Identification of Tumour Associated Antigens as Potential Targets for the Immunotherapy of B-cell Chronic Lymphocytic Leukaemia (B-CLL)

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To mom and dad with all my love and gratitude..
Acknowledgment

“As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them” John Kennedy

On the 27th of June at 6:06 am, the last word of this thesis was written down and the challenge was overcome. Now it is the time to acknowledge all the people who contributed directly or indirectly in the accomplishment of this work, without whom this thesis would not have been completed.

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Abstract

Identification of Tumour Associated Antigens as Potential Targets for the Immunotherapy of B-cell Chronic Lymphocytic Leukaemia (B-CLL)

Hind Abdulrazak Yassin Abdulmajed

B-CLL, the most common adult B-cell malignancy in USA and the western world, is characterized by the accumulation of CD5+ mature B-cells in blood, bone marrow and lymphoid tissues. Many cases require no treatment because of an indolent course, while other patients become symptomatic or develop signs of rapid progression. Treatment is usually non-curative and is directed at reducing the symptoms. However, new therapies are currently under investigation aiming to achieve a curative therapy for B-CLL. The increasing understanding of how peptide antigens are processed, transported and presented by HLA, the identification of tumour antigens recognised by cytotoxic T lymphocytes and the identification of T-cell epitopes on human tumor cells, have opened the routes for the development of more efficient strategies for tumour immunotherapy. Several antigens have been characterized as tumour associated antigens (TAA) in B-CLL, with the potential to elicit specific anti-tumour responses.

This thesis addresses the role of survivin, a member of the family of inhibitor of apoptosis proteins, as a potential target for the immunotherapy of B-CLL. Data on survivin expression showed no/ little expression of survivin in resting B-CLL, which was upregulated by in vitro activation with CD40 ligation, CpG-oligodeoxynucleotides (ODN), and with both stimulators together. CD40 ligation resulted in greater B-CLL cell activation, survivin expression, upregulation of activation markers (CD54, CD80 and CD86), and in enhanced activity of B-CLL cells to stimulate allogeneic T cell proliferation, compared to the other two ways of stimulation.

Studying the induction of autologous tumour specific T cell responses in vitro, CD40L activated B-CLL cells enhanced CTL responses compared to unactivated cells. No significant difference in response of B-CLL patients T cells to survivin peptide pulsed T2 cells was found between HLA-A2+ and HLA-A2- patients, and no clear peptide specific responses were seen in either group. After investigating survivin peptides for their potential to induce cytotoxic T cell responses, generating survivin specific CTL line from a healthy donor was attempted using peptide pulsed autologous monocyte derived dendritic cells, but was unsuccessful suggesting that no T cells in the culture were able to recognise survivin peptides, or that the culture conditions used were inappropriate for the generation and maintenance of such responses.

Finally, in investigation of other TAA expression in B-CLL cells, no expression of MAGE-A1, MAGE-A3, Proteinase-3, WT-1 was detected pre- and post CD40 activation. Positive PRAME expression was detected in 8 out of 20 CD40L activated CLL cells whilst NY-ESO-1 showed an upregulation in one sample. Overall results indicate that much work is still needed to study the potential of immunotherapy in the treatment of B-CLL.
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List of Abbreviation

ADCC: Antibody dependent cell mediated cytotoxicity
Ag: Antigen
ALCL: Anaplastic large cell lymphomas
ALK: Anaplastic lymphoma kinase
ALL: Acute lymphoblastic leukaemia
AML: Acute myeloid leukaemia
APCs: Antigen presenting cells
BCG: Bacille Calmette- Guerin
B-CLL: B- cell chronic lymphocytic leukaemia
BcR: B cell receptor
BIR: Baculovirus IAP Repeat
BM: Bone marrow
CARD: Caspase recruitment domain
CD: Cluster of differentiation
CD40L: CD40 ligand
CDR3: Complementary determining region-3
CLL: Chronic lymphocytic leukaemia
CML: Chronic myeloid leukaemia
CpG-ODN: Cytidine phosphate Guanosine- oligodeoxynucleotides
cpm: counts per minute
CR: Complete remssion
CTA: Cancer/ testis antigens
CTL: Cytotoxic T lymphocytes
DCs: Dendritic cells
DLI: Donor lymphocyte infusion
DNA: Deoxyribonucleic acid
dNTP: deoxynucleotides
EBV: Epstein Bar virus
ECL: Electrogenerated chemiluminescence
ELISpot: Enzyme Linked ImmunoSpot
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated mitogen-activated protein kinase
FACS: Fluorescence activated cell sorting
FcR: Fc receptor
FCS: Fetal calf serum
FISH: Fluorescence in situ hybridisation
FITC: Fluorescein isothiocyanate
FMOD: Fibromodulin
FSC: Forward scatter
GalCer: Galactosylceramide
GC: Germinal centre
GM-CSF: Granulocyte macrophage colony stimulating factor
Hb: Haemoglobin
HBV: Hepatitis B virus
HCC: Hepatocellular carcinoma
HCV: Hepatitis C virus
HLA: Human leukocyte antigen
HPLC: High performance liquid chromatography
HRP: Horseradish peroxidase
IAP: Inhibitor of apoptosis protein
IFN-γ: Interferon-gamma
Ig: Immunoglobulin
IgVH: Immunoglobulin V region
IL: Interleukin
IMTs: Inflammatory myofibroblastic tumours
Jak3: Janus kinase 3
JNK: Jun amino-terminal kinase
LAA: leukaemia associated antigens
mAbs: Monoclonal antibodies
MAGE: Melanoma antigen
MAPK: Mitogen-activated protein kinase
MCA: Methylcholanthrene
MDCs: Myeloid derived DCs
MDM2: Murine double-minute 2 oncoprotein
MFI: Mean fluorescence index/intensity
MHC: Major histocompatibility complex
MIIC: MHC class II compartments
ml: milliliter
MLR: Mixed lymphocyte reaction
Mo: Monocytes
MUC-1: Mucin
MVA: modified vaccine virus Ankara
Mφ: Macrophage
NCI: National cancer institute
NK: Natural Killer
NKR-P1: Natural killer receptor-P1
NSCLC: Non small cell lung cancer
ODN: Oligodeoxynucleotides
OR: Overall remission
PBMC: Peripheral Blood Mononuclear Cells
Pc5: Phycoerythrin- Cyanine-5
PDCs: Plasmacytoid DCs
PE: Phycoerythrin
PR-3: Proteinase-3
PRAME: Preferentially expressed antigen of melanoma
qRT-PCR: quantitative real time PCR
RNA: Ribonucleic acid
rpm: revolutions per minute
RPMI: Roswell park memorial institute
RT-PCR: Reverse transcriptase-polymerase chain reaction
RT: Room Temperature

