface CD25, CD28 and CD152 on normal T cells after B cell depletion and OKT3 stimulation in 50% CLL AB serum and 50% normal AB serum

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>CLL AB serum (median)</th>
<th>Normal AB serum (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>CD25</td>
<td>73.5% (65.8 - 78.4%)</td>
<td>19.8% (12.1 - 43.8%)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>87.0% (66.0 - 91.9%)</td>
<td>88.9% (70.0 - 95.4%)</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>13.6% (7.1 - 32.5%)</td>
<td>4.6% (1.6 - 10.7%)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* = range

Table showing expression of surface CD25, CD28 and CD152 in normal T cells (n=6) after magnetic depletion of CD19+ve B cells and stimulation with OKT3 for 48 hours in either 50% CLL or normal AB serum. There was a significantly higher expression of CD25 and CD152 on the T cells activated in the presence of CLL AB compared to activation in normal AB serum. No significant difference was observed in expression of CD28.

ns: not significant

Table 24: Expression of CD25, CD28 and CD152 on CLL T cells after B cell depletion and OKT3 stimulation in 50% CLL AB serum and 50% normal AB serum

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>CLL AB serum (median)</th>
<th>Normal AB serum (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>CD25</td>
<td>58.5% (40.5 - 81.0%)</td>
<td>14.0% (9.0 - 22.2%)</td>
<td>0.04</td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>58.9% (56.5 - 63.3%)</td>
<td>66.7% (65.4 - 68.8%)</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>4.5% (1.2 - 7.5%)</td>
<td>1.2% (0.0 - 2.4%)</td>
<td>ns</td>
</tr>
</tbody>
</table>

* = range

Table showing expression of surface CD25, CD28 and CD152 in CLL T cells (n=5) after magnetic depletion of CD19+ve B cells and stimulation with OKT3 for 48 hours in either 50% CLL or normal AB serum. There was a significantly higher expression of CD25 on the T cells activated in the presence of CLL AB serum compared to activation in normal AB serum. No significant difference was observed in expression of CD28 or CD152.

ns: not significant
CLL AB serum results – summary

When normal, B cell depleted PBMCs are cultured in OKT3 supplemented with 50% CLL AB serum, significantly more T cells express surface CD25 and CD152 when compared to culture in normal AB serum. In addition, when CLL B cell depleted PBMCs are cultured in OKT3 with 50% CLL AB serum, the number of T cells expressing CD25 is significantly increased, compared to culture in normal AB serum. This suggests that there may be either a soluble factor present in the CLL AB serum that increases activation, or an absence of an inhibitory factor that prevents it. The total amount of both CLL AB serum and normal AB serum used was 50%. In previous experiments, only 10% normal AB serum was used. Increasing to 50% AB serum allowed any effects of the CLL serum to be amplified. This may help to explain why results for normal controls were lower than normally seen, particularly for CD152. Previous experiments with normal T cells have shown the median CD152 expression to be 17.3% in 10% normal AB serum (table 20), whilst results in this experiment show a median CD152 expression of 4.6% in 50% normal AB serum (table 23). The difference in total amount of AB serum used is likely to be responsible for the changes in surface marker expression seen in normal T cells, although variation in patient samples is considered in more detail in the discussion.

The increase in numbers of CD25 expressing T cells seen in the CLL patients was striking, although not as high as that seen in normal controls. It is possible that the B cells are producing a soluble factor that enhances their own survival, whilst providing T cells with a low grade, continuous activation signal that may ultimately effect the T cells ability to respond efficiently to a given mitogen. Alternatively, the T cells themselves may be producing a soluble factor in response to the malignant B cells that increases their activation.

Although there was no evidence of a monoclonal antibody or any unusual chemistry in the CLL patients’ AB serum that may have caused this response (all routine immunology and
chemistry results carried out on the sample were normal), it would have been useful to repeat these experiments with different CLL AB serum. However, this was not possible, as AB Rh (D)+ is a rare blood type (approximately 3% of the population) and none of the other patients suitable for this study were AB Rh (D)+.

In an attempt to try and reproduce the results seen with CLL serum, recent work involving interleukin-15 (IL-15) was considered (Campbell JD et al 2001). This work has shown that in multiple myeloma patients, T cells cultured with IL-15 were able to proliferate and respond to IL-2 normally, even in the presence of tumour cells. IL-15 does not use the high affinity IL-2 receptor (CD25), and as the results presented earlier in this study have shown that T cells expressing CD25 are reduced, IL-15 may be able to restore some elements of T cell function, such as up-regulating CD25 expression, as the results with the CLL AB serum had shown.
4.7 Expression of CD25, CD28 and CD152 on CLL T cells after B cell depletion and OKT3 stimulation with interleukin-15 (IL-15).

When CLL T cells (n=3) were depleted of B cells and cultured in OKT3 with 10ng/ml of IL-15, there was no significant change in the expression of CD25 (p = 0.2), CD28 (p = 0.5) and CD152 (p = 0.3) when compared to culture without IL-15. Three CLL patient samples were analysed after culture with IL-15 and although there was a slight increase in the expression of each surface marker, the differences were not significant (table 25).

Table 25: Expression of CD25, CD28 and CD152 on CLL T cells after B cell depletion and OKT3 stimulation and culture with interleukin 15 (IL-15).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>OKT3 + IL-15 (median)</th>
<th>OKT3 + O (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>CD25</td>
<td>77.1% (72.1 - 90.0%)*</td>
<td>66.7% (65.3 - 90.0%)*</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>64.2% (59.1 - 87.4%)*</td>
<td>62.7% (50.7 - 89.3%)*</td>
<td>ns</td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>15.3% (11.5 - 15.3%)*</td>
<td>9.9% (7.6 - 17.1%)*</td>
<td>ns</td>
</tr>
</tbody>
</table>

* = range

Table showing expression of surface CD25, CD28 and CD152 on CLL T cells (n=3) after magnetic depletion of CD19+ve B cells and stimulation with OKT3 with and without IL-15 for 48 hours. There was no significant difference in the expression of any of the surface markers after culture with IL-15 ns: not significant
Culture with interleukin-15 (IL-15) – summary

When B cell depleted CLL PBMCs were cultured with OKT3 in the presence of IL-15, no difference was observed in the number of T cells expressing CD25, CD28 or CD152 compared to culture without IL-15. IL-15 was therefore unlikely to be responsible for the effects observed when T cells were cultured in CLL AB serum. If time had permitted, it may have been useful to further investigate this soluble factor in the CLL AB serum. However, the possibilities were vast and it was decided that it would be more beneficial to continue to investigate ways in which the observed T cell defects could be reversed.

Results presented here also show that there is a large range of expression of cell surface markers between patient samples. When results from table 25 for OKT3 stimulated T cells without IL-15 are compared to T cells cultured in an identical manner but on a different date, as shown in table 20, expression of CD28 ranges from a median 49.9% (range 10.3 – 61.5%) to a median 62.7% (range 50.7 – 89.3%). Similarly, results in table 20 show that CD152 expression also differs from those given in table 25, with a median 4.9% (range 3.1 – 14.3%) to a median 9.9% (range 7.6 – 17.7%). This anomaly is discussed in greater depth in the discussion and reference is made to the effect that clinical disease parameters, patient age and previous treatment may have had on these results.
4.8 Expression of CD25, CD28 and CD152 on CD2+ CLL T cells from a patient in clinical remission

T cells were enriched, as described in 2.4.5, from one patient who was previously diagnosed with CLL 6 years ago but who has now returned to normal blood counts and is in clinical remission. When stimulated for up-regulation of CD25, CD28 and CD152, as described in 2.4.3, levels of expression are lower than those seen in normal subjects, but, in keeping with those seen in other B-CLL patients, CD25 expression in was reduced at 61.7% compared to a median expression of 76.5% by normal T cells. CD28 expression was 77.2% compared to a normal median of 92.2% and CD152 expression in the patient was 7.2%, with the normal median being 17.3% (median data taken from figures presented in this thesis for normal controls after B cell depletion and stimulation, table 20)

Patient in remission - summary

When T cells from a patient who had been in clinical and haematological remission for 6 years were cultured in OKT3, there were less T cells expressing CD25, CD28 and CD152 compared to the average numbers in normal controls. This suggests that even in a patient in clinical remission, with normal blood counts, the T cell dysregulation may persist, raising the question of whether this observed dysfunction is primary or secondary to the disease. However, it must be pointed out that only one patient was studied due to time restrictions. More remission patients would have given a more detailed picture. In addition, normal controls show a range of expression of CD25 (43.0 - 95.1%), CD28 (56.4 - 92.4%) and CD152 (6.4 - 24.4%) and whilst this patient was below the average for normals, they were at the lower end of the normal range and it is difficult to draw conclusions without additional data.
CHAPTER FIVE: RESULTS - CYTOKINE STUDIES

All statistical analysis was carried out using the Mann-Whitney (Wilcoxon) comparison of medians. The number of patients used in each group was too small to assess normality and as a result, the non-parametric Mann-Whitney test has been used throughout.

5.0 Secretion of cytokines and serum factors

5.1 Secretion of Interleukin - 2 (IL-2) and Interferon-γ (IFN-γ) by T cells in a mixed lymphocyte reaction (MLR).

T cells were isolated from 5 CLL patients and 5 normal controls and cultured with normal allogeneic, irradiated PBMCs as described in 2.4.8. Cytokine secretion of IL-2 and IFN-γ was measured using ELISA's as described in 2.4.9 and 2.4.10 respectively. Secretion of IL-2 was significantly lower from the T cells of CLL patients compared to normal T cells (326.4pg/ml v 662.9pg/ml: p = 0.04). IFN-γ secretion was unchanged between CLL patients and normal controls (1408.6pg/ml v 1176.5pg/ml: p = 0.3) (Table 26) (figure 18).

All results were indicative of an MLR having taking place, as results for T cells alone without the allogeneic PBMCs were so low as to be unreadable by the plate-reader for each sample on the IL-2 ELISA. The range of IFN-γ expression was 5.7 - 11.1pg/ml for normals and 8.6 - 40.1 pg/ml for CLL patients. The results for IFN-γ expression in the control group of T cells with no allogeneic PBMCs show that the level of background IFN-γ in CLL patients was significantly higher than normal controls (p = 0.01) (table 27) (figure 19).
Table 26: Secretion of Interleukin-2 and Interferon-γ from normal and CLL T cells in an MLR

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>B-CLL (n=5) (median)</th>
<th>Normal (n=5) (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-2</td>
<td>326pg/ml (153.6 - 602.5)*</td>
<td>662.9pg/ml (157.4 - 1206.1)*</td>
<td>0.04</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>1408.6pg/ml (239.8 - 1696.2)*</td>
<td>1176.5pg/ml (133.1 - 3131.1)*</td>
<td>ns</td>
</tr>
</tbody>
</table>

* = range

$r^2$ for IL-2 ELISA = 0.993
$r^2$ for IFN-γ ELISA = 0.976

All samples measured on the same ELISA plate to avoid interassay variation

Table showing results of IL-2 and IFN-γ secretion from normal and CLL T cells in an MLR. There was a significant reduction in expression of IL-2 from CLL T cells compared to normals. There was no significant difference in IFN-γ expression between CLL patients and normals.

Table 27: Secretion of IFN-γ from normal and CLL T cells; background readings

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>B-CLL (n=5) (median)</th>
<th>Normal (n=5) (median)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon-γ</td>
<td>20.1pg/ml (8.6 - 40.1)*</td>
<td>8.5pg/ml (5.7 - 11.1)*</td>
<td>p = 0.01</td>
</tr>
</tbody>
</table>

* = range

$r^2$=0.976

All samples measured on the same ELISA plate to avoid interassay variation

Table showing secretion of IFN-γ from CLL and normal T cells in the unstimulated wells of the MLR. In the absence of stimulator cells, CLL T cells produce significantly higher levels of IFN-γ than normal controls.
Mixed Lymphocyte Reaction (MLR) – summary

When CLL T cells are cultured with normal, irradiated B cells in an MLR, they secrete less IL-2 compared to normal T cells. Production of IFN-γ is not reduced from CLL T cells compared to normal controls. The reduced IL-2 production observed by CLL T cells suggests that the dysfunction observed in the expression of cell surface and intracellular markers may persist through to the production of some cytokines. This observed reduction in IL-2 may be a cause of the decreased numbers of T cells expressing activation markers that is preventing the T cells from entering a full state of activation and thereby IL-2 production is reduced. Alternatively, if T cells are unable to produce sufficient IL-2, up-regulation of the key activation and interaction markers may not occur efficiently.