XI
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
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<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
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<tr>
<td>SCT</td>
<td>Stem Cell Transplantation</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
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<tr>
<td>SEREX</td>
<td>Serologic screening of recombinant expression libraries</td>
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<tr>
<td>TAA</td>
<td>Tumour associated antigens</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T_H</td>
<td>helper T cells</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour infiltrating lymphocyte</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>TRAF</td>
<td>TNF-R associated factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>WBC</td>
<td>White blood cells</td>
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<tr>
<td>WT-1</td>
<td>Wilm tumour antigen-1</td>
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<tr>
<td>β2M</td>
<td>β2 microglobulin</td>
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1. Introduction
1.1 B-cell chronic lymphocytic leukaemia (B-CLL)

Leukaemias are a group of heterogeneous neoplastic disorders of white blood cells (WBC). Based on their origin, myeloid or lymphoid, each of which can be acute or chronic, they can be divided into four major types: acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myeloid leukaemia (CML) and chronic lymphocytic leukaemia (CLL). CLL represents a monoclonal expansion of lymphocytes. In 95% of cases, CLL is a malignant clonal disorder of B lymphocytes. The remainder is secondary to a T cell clone. There are also many subtypes of the four major varieties of leukaemia, and the outlook is highly variable (Guipaud et al., 2003).

B-CLL is the most common adult leukaemia in the Western world. The current view of the immunobiology of B-CLL has changed considerably in recent years. Previously, it was believed that the B-CLL cells were functionally immature (Gale and Foon, 1987; Rosenblatt et al., 1991), minimally self-renewing and with defective apoptosis mechanisms (Chiorazzi et al., 2005; Ghia and Caligaris-Cappio, 2006; Raval et al., 2006). B-CLL is now viewed as a malignant disease of antigen experienced mature B lymphocytes that escape programmed cell death due to interactions with factors produced by other cells, including T cells (Chiorazzi et al., 2005; Ghia and Caligaris-Cappio, 2006; Raval et al., 2006). This disease arises from the accumulation of monoclonal mature B-lymphocytes in the peripheral blood, bone marrow, lymph nodes and spleen (Schroers et al., 2004). B-CLL is phenotypically characterized by cell surface co-expression of CD19, CD20, CD5, and CD23 (Jurlander, 1998) but low or absent expression of CD22, FMC7, CD79b and surface immunoglobulins (IgM and IgD) (Guipaud et al., 2003).

1.1.1 Epidemiology of B-CLL

B-CLL represents 22-30% of all leukaemia cases with a worldwide incidence projected to be between <1 and 5.5 per 100,000 people. Higher rates are found in Australia, North America and Europe (especially Ireland, Italy and Switzerland) (Redaelli et al., 2004). It is extremely low in Japan and Southeast Asia and the incidence of CLL in Africa is apparently not as low as it is in Asia (Pangalia et al., 2002).
B-CLL is considered to be mainly a disease of the elderly, with a median presenting age of 64-70 years (Redaelli et al., 2004). Only 10% of patients diagnosed with B-CLL are under the age of 50, and 1-2% are younger than 40 (Kay et al., 2002). The incidence of B-CLL increases dramatically with age. In 2003 in the US, people over the age of 65 had an incidence of 21 per 100,000 while those under the age of 65 had an incidence of 1.2 per 100,000. Because many B-CLL patients are asymptomatic, the true incidence is unknown. B-CLL presents in adults at higher rates in males than in females (2 to 3 times more), and in whites more than in blacks. The mortality rate for B-CLL in the US in 2003 was 1.5 deaths per 100,000. Males (2.2 per 100,000) had a higher mortality rate than females (1.04 per 100,000) in 2003 (Redaelli et al., 2004).

Researchers have attempted to correlate environmental risk factors with the incidence of B-CLL, but the evidence has not been strong for any single factor yet. Exposure to pesticides, ionizing radiation, carcinogens, diets, alkylating agents, hepatitis C and Epstein Barr virus have been suspected, but not consistently associated with B-CLL. Living in a farming community and the exposure to electromagnetic radiation has also been associated with B-CLL, but the evidence is not strong (Redaelli et al., 2004).