Although levels of secreted IFN-γ are unchanged between CLL and normal T cells in an MLR, when control cell populations were measured (T cells cultured alone with no irradiated B cells), CLL T cells showed a significantly higher production of IFN-γ compared to normal T cells. IFN-γ is produced in response to tumour cells (Ferrantini M and Belardelli F 2000). It is possible that the continued exposure to malignant B cells over long periods of time could result in a low grade but continued secretion of IFN-γ by the T cells as a response to the tumour cells. It has been suggested that 50% of CLL B cells express CD86, which is normally only up-regulated following activation and that expression of CD86 on these B cells can be further up-regulated by IFN-γ (Zheng Z et al 1998). This IFN-γ secretion could be a response to the tumour or stimulated directly by the B cells. Conversely, the B cells may produce a serum factor, which causes the increased IFN-γ response. CLL T cells appear to be able to increase IFN-γ production and the B cells benefit from this by reducing their susceptibility to apoptosis (Zaki M et al 2000) implying B cells may somehow be inducing the T cells into secreting cytokines that can directly aid their survival.
5.2 Secretion of Interleukin-2 (IL-2) by T cells in a mixed lymphocyte reaction supplemented with Interleukin-15 (IL-15)

T cells isolated from 2 CLL patients and 1 normal control were cultured with normal allogeneic, irradiated PBMCs and supplemented with IL-15 at 10ng/ml, 1ng/ml and 0ng/ml as described in 2.4.11. Secretion of IL-2 within the culture supernatant was measured using an ELISA. The higher the concentration of IL-15 was in the culture, the lower the expression of IL-2 (Table 29). Statistical comparison between one normal and two CLL subjects was not done.

<table>
<thead>
<tr>
<th></th>
<th>10ng/ml IL-15</th>
<th>1ng/ml IL-15</th>
<th>0ng/ml IL-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL 1</td>
<td>112.4</td>
<td>199.9</td>
<td>558.6</td>
</tr>
<tr>
<td>CLL 2</td>
<td>73.9</td>
<td>107.6</td>
<td>134.9</td>
</tr>
<tr>
<td>Normal 1</td>
<td>Result out of range – too low to read</td>
<td>67.0</td>
<td>213.3</td>
</tr>
</tbody>
</table>

$r^2 = 0.999$

All samples measured on the same ELISA plate to avoid interassay variation

Table showing secretion of IL-2 from CLL T cells after culture with 10ng/ml, 1ng/ml or no IL-15 in an MLR. As the levels of IL-15 in the MLR decreased, secretion of IL-2 increased.
Mixed Lymphocyte Reaction (MLR) with IL-15 – summary

When the original MLR was repeated in the presence of varying amounts of IL-15, the observed results appear to be “false”. IL-15 shares much homology with IL-2 (Giri JG et al 1995; Bulfone-Paus S et al 1997). In an attempt to see if IL-15 could increase IL-2 production, it is possible that the IL-15 from the culture medium bound to the monoclonal antibody for IL-2 pre-coated onto the ELISA plate. The higher the initial concentration of IL-15, the lower the IL-2 result. This does not give a true representation of IL-2 secretion in the presence of IL-15 in an MLR.

Results presented here also show that there is a large range of IL-2 secretion in CLL patients. Table 26 has a median of 326.4pg/ml (range 153.6 – 602.5pg/ml), yet cells cultured in an identical fashion in table 29 (no IL-15 added) show an IL-2 secretion of 558.6 and 134.9pg/ml. As discussed previously, these results are vastly different and will be considered in greater depth in relation to clinical disease parameters, patient age and previous treatment in the discussion.
6.0 Results summary

The results of this study suggest a profound dysregulation of the function of the CD2+ve T-cells in patients with B-CLL. The significantly reduced expression of the key activation and interaction markers CD4, CD5, LFA-1, CD25, CD28 and CD152 and significantly increased expression of HLA-DR on these cells also suggests partial activation of these T cells and dysfunctional interaction with B cells. Removal of B cells before cell culture did not correct these abnormalities. In contrast, expression of intracellular CD25 and CD152 was not reduced in activated T cells from B-CLL patients compared to normal controls and their expression was significantly greater in resting CLL T cells. Intracellular CD28 expression was reduced in activated, but not resting CLL T cells from B-CLL patients compared to normal controls.

When T cells from normal controls were cultured in CLL AB serum, levels of expression of CD25 and CD152 were significantly greater than with normal AB serum. T cells from B-CLL patients showed a similar response, but expression of CD25 and CD152 did not increase as much as in normals, suggesting that B-CLL serum contains a factor which can increase activation.

In a mixed lymphocyte reaction (MLR), T cells from B-CLL patients secreted significantly less IL-2 compared to normal controls. Levels of IFN-γ were similar for CLL T cells from B-CLL patients and normal controls. However, secretion of IFN-γ by resting, unstimulated T cells from B-CLL patients was significantly higher than secretion by those from controls. None of these defects could be corrected by the addition of exogenous IL-15. Finally, the abnormal expression of CD25 persisted at a level seen in untreated CLL in a patient who was in complete clinical and haematological remission, suggesting that these T cell changes may be persistent or even permanent.

There are two hypotheses that may explain this apparent dysregulation of T cell function in B-CLL. The first is immunosenescence and the second is anergy. Both immune
phenomena are well documented and will be discussed in relation to the findings in this study.

6.1 Range of expression in samples

6.1.1 Normal controls

There is evidence within the results presented here that there is a degree of variability within normal controls. Data presented in different tables from cells cultured in a similar manner but from different controls and on different dates show wide variation. Table 20 has a median CD152 expression of 17.3% (range 10.7 – 26.3%), whilst table 23 has a median CD152 expression of 4.6% (range 1.6 – 10.7%). Although parameters were slightly different (AB serum concentration was 10% in table 20 and 50% in table 23) these results are more varied than would normally be expected. If normal control data is re-analysed according to age (figure 21), it is possible to see that CD152 increases gradually with age. Previous work also suggests that CD152 expression increases with age (Elliott SR et al 1999). The age range of normal controls used in table 20 is 37-45, and for table 23, it is 23-63. Three normal controls (out of a total of six) were in their twenties and only one person was above 48. It is possible that one theory that could explain this variation in some of the normal control data is age of the subjects. Half of the controls with the low CD152 group were at the lower end of the age scale and this result could explain the large variation in patients results.

6.1.2 Patient samples

An intriguing finding in the experiments presented in this thesis have shown that there is often a case of high inter-assay variation amongst patient samples, as discussed in the results section. Cells cultured under identical conditions, but on different dates and from different patients, showed a wide variation in levels of expression of surface markers. In particular, tables 20 and 25 have cell populations treated in the same way. Both are
stimulated in the presence of OKT3 for 48 hours is the same culture medium, yet expression of CD28 ranges from a median 49.9% (range 10.3 – 61.5%) (table 20) to a median 62.7% (range 50.7 – 89.3%) (table 25). Similarly, results in table 20 show that CD152 expression also differs from those given in table 25, with a median 4.9% (range 3.1 – 14.3%) (table 20) to a median 9.9% (range 7.6 – 17.7%) (table 25). Within these particular groups, all patients were in stage A. The age range was 57-78 for table 20 and 55-70 for table 25. Prior treatment was similar; only 1 patient from each group had received prior treatment and in each case, treatment had consisted of chlorambucil + prednisolone.

To try and explain this anomaly further, patient data from different experiments were grouped according to age, prior treatment and stage. Although patient numbers were small and statistical analysis of each individual sub-group was not possible, graphical display of this data allows for any trends to become apparent that may help to explain the differences observed. Three groups of results were analysed; CD25, CD28 and CD152 as one group, TCR and CD4 as a second group and LFA-1 and ICAM-1 as the third group. Each group was examined for the same parameters.

6.1.2.1 Age

When patient data is organised into age and compared both within the CLL population and also to normal controls, results show that, compared to normals, CLL patients show a much greater range of expression as age increases, with no discernible trends. CD25 and CD28 data have no correlation with age and constantly fluctuate from the ages of 48 to 85 (figure 20). CD152 stays relatively constant across the same age group (figure 20). Replicative senescence (discussed in more depth in the next section) describes a continued decline in expression of both CD25 and CD28 as age increases. No such trend can be seen with CLL patient data that may explain the inter-assay variation. In comparison, graphical
data for normal controls, although not wholly constant, does show a trend of decreased expression of CD25 and CD28 as age increases (figure 21).

Previous work has shown that expression of CD152 is greater in adults when compared to neonates and in fact increases with age (Elliott SR et al 1999). CLL CD152 expression remains constant with age (figure 20), whilst normal controls show a small decline and then a gradual increase as age increases (figure 21).

Expression of TCR and CD4 generally increases with age in CLL patients (figure 22), whilst in normal controls, expression remains fairly constant (figure 23). CLL patients show an increase of expression of ICAM-1 and a slight increase then decline of LFA-1 as age increases (figure 24). In comparison, normal controls have a constant expression of LFA-1 and a slight increase in ICAM-1 as age increases (figure 25).

In summary, age does not seem to be a factor that affects surface marker expression in CLL and patients do not seem to follow normal – or expected – patterns of expression with age.

6.1.2.2 Prior treatment

When patients are grouped according to prior treatment regimes and surface marker expression is compared between the treatment groups, there are interesting results. Chemotherapy-induced T cell function (discussed in more depth shortly) suggests that T cells and T cell function can be profoundly affected by chemotherapy. As a result, when patients are grouped according to treatment history, one might expect to see a reduced expression of surface markers from those patients who had received previous treatment compared to treatment free groups. There is, in fact, a wide variation in the results. When chlorambucil treated patients are compared to non-treated groups, expression of CD25, CD28, CD152 and LFA-1 is higher in the treated group (figures 26 and 28). In comparison, expression of CD4 and TCR is reduced on cells from chlorambucil treated patients compared to non-treated groups (figure 27). If patients who had previously received
chlorambucil and prednisolone are compared to non-treated groups, expression of CD28, CD4 and LFA-1 is higher in the treated groups (figures 26, 27 and 28). In comparison, CD25, TCR and ICAM-1 expression is reduced. Prednisolone treatment alone gives an increase in expression of TCR and a decrease in expression of CD25, CD28 and TCR compared to non-treated groups (figures 26 and 27), whilst treatment with cyclophosphamide gives an increase in expression of CD25 and CD4 and a decrease in CD28 (figures 26 and 27).

Although this data gives some interesting results, there are no discernible trends that apply to each type of treatment regime and as a result, prior treatment is unlikely to cause the variation in patients results.

6.1.2.3 Stage of disease.

If patients are grouped according to the stage of their disease and expression of surface markers are re-analysed, more trends are discernible. In particular, expression of CD25 and CD28 decrease from stage A to stage B (figure 29). Expression of CD152 also declines from stage A to stage B, but this difference is not as striking (figure 29). In addition, expression of ICAM-1 and CD4 also decline as stage of disease progresses, whilst LFA-1 expression increases and TCR expression remains constant as disease progresses (figures 30 and 31). It is possible that some of the variability seen in patients samples could be due to stage of disease, although with reference to tables 20 and 25, where striking differences were seen, all patients were in stage A.

6.2 Chemotherapy-induced T cell dysfunction in previously treated patients

39% (16 out of 41) patients in this study had received previous chemotherapy to treat CLL. Of these 16 patients, the type of treatment received is shown in table 29 and the time since last treatment is shown in table 30.
### Table 29: Type of treatment received by patients

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Number of patients receiving treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>4</td>
</tr>
<tr>
<td>Chlorambucil + Prednisolone</td>
<td>4</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>1</td>
</tr>
<tr>
<td>Cladribine</td>
<td>1</td>
</tr>
<tr>
<td>Splenic radiotherapy</td>
<td>1</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>3</td>
</tr>
<tr>
<td>Combination (chlor, chlor + pred, purine analogues)</td>
<td>2</td>
</tr>
</tbody>
</table>

Table showing the type of treatment received by CLL patients and the number of patients receiving that particular type of treatment.

### Table 30: Length of time since last treatment regime

<table>
<thead>
<tr>
<th>Time since last treatment</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 years</td>
<td>1</td>
</tr>
<tr>
<td>6 years</td>
<td>3</td>
</tr>
<tr>
<td>5 years</td>
<td>1</td>
</tr>
<tr>
<td>4 years</td>
<td>3</td>
</tr>
<tr>
<td>3 years</td>
<td>2</td>
</tr>
<tr>
<td>2 years</td>
<td>3</td>
</tr>
<tr>
<td>1 year</td>
<td>3</td>
</tr>
</tbody>
</table>

Table showing the time lapsed since last treatment regime and the number of patients who last received treatment at that time.
Although treatment has not been shown to have any discernible effect when data is compared to none-treatment groups, it is important to discuss what effect chemotherapy may have on the T cell populations.

One of the most prominent findings of T cell response to chemotherapy is a reduction in total T cell numbers following treatment (Bernego MG et al 1984; Petrini B et al 1984; Emmendorffer CA and Pichler WJ 1985; Takamatsu Y et al 1993; Mackall CL et al 1997; Fagnoni FF et al 2002; Svane IM et al 2002). The T cell decline increases after each cycle of therapy, suggesting an accumulative effect of the drugs (Bernego MG et al) and the main cause of this decline is postulated to be CD4 T cells. In contrast, CD8 T cells are not affected in such a dramatic way and their regeneration after cessation of therapy is quicker than CD4 cells (Mackall CL et al 1997). This is believed to be because CD4 cells require a thymus dependent pathway for regeneration, whilst CD8 cells are capable of regeneration using a thymic-independent pathway. As a result of this, there is often a prolonged, inverse CD4/CD8 ratio and reduced total T cell numbers after chemotherapy, sometimes for as long as 2-3 years (Petrini B et al 1984). A reversed CD4/CD8 ratio is well documented in CLL (Kay NE et al 1979; Kay NE 1981; Semenzato G et al 1983; Totterman TH et al 1989; Kimby E et al 1989; Janssen O et al 1989; Burger T et al 1990; Goolsby CL et al 2000; Porakishvili N et al 2001) and it is possible that earlier studies may not have taken into account prior treatment before assessing CD4/CD8 numbers.

Older patients who are receiving chemotherapy may have a more pronounced T cell decline and take longer to recover CD4 numbers, as thymic function invariably decreases with age and an inverse relation between age and CD4 regeneration has been shown (Mackall CL et al 1995; Mackall CL et al 1997; Fagnoni FF et al 2002). If such theories are applied to CLL patients, one might expect to see a reduced expression of CD4 on previously treated patients compared to non-treated patients and also a more pronounced decrease in older patients who had received treatment. Within the patient group tested for CD4 expression in this study, one patient aged 76 had received cyclophosphamide 1 year
prior to test. CD4 expression was compared to a younger patient aged 56 who had received chlorambucil + prednisolone 4 years prior to test. Surprisingly, expression of CD4 was 100% for the 76 year old patient and only 35.8% for the 56 year old patient. Unfortunately patient numbers were too small to further evaluate this finding, but it would suggest that, in this instance, chemotherapy-induced T cell dysfunction may not be responsible for these findings. In contrast, CD8 numbers are unchanged from the normal control population and this would fit with findings for chemotherapy-induced T cell dysfunction.

Other important findings in patients treated with chemotherapy is the presence of cytokines produced in recovery phase after drug treatment. One study has shown that levels of IFN-γ and GM-CSF in both cultures and plasma are higher compared to normal controls (Takamatsu Y et at 1993). This suggests that T cells may be activated in vivo to produce haemopoietic cytokines during haematological recovery and that they may be actively involved in the induction of haemopoiesis. When data from this study is re-analysed according to prior drug treatment, the patient whose cells produced the highest levels of IFN-γ from a resting cell population had received chlorambucil 1 year prior to test and the amount of expression was almost double the amount produced by the next closest patient and five times higher than the lowest secreting patient. This suggests that in this experiment, the effects of chemotherapy may be in some part responsible for the results shown here.

Whilst it is important to take into account the effects of chemotherapy on T cell function and also to note that some findings in this thesis correlate with the findings of others, chemotherapy-induced T cell dysfunction cannot wholly explain the results observed in previously treated patients.

6.3 Immunosenescence or Replicative Senescence

Immunosenescence, also called replicative senescence, is described as a generalised, age-related decline in immune responses in the elderly (Globerson A and Effros RB 2000),
which may lead to an increased susceptibility to infectious disease, cancer and autoimmune phenomena (Solana A and Pawelec G 1998). It implies that, as lymphocytes age, they reach a point at which they cannot replicate (growth arrest) and as a result, their immune responses begin to diminish (Effros RB and Pawelec G 1997). This theory has recently been challenged because, while the immune system does show a reduced level of diminished response with age, it also undergoes dramatic restructuring that leads also to some enhanced functions (Globerson A and Effros RB 2000). It is possible that the results seen in this study may be further evidence of immunosenescence, as most patients who develop CLL are elderly. While every attempt was made to age match patients and normal controls, this was not always feasible as access to volunteers within the hospital was limited to those under retirement age. The majority of normal controls were between 30 and 50 years, this being the typical range of age of the hospital workforce. Careful comparison of abnormalities described as immunosenescence and those found within this study is therefore necessary.

6.3.1 CD25 and CD28 expression, IL-2 secretion

Recent studies reporting replicative senescence have consistently described reduced surface expression of CD28 on T cells (Effros RB and Pawelec G 1997; Pawelec G and Solana R 1997; Boucher N et al 1998; Globerson A and Effros RB 2000; Pawelec G and Solana R 2001). The results of the investigation for this thesis also show reduced expression of CD28. The reduced CD28 expression described in the initial studies was found on CD8^{+ve} T cells, and the CD8 subset of T cells is expanded in both replicative senescence (Effros RB and Pawelec G 1997; Pawelec G and Solana R 1997; Boucher N et al 1998; Globerson A and Effros RB 2000; Pawelec G and Solana R 2001) and also in CLL (Kay NE et al 1979; Kay NE 1981; Semenzato G et al 1983; Totterman TH et al 1989; Burger T et al 1990). Both of these findings suggest that replicative senescence may
be responsible for the observed reduction in T cells expressing CD28 and the observed increase in numbers of CD8+ T cells in CLL.

CD28 plays a critical role in upregulating expression of both IL-2 and the IL-2 receptor, CD25 (Jenkins M et al 1991; Powell JD et al 1998; Boonen GJJJC et al 1999; Boulougouris G et al 1999; Holdorf AD et al 2000). As a possible consequence of this reduced CD28 expression in replicative senescence, production of IL-2 and its receptor CD25 are reduced in the elderly (Antonaci S et al 1991; Ginaldi L et al 1999). This thesis also reports a significantly reduced expression of IL-2 from CLL T cells in the MLR.

However, while cell surface expression of CD28 is decreased in both CLL and replicative senescence, the results from this thesis show that although intracellular levels of CD28 are significantly decreased in activated CLL T cells, there is no difference in intracellular expression in resting CLL T cells compared to normals. If the decrease in surface CD28 in CLL T cells was a result of immunosenescence, then levels of intracellular CD28 should also be decreased in both activated and resting cells. But, as there is no significant difference in expression of intracellular CD28 in resting T cells, the decrease seen after activation is presumably a dysfunctional response to T cell stimulation.

Immunosenescence is also characterised by a decrease in surface expression of CD25. This thesis also shows a decrease in surface expression of CD25 on the T cells of patients with B-CLL. The combination of this reduced surface expression of CD25 with normal intracellular expression in activated T cells and the significant increase in expression of intracellular CD25 in resting T cells from these patients also suggests a dysfunctional response to T cell mitogens. This response cannot be explained on the basis of immunosenescence or replicative senescence alone.

6.3.2 CD152, CD154 and LFA-1 expression

One of the key findings described in this study was the reduced number of T cells expressing CD152 in CLL, with 50% of patients expressing no surface CD152. Studies
comparing key activation markers in neonates, children and adults have shown that expression of CD152 is greater in adults and less in neonates. CD152 expression increases with age (Elliott SR et al 1999). In older subjects, reduced expression of CD154, the ligand for CD40, and increased expression of adhesion markers such as LFA-1 (CD11a) have been described (Franceschi C et al 1995; Ginaldi L et al 1999). In comparison, this thesis finds no difference in surface expression of CD154 and a significant decrease in surface expression of LFA-1 comparing T cells from CLL patients with those from normal controls. All the findings discussed in 6.3.1 and 6.3.2 also suggest that the theory of immunosenescence does not apply to B-CLL.

6.3.3 HLA-DR expression, IFN-γ and TNF-α secretion

In this study significantly more T cells from CLL patients expressed HLA-DR on the cell surface after activation. Similarly, lymphocytes in the aged population show an increased number of T cells expressing HLA-DR, which is linked to an increase in serum secretion of TNF-α, IFN-γ and soluble IL-2R (Rea IM et al 1999). Previous studies have reported that CLL patients also have an increase of TNF-α in both the serum (Hulkkonen J et al 1998) and intracellularly (Mainou-Fowler T et al 2001). These results are all similar to the findings of T cell dysregulation presented in this thesis and those of other studies, and support the hypothesis that the T cell dysfunction may be one natural consequence of ageing.

Whilst both CLL patients and aged subjects have greater numbers of T cells expressing surface HLA-DR, there is a significant difference in the numbers of T cells that express HLA-DR. The highest number of T cells expressing HLA-DR reported in normal aged subjects was 25% of CD45RA T cells (Rea IM et al 1999). When HLA-DR expression was measured on CD4 and CD8 T cells, 27% of CD4 cells and 22% of CD8 cells expressed HLA-DR. This was a twofold increase compared to expression in younger subjects. In this thesis, an average of 90.7% of T cells from CLL patients expressed surface HLA-DR with
a range of expression from 63.1% to 100%, an increase in CLL which is unlikely to be the result of ageing or immunosenesence alone. This study also found an increased expression of the cytokine IFN-γ from resting CLL T cells compared to normals, whereas in contrast, IFN-γ was not found to be increased in resting T cells in normal elderly subjects presumed to show immunosenesence (Rea IM et al 1999; Ginaldi L et al 1999).

6.3.4 T cell numbers

During natural ageing, the absolute number of T cells decreases (Pawelec G et al 1995; Franceshi C et al 1995; Pawelec G et al 1999), whilst in CLL, the absolute number of T cells increases and the CD8 subset expands (Catovsky D et al 1974; Totterman H et al 1989; Burger T et al 1990; Caligaris-Cappio F and Hamblin T 1999; Stark P et al 1999; Caligaris-Cappio F et al 2001). The CD8 subset expands in both CLL and replicative senescence, but whilst CD4 absolute numbers have been observed to increase in CLL, a marked CD4 lymphopaenia is often observed in the elderly (Solana R and Pawelec G 1998). The results presented in this thesis show that the number of CD4 expressing T cells from CLL patients are significantly variable, suggesting that there is no set pattern to CD4 expression in the course of the disease.