1.1.2 Aetiology of B-CLL

The cause of B-CLL is not yet known, however a genetic aetiology may offer the best explanation. Some studies of large series of B-CLL patients demonstrated that in about half of the B-CLL cases, the immunoglobulin V region (IgVH) genes have undergone somatic mutations (Schroeder et al., 1994; Fais et al., 1998), and the unmutated cases were correlated to a worse clinical outcome (Damle et al., 1999; Hamblin et al., 1999). The presence of somatic mutations suggest that at least in the mutated patients, B-CLL cells had encountered an antigen and that the procedure triggered by this encounter had likely occurred in a germinal centre (GC) site (Volkheimer et al., 2007). However, based on other evidences, the unmutated cases could not be considered naïve B-cell driven and are similar to mutated ones, except for IgVH. Although slightly different, both subsets of B-CLL cells show a remarkable twist in IgVH genes usage, such as the overexpression of IgVH 1-69 and IgVH 3-21 (Fais et al., 1998). This bias appears to be disease related,
therefore suggesting a role for specific antigen or antigen-like elements in the genesis of this subset of B-CLL (Ghia and Caligaris-Cappio, 2006). In addition, both B-CLL subsets have surface phenotype of activated B cells similar to the phenotype of antigen experienced cells (CD23, CD25, CD69, and CD79) (Damle et al., 2002). The characteristics of B-CLL cells B cell receptors (BeR) look like those observed in normal B cells upon antigen interaction (Alfarano et al., 1999; Cragg et al., 2002). Finally, gene expression profiling revealed that both mutated and unmutated subsets share similarities with memory B-cells (Klein et al., 2001; Rosenwald et al., 2001). From all of these evidences, the impression has arisen that in B-CLL, regardless of the IgV<sub>H</sub> genes mutational status, the origin of cell appears to be an “antigen experienced B cell” (Ghia and Caligaris-Cappio, 2006).

Data of complementary determining region 3 (CDR3) sequences of the BCRs of the B-CLL cells are in line with the involvement of Ag or Ag-like element in the aetiology of B-CLL. Several studies showed that unrelated patients in different parts of the world express very similar, if not identical BCR (Ghia et al., 2005; Ghiotto et al., 2004; Messmer et al., 2004; Tobin et al., 2004; Widhopf et al., 2004).

The CLLU1 gene has been recently reported as a disease-specific gene due to its restricted expression pattern in unmutated B-CLL cells that is irrespective of trisomy 12 or large chromosomal rearrangements (Buhl et al., 2006). CLLU1 maps to a region on chromosome 12q22, surrounded by genes that are active in germinal center B cells and CLL cells, suggesting that the region is accessible for transcription in B cells. However, whether the CLLU1 is involved in the aetiology of B-CLL is not clear yet.

1.1.3 Clinical manifestations: symptoms and signs of B-CLL

B-CLL patients may have a wide range of symptoms with physical and laboratory abnormalities at the time of its diagnosis or later. Common findings in patients requiring treatment include lymphadenopathy (87%), splenomegaly (54%), hepatomegaly (14%), total white blood cell count > 100,000/μl (30%), haemoglobin concentration <11.0g/dL (31%) and platelet count <100,000/μl (16%) (Keating et al., 1998). However,
approximately 25% of patients feel well with no symptoms when a routine blood count reveals an absolute lymphocytosis, leading to a diagnosis of B-CLL (Redaelli et al., 2004). On the other hand, approximately 5 to 10% of patients showed typical symptoms defined by one or more of the following: increased fatigue, increasing inability to work, exercise or perform usual activities, excess sweating (night sweats), fever without the evidence of infection and weight loss (≥ 10% of the body within the previous six months) (Cheson et al., 1996). The presenting features may also relate to infections, autoimmune complications such as haemolytic anaemia or thrombocytopenia (Cheson et al., 1996). B-CLL patients are at higher risk for skin cancer (melanoma), soft tissue sarcoma, colorectal cancer, lung cancer, multiple myeloma, AML, and myelodysplastic syndrome. In very rare cases, B-CLL patients can develop ALL. The increased susceptibility to cancer could result from a loss of protective immune surveillance mechanisms or as a side effect of chemotherapy (Andritsos and Khoury, 2002).

### 1.1.4 Diagnosis of B-CLL

B-CLL is often discovered by chance when a patient has a routine blood test. It is important to distinguish B-CLL from other leukaemic lymphoproliferative disease e.g. mantle cell lymphoma, splenic lymphoma, marginal zone lymphoma, or hairy cell leukaemia (Table 1.1). Therefore, a minimum of two examinations is required to diagnose B-CLL:

1. Evaluation of blood lymphocyte count.
2. Immune phenotype of the leukaemia cells in the blood.

A crucial part of the B-CLL diagnosis is determining the immunophenotype of the leukaemia. Flow cytometry is a very accurate immunophenotyping tool that identifies the presence or absence of specific markers on blood or bone marrow cells, detect the mutated cells and determine their type. A minimal panel of cell surface markers to distinguish B-CLL from other entities include CD5, CD19, CD20, CD23 and surface Ig. Another characteristic of B-CLL is that the level of CD20, CD22 and CD79b typically are lower than that of normal B cells.
Histological examination of the bone marrow (BM) can access the extent and pattern (diffuse, non-diffuse) of the BM infiltration by B-CLL but it is not necessary for the diagnosis. A marrow biopsy and flow cytometry is also indicated to determine whether there is complete regression in the BM following treatment (Cheson et al., 1996; Binet et al., 2006). Additional investigations such as lymph node biopsy, cytogenetic/fluorescence in situ hybridisation (FISH) analysis, computed tomography scan and/or ultrasound may also be helpful in the diagnosis (Oscier et al., 2004).