6.3.5 T cell response to mitogen

The theory of replicative senescence is also based on the observation of an increase in the number of aged T cells unable to proliferate in response to known T cell mitogens (Boucher N et al 1998; Pawelec G et al 1999). Whilst the work for this thesis did not include measurement of proliferation of T cells from B-CLL patients, the finding of reduced expression of activation markers suggests that these T cells were unable to proliferate as efficiently as those from normal controls, in much the same way that aged T cells are unable to proliferate in response to mitogens.
However, T cells that have reached a stage of immunosenescence cannot respond to antigenic re-stimulation or T cell mitogens including anti-CD3 (Effros RB and Pawelec G 1997), suggesting that they would not be able to upregulate any activation markers. The results in this thesis show that T cells from B-CLL patients are able to mount a partial response to anti-CD3, distinguishing them from ageing immunosenescent T cells.

### 6.3.6 B cells

In elderly, healthy subjects, there is a general increase in the number of B cells expressing surface CD5 and producing auto-antibodies (Globerson A and Effros RB 2000). One of the key identifying factors of CLL is the expression of surface CD5 on the malignant B cell clone, the increase in production of autoantibodies and an increase in associated autoimmune clinical complications. Although the production of the auto-antibodies in CLL has not been directly linked to the malignant clone, the similarities that can be observed between a normal, healthy, elderly population and CLL are striking. It is possible that CLL may be a manifestation of an aged immune system which has perhaps undergone a higher than normal degree of challenge by external antigens resulting in a population of lymphocytes that have reached an immunosenescent end point. It is now quite normal for humans to live to at least 80 years or older and it is clear that the increase in CD5 expression and the production of autoantibodies in ageing is not entirely similar to the findings in CLL. Whilst there is an increase in CD5 expression on B cells with ageing, immunosenescence also results in a decrease in B cell numbers (Franceschi C et al 1995; Pawelec G et al 1999; Globerson A and Effros RB 2000). In contrast, in CLL there is a variable but often striking increase in B cell numbers. While the clonal malignant B cells also express CD5, they are not thought to be responsible for the production of the autoantibodies (Kobayashi R et al 1992; Kneitz C et al 1999). Experiments with animal models have also shown that younger animals are more susceptible to the induction of autoimmune disease than the old (Globerson A and Effros RB 2000).
The evidence discussed here would suggest that while CLL patients T cells do share many characteristics with immunosenescent T lymphocytes, there are too many variables which cannot be explained by the ageing process alone (table 31). But, it is possible that ageing may have an effect on their ability to respond effectively to the malignant B-cell clone as the disease progresses so this theory cannot be dismissed as entirely inapplicable to the immune dysfunction of CLL T cells.
Table 31: Differences and similarities between lymphocyte subsets in replicative senescence and CLL

<table>
<thead>
<tr>
<th>Cell status</th>
<th>Replicative Senescence</th>
<th>CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell CD28 surface expression</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td>T cell CD28 intracellular expression</td>
<td>decreased in activated cells decreased in resting cells</td>
<td>decreased in activated cells no change in resting cells</td>
</tr>
<tr>
<td>T cell CD25 surface expression</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td>T cell CD25 intracellular expression</td>
<td>decreased in activated cells decreased in resting cells</td>
<td>no change in activated cells increased in resting cells</td>
</tr>
<tr>
<td>T cell CD152 surface expression</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>T cell CD154 surface expression</td>
<td>decreased</td>
<td>increased (not significant)</td>
</tr>
<tr>
<td>LFA-1 expression</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>T cell HLA-DR expression</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>mean no. of T cells expressing HLA-DR</td>
<td>25%</td>
<td>90.7%</td>
</tr>
<tr>
<td>T cell IFN-γ production</td>
<td>increased in activated cells</td>
<td>increased in resting cells</td>
</tr>
<tr>
<td>T cell TNF-α production</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>T cell IL-2 production</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td>Total T cell numbers</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>Total B cell numbers</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>CD5 B cells</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>autoantibody production</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>CD5 B cell autoantibody production</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>T cell proliferative response to mitogen</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td>T cell activation response to anti-CD3</td>
<td>no</td>
<td>yes (partial)</td>
</tr>
</tbody>
</table>

Table showing the difference and similarities in expression of T cell markers, cytokine secretion and lymphocyte subsets in both replicative senescence and CLL.
6.4 Tolerance and clonal anergy

Complete activation of T cells requires two signals, one through the T cell receptor as the antigen binds, and the second through a costimulatory signal as the T cell interacts with the cell presenting the antigen. If the second signal is missing or reduced, the T cell is only able to make a partial response and will enter a state of unresponsiveness known as clonal anergy, in which the T cell is incapable of producing IL-2 on re-stimulation and becomes tolerant to activation signals (Schwartz RH 1990; Lechler R et al 2001; de St Groth BF 2001). There are a number of similarities that can be seen between CLL T cells and T cells tolerant through clonal anergy which will be described here.

6.4.1 CD28, CD25 and LFA-1 expression, IL-2 secretion

Recent evidence suggests that the critical second signal required from T cells to prevent their anergy and tolerance as a result of antigen presentation is expression of CD28 (Powell JD et al 1998). The evidence indicates that TCR engagement by antigen leads to progression of the cell cycle into G$_1$ when further cell cycle progression can be blocked by cell cycle inhibitors. However, CD28 ligation with its co-receptors CD80 and CD86 on B cells and other antigen presenting cells (APC), leads to the up-regulation of autocrine IL-2 production and the binding of IL-2 to its receptor down-regulates the cell cycle inhibitors so that the cell can progress through the rest of the cycle (Becker JC et al 1995). If, during antigen presentation, blocking antibodies to IL-2 are introduced \textit{in vitro}, anergy will also be induced (DeSilva DR et al 1991). Without the CD28 second signal from ligation with CD80/CD86 on B cells and APCs, T cells will remain in a partially activated state and be unable to fully respond to any further signals. Further stimulation with antigens may fail to cause T cell proliferation or IL-2 production and the T cells may remain anergic (Jenkins MK and Schwartz RH 1987).

The integrins such as LFA-1 and ICAM-1 have also been shown to have important roles in preventing anergy by promoting the adhesion between cells and facilitating the TCR signal...
after antigen binding (Powell JD et al 1998). CLL T cells appear to be in a state similar to anergy and tolerance, with reduced surface expression of both CD28 and LFA-1, as described in this thesis. This reduced expression could explain the state of partial activation of the CLL T cells. If the T cells have become tolerized \textit{in vivo}, their removal and subsequent activation with anti-CD3 in vitro would only result in a partial activation, due to the reduced expression of the second signal and their pre-existing tolerized state. In addition to the reduced expression of CD28 as the second signal and the reduced expression of LFA-1, this study has also shown that CLL T cells secrete less IL-2, another hallmark of the tolerized cell.

However, the increased expression of intracellular CD25 and CD152 found in resting CLL T cells suggests that some kind of response is made by the T cells and that there are factors present either within the CLL serum or the microenvironment that prevent their expression on the cell surface. After activation, expression of intracellular CD25 is lower. Continual antigenic stimulation from the serum or microenvironment \textit{in vivo} would repeatedly activate the T cells, even when in a resting state, and prevent a clear response when activated \textit{in vitro}.

This study has shown that when normal T cells are cultured in CLL AB serum, expression of CD25 is significantly higher than when cultured in normal AB serum, suggesting the presence of a serum factor from CLL patients that provides an activation signal. CLL T cells also have higher expression of CD25 when cultured in CLL AB serum compared to normal AB serum. While there is evidence of a tolerant state in the CLL T cells, it is possible that the \textit{in vivo} environment may in some way be responsible for this. Evidence suggests that anergy also persists after the removal of the induction signal, as shown in this study. Removal of the malignant B cells and subsequent activation of the remaining pure T cell population still results in reduced expression of CD28.
6.4.2 CD152 expression

Fewer T cells in CLL express surface CD152 and more express intracellular CD152 in their resting state than do normal controls. CD152 plays a critical role in down-regulating the T cell response following activation (da Rocha Dias S and Rudd CE 2001). In the absence of CD152, or if expression of CD152 was significantly reduced once activation had occurred, T cells would not receive signals to stop and may continue in a partial state of activation, rendering them more susceptible to autoimmunity and increasing T cell numbers as they resist the normal signals for apoptosis. The role of CD152 can be observed clearly in CTLA-4 knock-out mice which develop splenomegaly, lymphadenopathy, multi-organ lymphocytic infiltration and tissue destruction, dying within 3-4 weeks of birth (Tivol EA et al 1995). These findings suggest that CTLA-4 plays a critical role in down-regulating T-cell activation and preventing autoimmunity. Blocking antibodies to CD152 during activation produce a 4 to 5 fold reduction in the level of expression of CD95 (FasL), which induces antigen-induced cell death (AICD), a process that ensures the deletion of self-reactive T cells (da Rocha Dias and Rudd CE 2001). This suggests that under normal conditions of CD152 expression during T cell activation, ligation of CD152 with CD80/CD86 induces termination of the immune response by reducing IL-2 and IL-2R production, arresting cell cycle progression and inducing AICD to prevent the initiation of autoimmunity.

This reduced expression of CD152, however, does not correlate with theories of tolerance. Previous studies have shown that CD152 negative mice are resistant to tolerance induction, as demonstrated by proliferative responses, IL-2 production and progression into the cell cycle (Greenwald RJ et al 2001), confirming earlier experiments with CD152 negative mice (Tivol EA et al 1995). However, CLL T cells do not show normal proliferative responses and have low IL-2 production, suggesting that the expression of surface CD152 could be regulated by external factors, such as the serum factor referred to above.
Intracellular CD152, like intracellular CD25, is expressed in more resting CLL T cells than in normal T cells.

6.4.3 HLA-DR expression and IFN-γ secretion

The increased number of activated CLL T cells expressing HLA-DR and the increased secretion of IFN-γ from resting CLL T cells could be the result of long term, continual exposure to antigenic stimulation, resulting in a state of clonal anergy. IFN-γ is produced in response to tumour cells (Ferrantini M and Belardelli F 2000). It is possible that the continued exposure to malignant B cells over long periods of time could result in a low grade but continued secretion of IFN-γ as a response to the tumour cells. It has been suggested that 50% of CLL B cells express CD86, which is normally only up-regulated following activation and that expression of CD86 on these B cells can be further up-regulated by IFN-γ (Zheng Z et al 1998). This IFN-γ secretion, either as a response to the tumour or stimulated directly by the B cells, could either push the T cells into an anergic state or could simply be a result of pre-existing clonal anergy. Conversely, the B cells may produce a serum factor which causes the increased IFN-γ response. CLL T cells appear to be able to increase IFN-γ production and B cells benefit from this by reducing their susceptibility to apoptosis (Zaki M et al 2000) implying B cells may somehow be inducing the T cells into secreting cytokines that can directly aid their survival. The significantly higher number of T cells in CLL expressing HLA-DR, reported both here and by others (Garci-Suarez J et al 1991) could be a result of the higher expression of CD86, among other markers, on the malignant B cells (Zheng Z et al 1998).

Other evidence suggests that, at some point in the development of this disease, there may be a host T cell response directed against leukaemia-related antigens, demonstrated by the presence of clonal T cells (Wen T et al 1990; Serrano D et al 1997). These clonal T cells may have arisen from early T cells that attempted to respond to the malignant clone and support the theory that there may be a continuous, low grade T cell activation in response
to this. High IFN-γ secretion and increased numbers of T cells expressing HLA-DR could also be a result of this low grade activation.

6.5 Possible immunosuppression by CLL B cells

It is possible that some of the T cell defects described in this thesis may be caused by immunosuppression by the CLL B cells. In a normal immune system, T cells are more abundant than B cells, particularly in the peripheral blood. In CLL, B cell numbers increase dramatically and "dilute" T cells, originally causing what appeared to be a reduced T cell population. This increase in B cells numbers, the surface markers expressed on them and the possible factors secreted by them could all have a direct or indirect effect on T cell function. This should be taken into account when considering the T cell dysfunction presented in this thesis.

Key findings have shown that B cells are capable of suppressing the normal function of T cells. When CLL T cells are cultured with CLL B cells, the T cells show a reduced ability to co-operate in the production of immunoglobulins, a possible cause for the hypogammaglobulinaemia common to CLL patients (Callery RT et al 1980).

B cells may also suppress T cells by either absorbing or utilising cytokines essential for T cell function. If CLL T cells are cultured alone, they produce normal levels of IL-2. If CLL B cells are added to the system, levels of IL-2 produced are reduced and appear to be utilised by the B cells, suggesting a reversed immune control by B cells over the T cells (Zaknoen SL and Kay NE 1990).

Other methods of immune control by the CLL B cells include an ability to force T cells to up-regulate surface markers that would otherwise not normally be expressed. If CLL T cells are activated in the presence of CLL B cells, the T cells up-regulate surface CD30 (De Totero D et al 1999; Cerutti A et al 2001). This prevents up-regulation of CD40 on non-malignant B cells and prevents normal immunoglobulin production through T and B cell contact.
CLL B cells may also be capable of producing soluble factors that both suppress T cells and exert control over them. If T cells are cultured in the presence of supernatants from CLL B cells, a variety of T cell functions are inhibited, such as PHA-induced proliferation, PHA-stimulated entry of T cells into the cell cycle and PHA-induced production of IL-2 (Burton JD et al 1989). Control B cells and other malignant cell lines had little or no effect. Similarly, CLL B cells have been shown to secrete a protein called CCL22, which can actively induce the migration of T cells towards the B cells (Ghia P et al 2002). These T cells can then induce a strong chemokine production by the leukaemic clone, creating a vicious cycle that allows the clone to accumulate.

There is much evidence of CLL B cell control over T cells. Such facts should be taken into account when considering possible causes for the T cell dysfunction observed in this study. Even though the majority of experiments were carried out on T cell enriched populations, cell culture was only for 48 hours. It is possible that any suppression of the T cells by B cells may be long lasting, either as a prolonged down-regulation or in a partially activated state that prevents normal up-regulation of cell surface markers.

6.6 Conclusions

In CLL, T cells express an abnormal pattern of the normal activation and interaction markers. They do not respond to stimulation in the same way as normal T cells and more resting T cells express higher amounts of intracellular activation markers than activated T cells. The CLL T cell produces higher levels of the activation cytokine IFN-γ when resting, and when activated produces reduced levels of the activation cytokine IL-2. A soluble factor found in CLL AB serum causes significantly more normal T cells to express the activation markers CD25 and CD152 than when they are activated in normal AB serum. The patient who had been in clinical remission for 6 years had a continued reduced expression of CD25, suggesting that the T cell defect may persist even if the leukaemia is in clinical and pathological remission.
Replicative senescence has much in common with the CLL T cell, yet does not explain all the abnormalities described in this thesis. Tolerance also provides some interesting theories, but again cannot account for all of the findings. CLL is a slowly progressing disease. Chemotherapy-induced T cell dysfunction cannot wholly explain the findings for previously treated patients. The T cell will find itself surrounded by increasing numbers of malignant cells that may cause immunosuppression and until more is known about the B cells avoidance of immune recognition, it is difficult to understand how B and T cells fail to interact to prevent this. B and T cell interaction is critical and what is clear from this study is the reduced capability of the CLL T cell to express the markers and the cytokines necessary to do this efficiently. This T cell dysfunction may explain the partial response of these cells to B cell antigen presented to them in vitro via dendritic cells (Goddard RV et al 2001). Until ways are found to restore effective responsiveness, such attempts to use autologous T cells in adoptive immunotherapy will have only limited clinical value.
7.0 Future work

There are a number of experiments that could further enhance the findings of this thesis. Replicative senescence of the aged could not explain all of the results described here but further study may distinguish to what extent the CLL T cell defects are related to either the leukaemic process or the age of the patient. Replicative senescence is associated with a decrease in telomere length. With each subsequent replication of a cell, the telomeres shorten as the chromosome divides and telomere length is therefore indicative of the cells replicative status. CLL B cells do not show a significant increase in telomerase activity compared to solid tumours or other haematological malignancies such as hairy cell leukaemia (Trentin L et al 1999). It would have been useful however, to have measured telomere length in CLL T cells to evaluate if they had reached a state of replicative senescence when compared to normal age matched controls. In addition, T cells that have reached immunosenescence are only able to undergo a certain number of population doublings (PDs). After they have undergone continued replication, they are unable to continue with any further replications. Measuring the ability of CLL T cells to undergo PDs in vitro would provide useful information about their replicative age and further determine how important a role ageing plays in the CLL T cell.

The patient who had been in clinical remission for 6 years had a continued reduced expression of CD25, suggesting that the T cell defect may persist and be at least partly independent of the leukaemic process. Therefore, it may prove useful to study more patients in clinical remission with a greater array of monoclonals, in particular HLA-DR, as CLL patients have a significantly higher number of T cells expressing surface HLA-DR, while the number of T cells expressing other activation markers were significantly reduced. Secretion of IFN-γ from resting T cells in patients in remission would also help to show if the malignant B cells were responsible for the increased IFN-γ production by the T cells or if it is an independent CLL T cell phenomenon.
Determinants of IFN-γ production and its consequences could also provide more information. More normal T cells expressed activation markers when cultured in CLL AB serum. It would be useful to measure IFN-γ secretion from the resting T cells from the patient who supplied the AB serum. This may help to identify the serum factor that caused the increase in activation in normal T cells. Additional experiments to identify both the serum factor and the role of IFN-γ could include the addition of neutralising antibodies to the cultures of normal T cells and CLL T cells in CLL AB serum. If IFN-γ was present in the serum and did cause the increase in activation, neutralising antibodies would prevent this. HLA-DR expression has been associated with the presence of IFN-γ. To ascertain if the increase in HLA-DR on CLL T cells is due to IFN-γ, investigating the expression of HLA-DR on CLL T cells and normal T cells cultured in CLL AB serum with and without neutralising antibodies to IFN-γ may prove interesting. Finally, repeating the initial experiments of cell surface expression of all antigens studied in both CLL patients and normal controls with CLL AB serum may show if the serum factor could increase expression of these in the same way it increased expression of CD25 and CD152.

These experiments would all help in understanding if there is a factor in CLL serum that prevents full activation of CLL T cells, the possible identity of this serum factor and the role it may play in controlling the T cells themselves in vivo.
FIGURE 13: Percentage of cells expressing surface antigens that were significantly different between patients and normals

Bar chart showing the median expression of key surface markers on CD2+ T cells from CLL patients and normal controls
FIGURE 14: Percentage of stimulated T cells expressing intracellular antigens in patients and normals

Bar chart showing the median expression of intracellular CD25, CD28 and CD152 in OKT3 stimulated CD2+ T cells from patients and normal controls. A significant difference was observed in expression of CD28 ($p = 0.02$).
FIGURE 15: Percentage of resting T cells expressing intracellular antigens in patients and normals

Bar chart showing median expression of intracellular CD25, CD28, and CD152 in unstimulated CD2+ T cells from CLL patients and normals. A significant difference was observed in expression of CD25 (p = 0.01) and CD152 (p = 0.03).
Bar chart showing median expression of CD25, CD28 and CD152 on CD2+ T cells from normal controls after culture in either CLL or normal AB serum. Significant differences were found for CD25 (p = 0.002) and CD152 (p = 0.01).
FIGURE 17: Percentage of CLL T cells expressing surface antigens after cell culture in CLL and normal AB serum

Bar chart showing median expression of surface CD25, CD28 and CD152 on CD2+ CLL T cells after culture in CLL or normal AB serum. Significant difference was found for CD25 ($p = 0.04$)
Bar chart showing the median secretion of IL-2 and IFN-gamma in pg/ml from CD2+ T cells from patients and normals after a mixed lymphocyte reaction with normal, irradiated PBMCs. Significant difference was found for IL-2.
FIGURE 19: Secretion of IFN-g from resting patient and normal T cells

Bar chart showing median secretion of IFN-gamma in pg/ml from resting CD2+ T cells from CLL patients and normal controls.
Graph showing median expression of CD25, CD28 and CD152 in CLL patients in comparison to age. As the patients increase in age, there is no correlation to either an increase or a decrease in expression of any of the cell surface markers.
Graph showing median expression of CD25, CD28 and CD152 in normal controls. Whilst data shown is variable, it is more consistent than data for CLL patients and a gradual decrease in expression of CD28 can be seen as age increases.
Graph showing median expression of TCR and CD4 in CLL patients according to age. Although variable, data shows a subtle increase in expression of both with age.
FIGURE 23: Age comparison of TCR and CD4 expression in normal controls

Graph showing median expression of TCR and CD4 in normal controls. Expression is consistent with age and does not change considerably as age increases.
Graph showing median expression of LFA-1 and ICAM-1 in CLL patients according to age. LFA-1 appears to increase and then decrease as age progresses, whilst ICAM-1 expression steadily increases with age.
Graph showing median expression of LFA-1 and ICAM-1 according to age. Expression of LFA-1 is constant as age increases, whilst ICAM-1 shows a variable but general increase as age increases.
FIGURE 26: Mean expression of CD25, CD28 and CD152 in CLL patients according to prior treatment

Graph showing mean expression of CD25, CD28 and CD152 according to stage of disease. With small exceptions, all groups show expression of surface markers as equal or greater than the no treatment group.
FIGURE 27: Mean expression of TCR and CD4 in CLL patients according to prior treatment

Graph showing mean expression of TCR and CD4 according to prior treatment. Some treatment groups show higher surface marker expression than the none treated group.
FIGURE 28: Mean expression of LFA-1 and ICAM-1 according to prior treatment

Graph showing mean expression of LFA-1 and ICAM-1 according to prior treatment. LFA-1 expression is higher in the treated groups compared to none treated. ICAM-1 is slightly lower in treated groups.
FIGURE 29: Mean expression of CD25, CD28 and CD152 in CLL patients according to stage of disease.

Graph showing mean expression of surface markers according to stage of disease. Surface expression decreases from stage A to stage B.
FIGURE 30: Mean expression of TCR and CD4 in CLL patients according to stage of disease

Graph showing mean expression of TCR and CD4 according to stage of disease. CD4 expression decreases from stage A to stage B, whilst TCR expression remains constant.
Graph showing mean expression of LFA-1 and ICAM-1 according to stage of disease. LFA-1 expression increases from stage A to stage C, whilst ICAM-1 expression decreases.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICD</td>
<td>antigen-induced cell death</td>
</tr>
<tr>
<td>AIHA</td>
<td>autoimmune haemolytic anaemia</td>
</tr>
<tr>
<td>AITP</td>
<td>autoimmune thrombocytopenic purpura</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DBM</td>
<td>deleted in B-cell malignancy</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DLI</td>
<td>donor lymphocyte infusion</td>
</tr>
<tr>
<td>EBV</td>
<td>epstein barr virus</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EF</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cells</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in-situ hybridisation</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMZ</td>
<td>follicular mantle zone</td>
</tr>
<tr>
<td>GC</td>
<td>germinal centre</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>GVH</td>
<td>graft-versus-host</td>
</tr>
<tr>
<td>GVL</td>
<td>graft-versus-leukaemia</td>
</tr>
<tr>
<td>HLA-</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HVG</td>
<td>host-versus-graft</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>κ</td>
<td>kappa</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet haemocyanin</td>
</tr>
<tr>
<td>λ</td>
<td>lambda</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MIP-2</td>
<td>macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBSC</td>
<td>peripheral blood stem cell</td>
</tr>
<tr>
<td>PD</td>
<td>population doubling</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PWM</td>
<td>pokeweed mitogen</td>
</tr>
<tr>
<td>RAG</td>
<td>recombinant activating gene</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>sIg</td>
<td>surface immunoglobulin</td>
</tr>
<tr>
<td>sCD4</td>
<td>soluble CD4</td>
</tr>
</tbody>
</table>
sCD8: soluble CD8
SDF-1: stromal cell-derived factor-1
SLVL: splenic lymphoma with villous lymphocytes
TdT: terminal deoxynucleotidyl transferase
Th1: T helper 1
TCR: T cell receptor
TECK: thymus-expressed chemokine
TFS: treatment free survival
TNFR: tumour necrosis factor receptor
TNF-α: Tumour Necrosis Factor α
TGF-β: Transforming growth factor β
TRM: Transplant related mortality
VLA: very late antigen
VCAM: vascular cell adhesion molecule
VEGF: vascular endothelial growth factor
References