1.1.5 Staging of B-CLL

B-CLL is clinically staged based on two somewhat different major systems: the Rai system (1975) and the Binet systems (1981). Both of them are still in use throughout the world, although the first one is used more often in the USA while Binet system is used most often in Europe (Cheson et al., 2000). Both schemes demonstrate that more widespread lymphocyte infiltration is associated with a more advanced disease stage and a shorter survival time (Kalil and Cheson, 1999). These two classification systems are outlined in Table 1.2 and 1.3. They recognize early, intermediate and advanced disease. Parameters which participate in the formulation of these systems are the presence and

<table>
<thead>
<tr>
<th>Antigen</th>
<th>B-CLL</th>
<th>Mantle Cell Lymphoma</th>
<th>Splenic Lymphoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIg</td>
<td>Weak</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD5</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CD19</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD20</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD22</td>
<td>Weak or -</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD23</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD79b</td>
<td>Weak or -</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>FMC7</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.1 Immunophenotype of B-CLL and lymphoma that resemble it (Kay et al., 2002).
extent of lymphadenopathy by physical examination, the presence of splenomegaly and/or hepatomegaly, and the degree of anaemia and thrombocytopenia. Furthermore, the Rai and Binet staging systems do not discriminate between disease-related cytopenias and other cytopenias. Although the Rai and Binet systems by themselves are prognostic and help to guide treatment, there are other factors that recently have been shown to be superior predictive of disease prognosis such as those mentioned later in Table 1.4.

<table>
<thead>
<tr>
<th>Risk Level</th>
<th>Stage</th>
<th>Clinical features at diagnosis</th>
<th>Median survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0</td>
<td>Blood and marrow lymphocytosis</td>
<td>10 years</td>
</tr>
<tr>
<td>Intermediate</td>
<td>I</td>
<td>Lymphocytosis and lymphadenopathy</td>
<td>9 years</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Lymphocytosis and splenomegaly or hepatomegaly</td>
<td>7 years</td>
</tr>
<tr>
<td>High</td>
<td>III</td>
<td>Lymphocytosis and anaemia (haemoglobin&lt;11g/dL)</td>
<td>5 years</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>Lymphocytosis and thrombocytopenia (platelets&lt;100000/µl)</td>
<td>5 years</td>
</tr>
</tbody>
</table>

Table 1.2 Rai classification system for B-CLL (Rai et al., 1975; Redaelli et al., 2004)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical features at diagnosis</th>
<th>Median survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Blood and marrow lymphocytosis and less than three areas of palpable lymphoid involvement, Hb=10g/dL and platelets=100x10⁹/L</td>
<td>&gt;7-10 years</td>
</tr>
<tr>
<td>B</td>
<td>Same with three or more areas of palpable lymphoid involvement, Hb=10g/dL and platelets=100x10⁹/L</td>
<td>5-7 years</td>
</tr>
<tr>
<td>C</td>
<td>Same plus anaemia or thrombocytopenia, Hb less than 10g/dL and/or platelets less than 100x10⁹/L</td>
<td>&lt;2-5 years</td>
</tr>
</tbody>
</table>

Table 1.3 Binet classification system for B-CLL (Binet et al., 1981; Cheson et al., 1996; Redaelli et al., 2004)
1.1.6 Prognosis of B-CLL

It has been recognized for years that cases of B-CLL have variable clinical courses. This wide range in survival times for patients with B-CLL, from a few years to more than a decade, made therapeutic decisions difficult, particularly because some patients remained well even if they were not treated. This led to the development of the staging system, based on prognostic factors, to facilitate the choice of therapy for the patient. As B-CLL progresses slowly, early stage B-CLL is generally not treated since it is believed that early B-CLL intervention does not improve survival time or quality of life. Instead the condition is monitored over time. The decision to start B-CLL treatment is taken when the patient’s blood work and clinical symptoms indicate that the disease has progressed to a point in which the patient status needs to be treated. The objective criteria for treatment instigation are given in Table 1.5.

Clinical staging is still the most commonly used predictor of survival in B-CLL, but is not sufficient to estimate the prognosis reliably for patients with early stage disease (e.g. Binet stage A, Rai stages 0-II) (Binet et al., 2006). Therefore, additional parameters have been devised to more accurately evaluate the prognosis of patients with B-CLL. Table 1.4 lists the various prognostic factors in patients with B-CLL that have been identified by multivariable analysis.
In addition to the prognostic factors mentioned above, there are current helpful biologic paradigms that are linked to the B-CLL cells, including their low level of cell cycle activity, resistance to apoptosis, common recurring genetic defects, and defective antigen presenting capacity. Other less well known but relevant biologic aspects include the capacity of B-CLL cells to produce multiple cytokines that impact apoptosis or drug resistance and their ability to be induced to undergo signalling by these cytokines (Kay et al., 2002).

### 1.1.7 Treatment of B-CLL

Treatment for patients with B-CLL has been improved over the past several years, owing to advances in understanding the biology of the disease. So far no treatment regimen has been shown to be curative in patients with B-CLL. Because no therapy for B-CLL has been shown to be curative and toxicities associated with the therapeutic
regimens are expected, the decision to initiate therapy is an important consideration of B-CLL.