Aguilar-Santelises M, Loftenius A, Ljungh C, Svenson SB, Andersson B, Mellstedt H, Jondal M, (1992) Serum levels of helper factors (IL-1 alpha, IL-1 beta and IL-6), T-cell products (sCD4 and sCD8), sIL-2R and beta 2-microglobulin in patients with B-CLL and benign lymphocytosis Leukemia Research 16 (6-7) 607-613


lymphocytes in the elderly Immunopharmacology and Immunotoxicology 13 (1&2) 87-100


Bairey O, Zimra Y, Shaklia M, Rabizadeh E, (2001) Bcl-2 expression correlates positively with serum basic fibroblast growth factor (bFGF) and negatively with cellular vascular endothelial growth factor (VEGF) in patients with chronic lymphocytic leukaemia British Journal of Haematology 113 400-406


Barclay AN, Brady RL, Davis SJ, Lange G, (1993) CD4 and the immunoglobulin superfamily Philosophical Transactions of the Royal Society B: Biological Sciences 342 (1299) 7-12


Becker JC, Brabletz T, Kirchner T, Conrad CT, Brocker EB, Reisfeld RA, (1995) Negative transcriptional regulation in anergic T cells Proceedings of the National Academy of Science USA 92 2375-2378


Berendes HD, Meyer GF, (1968) A specific chromosome element, the telomere of Drosophila polytene chromosomes Chromosoma 25 (2) 184-197


Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB, (1995) CD28 co-stimulation can promote T cell survival by enhancing the expression of Bcl-xL Immunity 3 (1) 87-98


Borst J, van Dongen JJ, de Vries E, Comans-Bitter WM, van Tol MJ, Vossen JM, Kurrle R, (1990) BMA031, a monoclonal antibody suited to identify the T cell receptor alpha beta/CD3 complex on viable human T lymphocytes in normal and disease states Human Immunology 29 (3) 175-188


Braun M, Melchers I, Peter HH, Illges H, (1998) Human B and T lymphocytes have similar amounts of CD21 mRNA, but differ in surface expression of the CD21 glycoprotein *International Immunology* 10 (8) 1197-1202


224

Burrows PD, Cooper MD, (1993) B-cell development in man Current Opinion in Immunology 5 201-206

Burton JD, Weitz CH, Kay NE, (1989) Malignant chronic lymphocytic leukaemia B cells elaborate soluble factors that down-regulate T cell and NK function American Journal of Hematology 30 (2) 61-67


Campbell JD, Cook G, Robertson SE, Fraser A, Boyd KS, Gracie JA, Franklin IM (2001) Suppression of IL-2-induced T cell proliferation and phosphorylation of STAT3 and STAT5 by tumor-derived TGF beta is reversed by IL-15 The Journal of Immunology 167 (1) 553-561

morphology and resistance to conventional chemotherapy Cancer Genetics and Cytogenetics 90 (2) 118-124


Carmo AM, Castro MA, Arosa FA, (1999) CD2 and CD3 associate independently with CD5 and differentially regulate signalling through CD5 in Jurkat T cells The Journal of Immunology 163 (8) 4238-4245

Casey TP, (1968) CLL in a child presenting at the age of 2 years and 8 months Australian Annals of Medicine 17 70 - 74


Chen JR, Gu B, Dao LP, Bradley CJ, Mulligan SP, Wiley JS, (1999) Transendothelial migration of lymphocytes in chronic lymphocytic leukaemia is impaired and involves down-regulation of both L-selectin and CD23 British Journal of Haematology 105 (1) 181-189


Clark LB, Foy TM, Noelle RJ, (1996) CD40 and its ligand Advances in Immunology 63 43-78


Crossen PE, (1997) Genes and chromosomes in chronic B-cell leukaemia Cancer Genetics and Cytogenetics 94 (1) 44-51

Crump AL, Grusby MJ, Glimcher LH, Cantor H, (1993) Thymocyte development in major histocompatability complex-deficient mice: evidence for stochastic commitment to the CD4 and CD8 lineage Proceedings of the National Academy of Science USA 90 10739-10743


DeLuca D, Clark DR, (2002) Interleukin-7 negatively regulates the development of mature T cells in fetal thymus organ cultures *Developmental and Comparative Immunology* **26** (4) 365-384


DeSilva DR, Urdahl KB, Jenkins MK, (1991) Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation *The Journal of Immunology* **147** 3261-3267

De St Groth BF, (2001) DCs and peripheral T cell tolerance *Seminars in Immunology* **13** (5) 311-322


DiGiuseppe JA, Borowitz MJ (1998) Clinical utility of flow cytometry in the chronic lymphoid leukemias *Seminars in Oncology* **25** (1) 6-10


Dorshkind K, (1990) Regulation of haemopoiesis by bone marrow stromal cells and their products *Annual Review of Immunology* **8** 111-137


Duchosal M. A., (1997) B-cell development and differentiation *Seminars in Hematology* **34** (1 suppl.1) 2-12


229


Fanslow WC, Srinivasan S, Paxton R, Gibson MG, Spriggs MK, Armitage RJ, (1994) Structural characteristics of CD40 ligand that determine biological function Seminars in Immunology 6 267-278

Fearon DT, Carroll MC, (2000) Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex Annual Review of Immunology 18 393-422

Fernandez LA, MacSween JM, Langley GR, (1983) Normal T cell colony numbers in untreated patients with chronic lymphocytic leukaemia (CLL) British Journal of Haematology 57 97-104


during thymic maturation of human T lymphocytes International Immunology 11 (11)
1841-1849

Fitch FW, Lancki DW, Dialynas DP, (1984) Contributions of T cell clones to an understanding of the T cell receptor for antigen Transplantation 38 (3) 201-208

Fleury SG, Croteau G, Sekaly RP, (1991) CD4 and CD8 recognition of class II and class I molecules of the major histocompatibility complex Seminars in Immunology 3 (3) 177-185


Foa R, Giovarelli M, Jemma C, Fierro MT, Lusso P, Ferrando ML, Lauria F, Forni G, (1985) Interleukin 2 (IL-2) and Interferon-γ production by T lymphocytes from patients with B-chronic lymphocytic leukemia: Evidence that normally released IL-2 is absorbed by the neoplastic B cell population Blood 66 (3) 614-618


Foon KA, Gale RP, (1987) Staging and therapy of chronic lymphocytic leukaemia Seminars in Hematology 24 (4) 264-274


Fraser JD, Irving BA, Crabtree GR, Weiss A, (1991) Regulation of interleukin-2 gene enhancer activity by the T-cell accessory molecule CD28 Science 251 313316

Fratazzi C, Carini C, (1997) Interleukin-7 modulates CD23 and HLA-DR expression on CD4+ T cells and promotes a Th-2 type cytokine profile Allergol Immunopathology 25 (4) 189-198


*Trends in Immunology* 22 (4) 187-191


Gardby E, Lycke NY, (2000) CD19-deficient mice exhibit poor responsiveness to oral immunization despite evidence of unaltered total IgA levels, germinal centres and IgA-
isotype switching in Peyers patches European Journal of Immunology 30 (7) 1861-1867

Gardiner AC, Corcoran MM, Oscier DG, (1996) Different region of loss from chromosome 13q14 translocation in B-CLL determined by FISH using Yac probes British Journal of Haematology 92 (suppl. 1) 80

Germain RN, (2002) T-cell development and the CD4-CD8 lineage decision Nature Reviews Immunology 2 309-322


233


Goolsby CL, Kuchnio M, Finn WG, Peterson L, (2000) Expansions of clonal and oligoclonal T cells in B-cell chronic lymphocytic leukemia are primarily restricted to the CD3(+)CD8(+) T-cell population Cytometry 42 (3) 188-195


Green DR, Schuler M, (2000) T cell development: some cells get all the breaks Nature Immunology 1 (1) 15-17

Green JM, Karpitskiy V, Kimzey SL, Shaw AS, (2000) Coordinated regulation of T cell activation by CD2 and CD28 The Journal of Immunology 164 (7) 3591-3595


Gu B, Bendall LJ, Wiley JS, (1998) Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases Blood 92 (3) 946-951


Hamblin TJ, Oscier DG, (1997) Chronic lymphocytic leukaemia: the nature of the leukaemic cell Haematological Oncology 11 119-128

Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK, (1999) Unmutated Ig VH genes are associated with a more aggressive form of chronic lymphocytic leukaemia Blood 94 (6) 1848-1854

Han T, Dadey B, (1979) In vitro functional studies of mononuclear cells in patients with CLL: evidence for functionally normal T lymphocytes and monocytes and abnormal B lymphocytes Cancer 43 (1) 109-117


Hanada M, Delia D, Aiello A, Stadtmauer E, Reed J, (1993) Bcl-2 gene hypomethylation and high level expression in B-cell chronic lymphocytic leukaemia Blood 82 1820-1828


235
to sequence, message expression, gene structure and chromosomal location. *The Journal of Immunology* 147 (3) 1037-1044


236


Janeway CA Jr (1991) The co-receptor function of CD4 Seminars in Immunology 3 (3) 153-160


Jenkins MK, Taylor S, Norton SD, Urdahl KB, (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells The Journal of Immunology 147 2461-2466


237


Kappes DJ, Strominger JL, (1986) Structure and evolution of the HLA class II SX beta gene *Immunogenetics* 24 (1) 1-7


Kay NE, (1981) Abnormal T-cell subpopulation function in CLL: Excessive suppressor (Tγ) and deficient helper (Tμ) activity with respect to B-cell proliferation *Blood* 57 (3) 418-420


Kay NE, Johnson JD, Stanek R, Douglas SD, (1979) T-cell subpopulations in chronic lymphocytic leukaemia: abnormalities in distribution and in *in vitro* receptor maturation *Blood* 54 (2) 540-543


Kimby E, Mellstedt H, Nilsson B, Bjorkholm M, Holm G, (1989) Differences in blood T and NK cell populations between chronic lymphocytic leukemia of b-cell type (B-
CLL) and monoclonal B-lymphocytosis of undertermined significance (B-MLUS)
*Leukemia* 3 (7) 501-504


Kraut EH, Neff JC, Bouroncle BA, Gochnour D, Grever MR. (1990) Immunosuppressive effects of pentostatin Journal of Clinical Oncology 8 848-855


Kroft SH, Finn WG, Kay NE, Peterson LC. (1997) Isolated 13q14 abnormalities and normal karyotypes are associated with typical lymphocyte morphology in B-cell chronic lymphocytic leukaemia American Journal of Clinical Pathology 107 (3) 275-282


Lankester AC, van Scijndel GM, Cordell J, van Noesel CJ, van Lier RA. (1994) CD5 is associated with the human B cell antigen receptor complex European Journal of Immunology 24 812-816


Lenschow DJ, Walunas TL, Bluestone JA. (1996) CD28/B7 system of T cell costimulation Annual Reviews in Immunology 14 233-258

Letourneur F, Klausner RD. (1992) Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tails of CD3 e Science 255 79-82

Leveille C, Daccak R, Mourad W. (1999) CD20 is physically and functionally coupled to MHC class II and CD40 on human B cell lines European Journal of Immunology 29 (1) 65-74

240

Liew FY, (1994) Induction and regulation of CD4+ T cell subsets Ciba Foundation Symposium 187 170-175


Liu Y, (1997) Is CTLA-4 a negative regulator for T-cell activation Immunology Today 18 569-572


Lydyard PM, Jewell AP, Jamin C, Youinou PY, (1999) CD5 B cells and B-cell malignancies Current Opinion in Hematology 6 30-36

MacLennan IC, Casamayor-Palleja M, Toellner KM, Gulbranson-Judge A, Gordon J, (1997) Memory B-cell clones and the diversity of their members Seminars in Immunology 9 (4) 229-234


Mainou-Fowler T, Miller S, Proctor SJ, Dickinson AM, (2001) The levels of TNF alpha, IL-4 and IL-10 production by T-cells in B-cell chronic lymphocytic leukaemia (B-CLL) Leukemia Research 25 (2) 157-163


242


Moroy T, Karunsky H, (2000) Regulation of pre-T-cell development Cellular and Molecular Life Science 57 (6) 957-975


Mu X, Kay NE, Gosland M, Jennings CD, (1997) Analysis of blood T-cell cytokine expression in B-chronic lymphocytic leukaemia: evidence for increased levels of cytoplasmic IL-4 in resting and activated CD8 T cells British Journal of Haematology 96 733-735


Noelle E, Snow EC, (1990) Cognate interactions between helper T cells and B cells *Immunology Today* **11** (10) 361-368


Oscier DG, (1999) Chronic lymphocytic leukaemia British Journal of Haematology 105 (suppl. 1) 1-3


Osorio LM, Jondal M, Aguilar-Santelises M, (1998) Regulation of B-CLL apoptosis through membrane receptors and Bcl-2 family proteins Leukemia and Lymphoma 30 247-256


Otero DC, Omori SA, Rickert RC, (2001) CD19-dependent activation of Akt kinase in B-lymphocytes The Journal of Biological Chemistry 276 (2) 1474-1478

Parner EG (1999) Antigen presentation in the immune response to infectious diseases Clinical and Infectious Disease 28 (4) 714-716


Pawelec G, Solana R, (2001) Immunoageing—the cause or effect of morbidity? *TRENDS in Immunology* 22 (7) 348-349


Porakishvili N, Roschupkina T, Kalber T, Jewell AP, Patterson K, Yong K, Lydyard PM, (2001) Expansion of CD4+ T cells with a cytotoxic phenotype in patients with B-chronic lymphocytic leukaemia (B-CLL) *Clinical and Experimental Immunology* 126 (1) 29-36


Rassenti LZ, Kipps TJ, (2000) Expression of Ig-beta (CD79b) by chronic lymphocytic leukemia B cells that lack heavy-chain allelic exclusion Blood 95 (8) 2725-2727


Rea IM, Mcnerlan SE, Alexander HD, (1999) CD69, CD25 and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-α, IFN-γ and sIL-2R levels in aging Experimental Gerontology 34 (1) 79-93


Ridge JP, Di Rosa F, Matzinger PA, (1998) A conditioned dendritic cell can be a temporal bridge between a CD4+ T helper cell and a T-killer cell Nature 393 474-478


Cladribine combined with cyclophosphamide and mitoxantrone as front-line therapy in chronic lymphocytic leukaemia Leukemia 15 (10) 1510-1516


Robey E, Fowlkes BJ, (1994) Selective events in T cell development Annual Review of Immunology 12 675-705


Romagnani S, (2000) T-cell subsets (Th1 versus Th2) Annual Allergy and Asthma Immunology 85 (1) 9-18

Rossi E, Matutes E, Morilla R, Owusu-Ankomah K, Heffernan AM, Catovsky D, (1996) Zeta chain and CD28 are poorly expressed on T lymphocytes from chronic lymphocytic leukaemia Leukemia 10 494-497


best single prognostic parameter in chronic lymphocytic leukemia: a multivariate survival analysis of 329 cases Blood 64 642-648


Rudin CM, Thompson CB, (1998) B-cell development and maturation Seminars in Oncology 25 (4) 435-446


Samelson LE, Schwartz RH (1984) Characterization of the antigen-specific T cell receptor from a pigeon cytochrome c-specific T cell hybrid Immunology Review 81 131-144


Scholl PR, Geha RS, (1994) MHC class II signalling in B-cell activation Immunology Today 15 (9) 418-422


Schwartz RH, (1990) A cell culture model for T lymphocyte clonal anergy Science 248 1349-1356


Soderberg O, (1998) Growth and survival of B-chronic lymphocytic leukaemia cells Medical Oncology 15 (2) 73-78


Spertini F, Chatila T, Geha RS, (1992) Signals delivered via MHC class II molecules synergize with signals delivered via TCR/CD3 to cause proliferation and cytokine gene expression in T cells *The Journal of Immunology* 149 65-70


Stanglmaier M, Reis S, Hallek M, (2000) Stimulation of programmed cell death (PCD) and tyrosine kinases by monoclonal antibodies against CD20 and CD52 in B-CLL and B-cell lines in vitro *Blood* 11 (suppl. 1) 160a


Steinman RM, Cohn ZA, (1973) Identification of a novel cell type in peripheral lymphoid organs of mice I. Morphology, quantitation, tissue distribution *Journal of Experimental Medicine* 137 1142


Terhorst C, van den Elsen P (1985) The T-cell receptor/T3 complex Year Immunology 62-73


Thornton PD, Matutes E, Bosanquet AG, Lakhnai AK, Grech H, Ropner JE, Joshi R, Mackie PH, Douglas DC, Bowcock SJ, (2000) High dose methyl prednisolone (HDMP), alone or in combination with other drugs guided by the disc assay, as salvage treatment for CLL with p53 abnormalities Blood 11 (suppl. 1) 760a


252

Totterman TH, Carlsson M, Simonsson B, Bengtsson M, Nilsson K, (1989) T-cell activation and subset patterns are altered in B-CLL and correlate with the stage of the disease Blood 74 (2) 786-792


253
Viret C, Janeway CA, (1999) MHC and T cell development Reviews in Immunogenetics 1 (1) 91-104


Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA, (1994) CTLA-4 can function as a negative regulator of T cell activation Immunity 1 405-413


Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW, (1995) Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment *Immunity* 3 521-530


Zaki MH, Patten N, Bachinsky M, Nowell PC, Morre JS, (2000) TGFβ network alterations in B-CLL: overexpression of type II TGFβ receptor associated with G0/G1 cell cycle arrest in the non-neoplastic T cells *Blood* 96 (11) (suppl.1) 750a

Zaknoen SL, Kay NE, (1990) Immunoregulatory cell dysfunction in chronic B-cell leukemias *Blood Reviews* 4 165-174


Analysis of the expression of critical activation/interaction markers on peripheral blood T cells in B-cell chronic lymphocytic leukaemia: evidence of immune dysregulation

Sophie Scrivener,1 Edward R. Kaminski,1 Andrew Demaine,2 and Archie G. Prentice1

1Derriford Combined Laboratory, Derriford Hospital, and 2University of Plymouth, Plymouth, UK

Received 19 March 2000; accepted for publication 27 November 2000

Summary. B-cell chronic lymphocytic leukaemia (B-CLL) is characterized by an accumulation of clonal malignant B cells. The intrinsic characteristics that permit this accumulation have been extensively studied and described. However, it is possible that proliferation and survival of this malignant clone is facilitated by a disruption in the interaction between B and T cells that normally regulate the immune system. In this study, using flow cytometry and cell culture techniques, marked abnormalities of the expression of certain key activation molecules on the peripheral blood T cells of patients with B-CLL were demonstrated. In particular, on comparison with normal controls, there was a marked reduction in the number of circulating T cells expressing CD25 (interleukin 2 receptor) \((P = 0.007)\), CD28 \((P = 0.01)\) and CD152 (CTLA-4) \((P = 0.001)\). There was also a reduction in the number of circulating T cells expressing CD4 \((P = 0.03)\), CD5 \((P = 0.05)\) and CD11a \((P = 0.01)\). There was no difference in the number expressing T-cell receptor \(\alpha\beta\) \((P = 0.1)\), CD8 \((P = 0.4)\), CD54 \((P = 0.4)\) and CD154 \((P = 0.5)\), and the only marker expressed on a greater number of circulating T cells in B-CLL patients was HLA-DR \((P = 0.05)\). These results suggest that there is a profound T-cell dysregulation that may contribute to the survival of the malignant B cells in patients with B-CLL and to the related autoimmune phenomena of the disease.

Keywords: B-CLL, T cells, immunophenotyping.
The binding of LFA-1 (CD11a)/ICAM-1 (CD54) allows the cells to be brought into close enough contact for antigen recognition to take place via the T-cell receptor (TCR)/CD3 complex. The immune response continues via signalling between HLA antigens and the TCR, a process that is facilitated by the CD3 complex and strengthened by CD4 (MHC class II) or CD8 (MHC class I) binding, thus triggering the T-cell activation cascade (Benjamins et al. 1996). Signalling through the TCR/CD3 complex leads to the initiation of key T-cell immune responses, including cytokine production and surface marker upregulation. The cell surface marker CD28 on the T-cells interacts with the CD80/CD86 receptors on the B cells and upregulation of CD154 on the T-cell leads to binding with its appropriate ligand, CD40, on the B-cell surface (Durie et al. 1994; Clark et al. 1996; Grewal & Flavell, 1996; 1997; Lenschow et al. 1996). Production of interleukin 2 (IL-2) is initiated and receptors for this cytokine (CD25/IL-2R) are constitutively expressed approximately 48 h post activation, facilitating recruitment of T cells and their continued activation and clonal expansion (Waldmann, 1986, 1991; Taniguchi & Minami, 1993). The final key stage in this cascade is the expression of CTLA-4 (CD152), believed to send a negative 'off' signal to the T cell and to control the immune response, either by terminating T-cell proliferation or by inducing apoptosis (Walunas et al. 1994; Krummell & Allison, 1995; Tivol et al. 1995; Schweitzer & Sharpe, 1998).

This study was therefore designed to examine, in vitro, T cells from the peripheral blood of patients with B-CLL, in five stages. The first was to confirm a previous report of reduced T-cell CD154 expression (Cantwell et al. 1997). The second was to examine expression of other key molecules involved in antigen recognition and T-cell activation: CD25 (IL-2R), CD28, CD152 (CTLA-4), TCRβ, CD4, CD5, CD8 and HLA-DR. The third was to examine the expression of the adhesion molecules critical for cell to cell contact: LFA-1 (CD11a) and ICAM-1 (CD54). The fourth stage involved the removal of the malignant B-cell clone using magnetic bead separation and the subsequent stimulation of the remaining T cells for analysis of two key surface antigens, CD28 and CD152. The final stage involved depleting the B cells, stimulating with OKT3 and permeabilizing for analysis of internal CD25, CD28 and CD152.

PATIENTS AND METHODS

This study was approved by the local research ethics committee.

Patients. Between September 1997 and November 1999, 33 blood samples were taken with informed consent from 27 patients with classic B-CLL who were either untreated or not treated for the preceding 6 months (19 men and eight women, median age 72 years. range 49–86 years). None of the patients had received purine analogues prior to this study. In addition, 26 control samples were taken from 13 age-matched normal subjects.

Cell isolation. Peripheral blood mononuclear cells (PBMCs) were isolated from sodium heparin anticoagulated blood by density gradient centrifugation with the lymphocyte separation medium Lymphoprep (Nycomed Amersham, UK). Thereafter, PBMCs were washed twice in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco Life Technologies, Paisley, UK), counted, resuspended in RPMI-1640 medium supplemented with 10% human AB serum (National Blood Transfusion Service, Bristol, UK).

Cell culture. A controlled comparison between the mitogenic effects of phytohaemagglutinin (PHA) and OKT3 (anti-CD3) was carried out for expression of each of the cell surface markers. In these experiments, the most effective method of upregulating each marker was determined prior to data collection. PBMCs for CD28 and CD154 analysis were cultured for 4 h in RPMI-1640 medium (Gibco) with 10% human AB serum at 37°C in 5% CO2. The culture...
plates (Nycomed) were coated with OKT3 (Janssen-Cilag, High Wycombe, UK) for 2 h at room temperature at a concentration of 1 mg/ml in 500 ml of phosphate-buffered saline (PBS) per well and then washed five times in excess PBS prior to the addition of the cells at a concentration 1 x 10^6 per well.