B-CLL treatment focuses on controlling the disease and its symptoms rather than on a complete cure. Initial B-CLL treatments vary depending on the exact diagnosis and the progression of the disease. National Cancer Institute (NCI) investigators have published criteria for treatment initiation within the context of clinical trials, applicable either at diagnosis or during the clinical course of the disease, as listed on Table 1.5.

1. Presence of at least one of the following disease related symptoms:
   a. Weight loss $\geq 10\%$ within the previous 6 months
   b. Extreme fatigue
   c. Fever of greater than 38°C for $\geq 2$ weeks without evidence of infection
   d. Night sweats without evidence of infection

2. Evidence of progressive marrow failure as manifested by the development of, or worsening of, anaemia and/or thrombocytopenia.

3. Autoimmune anaemia and/or thrombocytopenia poorly responsive to corticosteroid therapy

4. Massive ($>6$cm below the left costal margin) or progressive splenomegaly

5. Massive nodes or clusters (i.e. $>10$cm in longest diameter) or progressive lymphadenopathy

6. Progressive lymphocytosis with an increase of $>50\%$ over a 2 month period or an anticipated doubling time of less than 6 months

7. Marked hypogammaglobulinaemia or the development of a monoclonal protein in the absence of any of the above criteria for active disease

Table 1.5 National Cancer Institute (NCI) criteria for treatment initiation in B-CLL patients (Cheson et al., 1996).

Patients at a very early stage may not require chemotherapy at all. In fact, most patients do not receive treatment after their initial diagnosis, and could be followed without any evidence of progressive development (Dreger and Montserrat, 2002). On the other hand, some other patients develop early symptoms of bone marrow failure, repeated infection and transformation into more aggressive forms of the disease. Infection is a major consequence of B-CLL disease itself, as well as a side effect of treatment. In 20-100% of B-CLL cases, patients have a defective immune system because of dysfunctional B-cells and T cells (Andritsos and Khoury, 2002).
1.1.7.1 *Single-agent therapies*

Historically, the approach to the treatment of B-CLL was based on the **alkylating agents**, most commonly chlorambucil (Keating *et al.*, 2003). With such therapy, the complete remission (CR) rate is however not more than 10%, and the overall response (OR) rate is approximately 50%-60% with median survival of 48 to 60 months. Given its lower toxicity, lower cost, oral administration and comparable survival rates, chlorambucil is an effective first line treatment (Hamblin, 2001). Although it is usually given to patients who cannot tolerate chlorambucil, cyclophosphamide is another alkylation agent used to treat B-CLL, and is effective too (Kipps, 2000)

**Glucocorticoids** such as oral prednisone or intravenous methylprednisolone were also reported to be useful in controlling the disease in 10% of patients. However, treatment with any of the agents above as well as purine analogs mentioned below has been associated with increased risk of infection.

**The purine analogue:** the introduction of the purine analogs during the mid 1980s rejuvenated research in B-CLL. These agents include the adenosine deaminase inhibitor pentostatin and fludarabine and cladribine (Hamblin *et al.*, 2002). Fludarabine is the most effective drug for the treatment of B-CLL (Hamblin, 2001). As a first line therapy, overall response rates are about 70%, with 20% to 63% complete response. However, a survival benefit has not been noted. Fludarabine may also be used after alkylating agent failure, as response rates are as high as 45%. Patients who fail three courses of fludarabine should be treated with another drug. Factors associated with a poor response include advanced disease stage, prior treatment, old age and low albumin (Cheson, 2000).
Cladribine is deoxyadenosine analogue that may not be as effective as fludarabine. However, this agent is more popular in Europe than in the USA (Hamblin, 2001). Pentostatin is also a purine analogue but its response of only 25% makes it the least effective for the treatment of B-CLL (Redaelli et al., 2004). Purine analogues are typically stopped after two cycles if there is no benefit because side-effects are so significant (Cheson, 2000).

In a recent publication of a phase II study, it was demonstrated for the first time that single agent lenalidomide is clinically active in B-CLL. Lenalidomide is an immunomodulating drug with antitumour activity. 47% of patients with relapsing or refractory B-CLL attain overall response or complete remission to lenalidomide. Antileukaemic effects were noted 7 days after the therapy, with incremental responses with extended therapy. Lenalidomide also has T-cell stimulatory effect (Chanan-Khan et al., 2006).

**Monoclonal antibodies**

Monoclonal antibodies (mAb) are emerging as attractive agent in the treatment of B-CLL (Lundin and Österborg, 2004). The first mAb approved for use in lymphoid malignancies was Rituximab (Keating et al., 2003). Rituximab (Rituxin) is a chimeric anti-CD20 monoclonal antibody. The overall response rate is 30-50% in previously treated patients, but may be higher in patients receiving escalated doses, and much higher (up to 85%) in previously untreated patients (Pangalis et al., 2002). Unlike Alemtuzumab, Rituximab has less myelosuppresion and potential for cellular immune suppression and is therefore a good candidate to combine with other active agents in B-CLL. In addition, it has been demonstrated that Rituximab monotherapy down regulates that anti-apoptotic proteins mcl-1 and XIAP expression in B-CLL cells in vivo offering in this way an enhanced response to fludarabine based therapy (Kay et al., 2002).
Alemtuzumab (Campath-1 H) is a humanized anti-CD52 mAb that binds to the cell membrane of >95% of all normal human blood lymphocytes, particularly on malignant B- and T- cells in all lymphoid malignancies and on monocytes and macrophages (Pangalis et al., 2002). Side effects include fever, nausea, vomiting, urticaria, hypotension, severe myelosuppression. However, the main side effect when using this mAb is depletion of normal lymphocytes and thereby an increased risk of infections (Redaelli et al., 2004). Due to the expression of CD52 on normal lymphoid cells, long-lasting lymphocytopenia occurs in all patients (Keating et al., 2002; Lundin et al., 2004). The most profound effect are on CD4 T cells, which reach their trough value after about 4 weeks of therapy and thereafter show a slow but steady improvement (Keating et al., 2002; Lundin et al., 2004). The rise in CD4 T cells after Alemtuzumab therapy may partly be explained by expansion of a small population of pre-existing CD52 negative T cells (Lundin et al., 2004).

Other antibodies that target B-CLL cells include (Kay et al., 2002):
- HU1D10: a humanised mAb that targets a β-chain epitope of HLA-DR and appears to induce apoptosis via a novel pathway as compared to other mAbs.
- IDEC-152: a mAb that targets CD23 and appears to cause apoptosis.

1.1.7.2 Combination therapy

The effectiveness of combination therapy versus single agent monotherapy protocols such as fludarabine is debated. A variety of combination chemotherapy studies with the purine analogs have yielded mixed results. Yamauchi et al. (2001) data suggested synergistic interaction between DNA damaging agents and the purine analogs. Such synergy likely occurs as a consequence of alkylator induced DNA damage and subsequent inhibition of DNA repair by the purine analog (Hamblin et al., 2002). Combination studies with alkylating agents and each of the purine analogs have been performed (Byrd et al., 2000; Robak et al., 2001). However, many side effects were noted including: myelosuppression, nausea, vomiting, alopecia, secondary cancers, hemorrhagic cystitis, cardiomyopathy and neuropathy (Redaelli et al., 2004).
1.1.7.3 Stem cell transplantation

B-CLL is theoretically ideal for bone marrow or stem cell transplantation (SCT). Currently, SCT is preferred over bone marrow harvest. However, SCT was not widely used in the management of B-CLL for a number of reasons. Prior to the development of the purine analogs, adequate clearing of blood and bone marrow to collect uncontaminated stem cells for autologous transplantation was not possible. In addition, the advanced age of the patient population with B-CLL limited the allogeneic SCT approach. Therefore, SCT is usually reserved for younger patients with more advanced disease or those who have relapsed or failed initial drug treatments. Allogeneic SCT has been shown to be effective in 20-55% of patients although it can be associated with considerable mortality (Toze et al., 2000). In allotransplant series there is a survival plateau of 40-60%, the relapse rate being 10-25%. This suggests that a fraction of patients is cured (Rai et al., 2001). On the other hand, autologous SCT has not been found to be curative. An important problem with autologous transplants is that in most cases, harvested stem cells are contaminated with residual B-CLL cells. This has encouraged the investigation of different in vitro removal techniques in order to reduce the risk of relapse due to the re-infusion of tumour cells. Several methods, such as B-cell negative selection, CD34+ positive selection, or double selection (i.e. positive selection of CD34 cells followed by B-cell negative selection) are available (Rai et al., 2001). In contrast to autologous transplantation, allografting is a potentially curative procedure in B-CLL, based on the long term disease free survival of some patients who have achieved a complete remission. The inferior 4-year overall survival in registry data of patients who have undergone allogeneic compared with autologous transplantation, reflects both the higher treatment related mortality associated with allografting and the selection of higher risk drug resistant patients (Oscier et al., 2004). Allogeneic SCT which also employs the graft versus leukaemia effect, should probably be reserved for patients with advanced, poor risk disease and a low probability of successful autologous SCT (Dreger et al., 2000). Nonablative allogeneic transplants have increasingly been performed to explore the graft versus leukaemia effect (Keating et al., 2003).
1.1.7.4 Non pharmacological treatment for B-CLL

Other medical procedures sometimes used in B-CLL include splenectomy, radiation, leukapheresis (blood filtering to remove excess white blood cells). Splenectomy is typically used only to reduce the effect of clinical symptoms or to lessen thrombocytopenia or anaemia.

For patients who were drug resistant undergoing splenectomy to relieve anaemia or thrombocytopenia, the response rates were 50-77% and 61-88% respectively (Oscier et al., 2004). Radiation is used as painkilling care or to minimize symptoms when lymph nodes are compressing other organs. Splenic irradiation is used in patients unable to undergo surgery and has become restricted to the treatment of symptomatic splenomegaly unresponsive to chemotherapy. It remains a useful, generally well tolerated and effective treatment with 50-90% of patients experiencing a reduction in splenic size and relief of abdominal pain and discomfort (Oscier et al., 2004). Leukapheresis can reduce organ involvement and help haemoglobin and platelets level, but has not been shown to have an impact on survival (Andritsos and Khoury, 2002).