PBMCs for CD25, CD152 and HLA-DR analysis were cultured for 48 h in RPMI-1640 medium with 10% AB serum at 37°C in 5% CO2, PHA (5 μl) (Murex, Maidenhead, UK) was added to 1 x 10^6 cells at a final concentration of 1 mg/ml for CD25 activation. OKT3 was used as described above for CD152 and HLA-DR analysis.

**B-cell depletion.** Sodium heparin anticoagulated blood was added 1:1 with cold PBS and layered onto Lymphoprep as described above. Cells were resuspended in buffer (PBS + 2 mmol/l EDTA; Sigma-Aldrich, Poole, UK) and 0.5% bovine serum albumin (BSA: Lorne Laboratories, Reading, UK). CD19 microbeads (Miltenyi-Biotec, Bergisch Gladbach, Germany) were added at a concentration of 1 mg/ml for CD25 activation.

**ICAM-1** and **CD28** expression varied significantly between patients and normals (Table I). The Mann–Whitney (Wilcoxon) comparison of medians was used for statistical analysis.

**RESULTS**

**CD154 expression on CD25+ T cells**

A greater number of CD25+ T cells from patients with B- CLL expressed the CD40 ligand CD154 than normals after stimulation (16.6% ± 14 vs. 11.1% ± 4; P = 0.5), although this difference was not significant (Table I). The use of an anti-CD3 mitogen followed by an anti-CD3 monoclonal antibody may effect results previously described by others (Cantwell et al. 1997). OKT3 triggers stimulation of T cells by binding to and occupying the CD3 receptor (Sgro, 1995; Bonnefoy-Berard & Revillard, 1996; Reinke et al. 1997; Brusa et al. 1998). We have shown that if a CD3 monoclonal antibody is subsequently used to identify the T-cell population, a false low number is obtained. OKT3 receptor occupation will prevent and/or reduce CD3 monoclonal antibody (mAb) binding. We therefore used CD2 as our pan T-cell marker and subsequently showed that we were able to identify a greater number of T cells than with CD3 (81.2% ± 3.1 vs. 71.3% ± 5.6; CD3, P = 0.04).

**CD25, CD28, CD152, TCRαβ, CD4, CD8 and HLA-DR expression on CD28+ T cells**

Significantly fewer T cells from B-CLL patients than normals expressed the markers CD25 (IL-2R) (30.7% ± 27 vs. 76.5% ± 18; P = 0.007), CD28 (52.8% ± 23 vs. 79.9% ± 12; P = 0.01) and CD152 (CTLA-4) (1.9% ± 2 vs. 15.5% ± 7, P = 0.001) after stimulation. Fifty percent of B-CLL patients failed to express any surface CD152 following OKT3 activation (range 0–7.5%), while the percentages of CD28+ T cells expressing CD25 after activation varied greatly between patients (range 18–91.2%) (Table II).

In unstimulated T cells from patients with B-CLL compared with those from normals, there was a significant reduction of the percentages of cells expressing the cell surface markers CD4 (47.4% ± 27 vs. 66.2% ± 4; P = 0.03) and CD8 (85.0% ± 10 vs. 99.2% ± 1,

© 2001 Plymouth Postgraduate Medical School, *British Journal of Haematology* 112: 959–964
Table II. Expression of cell surface markers that were significantly different between patients and normals.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell marker</th>
<th>B-CLL (mean ± SD)</th>
<th>Normal (mean ± SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>HLA-DR</td>
<td>90·7% ± 18·5</td>
<td>65·9% ± 18·7</td>
<td>0·05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(63·1–100%)</td>
<td>(68·6–89·2%)</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD4</td>
<td>47·4% ± 27·4</td>
<td>66·2% ± 4·9</td>
<td>0·03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(16·6–100%)</td>
<td>(58·7–71·3%)</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD5</td>
<td>85·9% ± 10·4</td>
<td>99·2% ± 1·4</td>
<td>0·05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(74·5–100%)</td>
<td>(99·6–100%)</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD23</td>
<td>30·7% ± 27·9</td>
<td>76·5% ± 18·4</td>
<td>0·007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1·8–91·2%)</td>
<td>(43·0–95·1%)</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>CD11a</td>
<td>77·9% ± 22·5</td>
<td>99·9% ± 0·08</td>
<td>0·01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(43·6–100·0%)</td>
<td>(99·8–100·0%)</td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>CD28</td>
<td>52·8% ± 23·2</td>
<td>79·9% ± 12·1</td>
<td>0·01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(19·5–100·0%)</td>
<td>(56·4–92·4%)</td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>CD152</td>
<td>1·9% ± 2·7</td>
<td>15·5% ± 7·6</td>
<td>0·001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0·0–7·5%)</td>
<td>(6·4–24·4%)</td>
<td></td>
</tr>
</tbody>
</table>

*P = 0·05, with a wide range of expression between patients (Table II).

Although percentages of unstimulated CD3+ cells from B-CLL patients expressing CD8 and CD38 were lower, these were not significant differences (Table I).

In contrast, significantly greater numbers of stimulated CD3+ cells from B-CLL patients expressed HLA-DR compared with normals (90·7% ± 18 vs. 65·9% ± 18, P = 0·05) (Table II). Expression of adhesion molecules LFA-1 (CD11a) and ICAM-1 (CD54). Significantly fewer T cells of patients with B-CLL expressed the adhesion molecule LFA-1 (CD11a) than normals (77·9% ± 22 vs. 99·9% ± 0·08, P = 0·01) (Table II) in unstimulated cells. Expression of ICAM-1 (CD54) was not significantly different between patients and normals (Table I).

Expression of CD28 and CD152 on stimulated CD2+ T cells after depletion of B cells

T cells from patients with B-CLL continued to show a reduction in the expression of the two surface antigens CD28 (45·0% ± 19·3 vs. 88·3% ± 8·6, P = 0·01) and CD152 (6·8% ± 5·1 vs. 15·7% ± 8·1, P = 0·05) compared with normal controls after depletion of the malignant CD19+B-cell population and stimulation of the remaining cell population (Table III). CD19 cells were depleted from PBMCs and any remaining B cells were counted using CD20. The mean CD20 count after B-cell depletion was 3·2% for B-CLL patients and 2·3% for normal controls.

Expression of intracellular CD25, CD28 and CD152 on stimulated and unstimulated CD2+ T cells after B-cell depletion

After permeabilization of stimulated CD2+ cells, intracellular CD25 was expressed in significantly fewer cells in B-CLL patients than in normals (46·2% ± 18·0 vs. 65·9% ± 6·7, P = 0·02) (Table IV). CD25 and CD152 positivity were also reduced on stimulated T cells, but not significantly. In contrast, significantly more unstimulated CD2+ cells from CLL patients expressed intracellular CD25 (7·3% ± 3·3 vs. 3·3% ± 2·2, P = 0·01) and intracellular CD152 (9·2% ± 4·1 vs. 5·4% ± 2·6, P = 0·03) than normals. Intracellular CD28 positivity was reduced in unstimulated CD2+ cells from CLL patients, but this difference was not significant.

When levels of intracellular CD25, CD28 and CD152 were compared in activated and unactivated cells in normals and patients, there was a significant difference seen in the expression of CD25 and CD152 after activation. In contrast, levels of CD28 were not significantly different in either patients or controls after activation.

**DISCUSSION**

These results suggest that the circulating T-cell compartment in the peripheral blood is profoundly dysregulated in patients with B-CLL. Expression of key cell surface activation and interaction markers is markedly reduced after activation, with the exception of HLA-DR and CD154, the ligand for B-cell CD40. Each of the key markers discussed here plays a major role in stimulating not only the T cell on which it is expressed, but also in the activation of and interaction with many other key immune responders. The complete lack of expression of surface CD152 (CTLA-4) on the circulating T cells of half the patients with B-CLL, the persistence of its reduced expression after B-cell depletion and the increased intracellular expression in unstimulated cells are of interest. CD152 plays a pivotal role in immune regulation by effectively providing a negative feedback ('switch-off') signal to the T cell once an immune response has been initiated and completed (Walunas et al. 1994; Krummel et al. 1995; Tivol et al. 1995; Schweitzer & Sharpe, 1998).

The finding of increased expression intracellularly in unstimulated cells suggests that these T cells may be in a partial state of activation, yet are unable to effectively express the antigen or signal externally. Without the external expression, failure to 'switch off' the T-cell compartment could lead to the survival of a clone of T cells in this partial state of activation, able to make weak immune responses; possibly against self-antigen, yet unable to mount an effective response to known T-cell mitogens. CD152 knock-out mice display a clinical and pathological syndrome that is similar to that seen in the B-CLL patient (Tivol et al. 1995). This includes spontaneous lymphoproliferative disease with lymphocytic infiltrates in many

© 2001 Plymouth Postgraduate Medical School, *British Journal of Haematology* 112: 959–964
tissues, splenomegaly, lymphadenopathy and higher rates of autoimmune phenomena than in CD152+ mice. Failure to complete a T-cell response or a continued partial response to malignant B-cell antigens could facilitate their proliferation in these mice and in human subjects. The lack of expression of CD152 may result in a failure to delete autoreactive T cells or prevent antigen-specific apoptosis of activated T cells. Anderson et al (2000) have recently shown that, if T cells are not fully activated or the TCR signal is weak, blockade of CD152 paradoxically inhibits immune responses. The lack of expression of CD152 demonstrated in the CLL T cells may equate to blockade in this respect.

Reduced expression of surface and intracellular CD28 after activation suggests a relative inability to interact effectively with any cell that expresses the CD28 ligands CD80 and CD86, including B cells, and thus an impaired ability to promote antigen presentation and processing. Cell–cell adhesion may also be impaired because of reduced expression of LFA-1 (CD11a). A reduced expression of surface CD25 after activation suggests a relative inability to respond to II-2 and, thus, an impaired ability to control T-cell activation. However, an increased expression of intracellular CD25 in unstimulated T cells correlates with the finding of increased internal CD152. Both results imply that the T cells are in a partially activated state. Reduced expression of CD4 implies that the initial activation signal generated by MHC class II antigen presentation through the CD3/TCR/CD4 complex on the T-cell surface may be weakened, although reduction in expression of TCRaβ is not statistically significant. CD4 plays an important role in both adhesion between B and T cells and also in generating unique and rapid signals to the cell nucleus for activation (Benjamini et al. 1996). A reduction or absence of this antigen may impair or prevent transmission of signals for T-cell activation.

In contrast to our findings of reduced expression of these markers and to the findings of others (Cantwell et al. 1997), we have shown that surface CD154 (CD40L) is expressed equally on T cells in normal subjects and B-CLL patients. By using CD2 as the pan T-cell marker after activation instead of CD3, we may have included T cells that would not have been detected owing to CD3 receptor occupation by OKT3 or other anti-CD3 activators. OKT3 is believed to occupy and modulate the CD3 antigen and to either become endocytosed into the cell or block the receptor, preventing antibody binding and thus reducing the total number of T cells that will stain positively with CD3 (Sgro. 1995; Bonnefoy-Berard & Revillard, 1996; Reineke et al., 1997; Brusa et al. 1998).

We have also shown that HLA-DR is expressed on significantly more T cells in B-CLL patients than in normal controls. This anomaly of normal or increased expression of CD154 and HLA-DR compared with reduced expression of the other antigens in the activation pathway requires further investigation.

These abnormalities of cell surface antigen expression on B-CLL T cells are not altered by depletion of the malignant B-cell clone. This implies that either the effect of the malignant cells is long-lived or that the T-cell abnormality is a primary one. The former explanation is the more probable, suggesting a chronic but not necessarily irreversible dysfunction of the T-cell compartment. Studies are currently underway to address this question.

ACKNOWLEDGMENTS

This work was supported by grants from the Cornwall Leukaemia Trust and the Plymouth and District Leukaemia Fund. We thank Dr Anton Kruger for his encouragement and support, Nick Hurlock for excellent technical assistance and Dr Adrian Copplestone for patient selection.

REFERENCES


Chiorazzi, N., Fu, S.F., Montazeri, G., Kunkel, H.G., Rai, K. & Gee, T.