The lack of a curative therapeutic regime for B-CLL has led to interest in developing alternative forms of treatment such as immunotherapy.
1.2 Tumour associated antigens expressed in B-CLL

Tumour associated antigens (TAAs) are molecules preferentially expressed by tumour cells, but not in normal tissues, that can be recognised by the adaptive immune system. The description of the first human TAA recognised by cytotoxic T cells over a decade ago was a groundbreaking step in cancer immunology (Boon and van der Bruggen, 1996). During the last few years, effective strategies to identify TAAs recognized by T cells and/or B cells have been developed and have led to the characterization of various families of TAAs. These antigens are proteins that could serve as distinctive molecular markers of disease as well as possible specific targets for immunotherapies (Jäger et al., 2003). Synthetic peptides corresponding to defined antigenic epitopes for tumour specific CTLs represent an approach currently being developed to treat various types of cancers. In this development, the choice of the TAA is critical in its design, which must clearly distinguish cancer cells from normal cells. Within the past decade, several TAAs have been identified on the basis of the antigen specificity of infiltrating T cells in tumours of cancer patients (Renkvist et al., 2001). Unfortunately, the use of TAAs in cancer immunotherapy has brought with it a number of challenges. These challenges include generating CTLs that exhibit weak tumour killing activity or the possibility of inducing autoimmunity and destruction of normal cells to widely expressed self tumour antigens (Restifo, 2001). Several matters must be considered for the design of effective peptide based cancer immunotherapy treatments for use in clinical trials. First, identifying novel tumour antigens expressed on various tumour types can facilitate their use in clinical trial settings. Secondly, CTL epitopes need to be identified for a range of different HLA-types. Thirdly, identifying whether the tumour antigens are expressed only on tumour cells or are expressed at low levels on normal cells (Pouniotis et al., 2004) (Figure 1.2).
The number of potential target tumour antigens is increasing. Target antigens of haematological malignancies are expressed in different molecular forms with distinct immune effector pathways appropriate for each. Different categories of cancer antigens have been identified that induce CTL responses:

1.2.1 Cancer testis antigens

The identification of tumour antigens capable of inducing an immune response in cancer patients and the development of immunogenic cancer vaccines targeting these antigens represent a challenging task for the tumour immunologists. In the early 1990s, Boon and his colleagues reported the first successful cloning of a human tumour antigen, termed melanoma antigen-1 (MAGE-1) which elicited a CTL response in the autologous melanoma patient (van der Bruggen et al., 1991; Traversari et al., 1992). Several analyses showed MAGE-1 to be expressed exclusively in testis among normal tissues.
However, amongst tumours its expression was not restricted to melanoma but was also found in a percentage of various cancer types. The specific lysis of cultured tumour cells by autologous CTL \textit{in vitro} was first observed in melanoma systems. The use of genetic approach of T cell epitope cloning also led to the identification of the BAGE and the GAGE antigens, both of which are recognized by CTL of melanoma patients. This approach required the establishment of autologous CTL lines and tumour cell lines from the same patient, which is not easy to achieve for most epithelial tumour types.

Early studies of CT antigens defined common features of these antigens (Chen \textit{et al.}, 1998):

- Expression restricted to gametogenic tissues and cancer.
- Presence of multigene families.
- Immunogenicity in cancer patients.

Following studies have since identified other characteristics shared by this group of genes and their products while also identifying exceptions of each rule. Additional features that have since known include (Scanlan \textit{et al.}, 2002):

- Heterogenous protein expression in cancer.
- Correlation of mRNA expression with tumour progression and with tumours of higher malignant potential.
- \textit{In vitro} activation by hypomethylation and/or histone deacetylase inhibitors.
In summary, CT antigen genes or gene families that have been identified to date are listed below:

<table>
<thead>
<tr>
<th>Gene Family</th>
<th>Family member</th>
<th>Gene family</th>
<th>Family member</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGE-A</td>
<td>MAGE-A</td>
<td>CSAGE</td>
<td>CSAGE, TRAG3</td>
</tr>
<tr>
<td></td>
<td>(1,2,3,4,5,6,8,9,10,11,12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAGE</td>
<td>BAGE- (1,2,3,4,5)</td>
<td>MMA1</td>
<td>MMA1- (a,b)</td>
</tr>
<tr>
<td>MAGE-B</td>
<td>MAGE-B- (1,2,5,6)</td>
<td>CAGE</td>
<td>CAGE</td>
</tr>
<tr>
<td>GAGE-1</td>
<td>GAGE- (1,2,3,4,5,6,7,8)</td>
<td>BORIS</td>
<td>BORIS</td>
</tr>
<tr>
<td>SSX</td>
<td>SSX- (1,2a,2b,3,4)</td>
<td>HOM-TES-85</td>
<td>HOM-TES-85</td>
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<tr>
<td>NY-ESO</td>
<td>NY-ESO-1</td>
<td>AF15q14</td>
<td>AF15q14</td>
</tr>
<tr>
<td></td>
<td>LAGE-1a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAGE-1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAGEC1</td>
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<td>SYCP1</td>
<td>SYCP1</td>
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<td>BRDT</td>
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<td>MORC</td>
</tr>
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</tr>
<tr>
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<td>TPX1</td>
<td>TPX1</td>
</tr>
<tr>
<td>HAGE</td>
<td>HAGE</td>
<td>NY-SAR35</td>
<td>NY-SAR35</td>
</tr>
<tr>
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<td>SAGE</td>
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<tr>
<td>ADAM2</td>
<td>ADAM2</td>
<td>NXF2</td>
<td>NXF2</td>
</tr>
<tr>
<td>PAGE-5</td>
<td>PAGE-5</td>
<td>TDRD1</td>
<td>TDRD1, NY-CO45</td>
</tr>
<tr>
<td>LIPI</td>
<td>LIPI</td>
<td>TEX15</td>
<td>TEX15</td>
</tr>
<tr>
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<td>NA88A Pseudogene</td>
<td>FATE</td>
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</tr>
<tr>
<td>Pseudogene</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CTAGE-1</td>
<td>CTAGE- (1,2)</td>
<td>TPTE</td>
<td>TPTE</td>
</tr>
</tbody>
</table>

Table 1.6 List of known CT antigen families and their antigens
(http://www.cancerimmunity.org/CTdatabase/)
Out of these CT antigens, MAGE-A1, MAGE-A3 and NY-ESO-1 attracted our attention to check whether they are expressed on B-CLL cells or not. NY-ESO-1 was originally cloned from an oesophageal cancer by the SEREX, an approach based on the screening of recombinant tumour cDNA libraries for specific interactions with autologous serum antibodies. Because NY-ESO-1 and members of MAGE-A family are frequently expressed in tumours of different histological type, they are attractive targets for antigen specific immunotherapy of cancer.

1.2.2 Melanocyte differentiation antigens

This group of antigens, first cloned from the SK-MEL-29 tumour, expressed during melanocyte differentiation was identified as targets for autologous CTL in melanomas (Brichard et al., 1993; Bakker et al., 1994; Coulie et al., 1994). Epitopes derived from self antigens such as Melan-A/MART-1, gp100, and tyrosinase have been found to be targets for CTL and tumour infiltrating lymphocytes (TIL) in the context of HLA-A2 and other MHC class I molecules (Wölfel et al., 1994; Kang et al., 1995). These are TAA that are expressed by both normal and tumour cells. Other examples of this type of TAA include the prostate specific antigen in prostate cancer, and proteinase-3 in CML (Gordan and Vonderheide, 2002).

These antigens are among the best described in humans, and have created an interesting debate about the available effector cells to target them, particularly in the case of Melan-A/MART-1. Early studies of melanoma patients revealed high levels of recognition (Her et al., 1996); unexpectedly, many healthy controls also showed anti Melan-A/MART-1 reactivity (Pittet et al., 1999). These antigens are a class of TAA that is promising for tumours of non vital cell types, such as melanocytes and prostatic epithelia, but may be less advantageous for other tumour types, such as lung and renal cancers (Gordan and Vonderheide, 2002).
1.2.3 Over-expressed tumour antigens

The antigens proteinase-3, Wilm’s tumour gene encoded transcription factor-1 (WT-1) and mucin-1 (MUC-1) are markedly overexpressed in different types of haematological tumours. Proteinase-3, a primary neutrophilic granule protein, is overexpressed in leukaemic progenitors from patients with AML and CML but is minimally expressed by normal marrow progenitors. Molldrem et al. (1997) have identified proteinase-3 peptides that bind common HLA alleles. These peptides have been used to stimulate T-cell populations leading to the generation of proteinase specific cytotoxic T cells able to recognize and kill unmodified AML cells. Furthermore, by using proteinase/HLA-A*0201 tetrameric complexes, CD8+ T cells specific for a peptide derived from proteinase-3 have been identified in CML patients in remission following allogeneic bone marrow transplantation or interferon treatment (Molldrem et al., 2000).

Another candidate antigen is WT-1 which is also overexpressed by many human leukaemias including AML, CML and ALL. An HLA-A*0201 restricted epitope of WT-1 has been identified. T cells specific for this epitope were generated and shown to lyse leukaemia cell lines and inhibit colony formation by transformed CD34+ progenitor cells from CML patients, while not affecting normal CD34+ progenitors (Ohminami et al., 2000; Gao et al., 2000). Gaiger et al. (2001) demonstrated that antibodies against WT-1 are present in the serum of patients with AML, making this antigen a target for vaccine strategies aimed to generate both cellular and humoral antileukaemic immune responses.

MUC-1, an immunogenic epithelial mucin, is present in an underglycosylated form on solid tumours such as breast, pancreatic and ovarian carcinomas. Also, it has been found to be overexpressed in multiple myeloma (Takahashi et al., 1994). MUC-1 exposes an epitope which is recognized by both B- and T-cells of the immune system.
1.2.4 Mutated TAA

Mutated TAA are appealing targets and defined by point mutations of constitutive cellular proteins, leading to strong CTL responses against tumour cells in patients with cancer (Coulie et al., 1995). P53, Ras and HER-2/NEU are examples for mutated TAA for tumour immunotherapy (Gordan and Vonderheide, 2002). However, these TAA are usually specific to individual patient’s tumour which limit a broader application in immunotherapy regimes.

1.2.5 Viral antigens

Viral diseases are associated with different malignancies in humans. For example, Epstein Bar virus (EBV) with Burkitts lymphoma, hepatitis B and C viruses (HBV, HCV) with hepatocellular carcinoma, human papilloma virus (HPV) with cervical and anal carcinoma, and human T lymphotrophic virus (HTLV) with T cell leukaemia. Independent of whether the viral infection is the oncogenic agent, it was shown that viral antigens are expressed in the associated tumours and can be used as targets for preventive or therapeutic vaccination (Gordan and Vonderheide, 2002).

1.2.6 Universal tumour antigens

The search for universal tumour antigens can be seen an attempt to combine the differential TAA, cancer/testis TAA and mutated TAA. This type of antigens should be expressed by all tumours and by no normal tissues, and its expression should be necessary for the progression of that tumour. Survivin, CYP1B1 and MDM2 are known as universal TAA.
1.3 Survivin: the inhibitor of apoptosis protein

1.3.1 Survivin structure

Survivin is a 16.5 kDa intracellular protein that belongs to the inhibitor of apoptosis protein (IAP) family. IAP molecules are characterized by the presence of 1-3 copies of a ~70 amino acid zinc finger fold, designated the baculovirus IAP repeat (BIR). Other structural motifs found in certain IAPs include a caspase recruitment domain (CARD), a ring finger, a ubiquitin-conjugating domain and a nucleotide binding P-loop motif (Deveraux and Reed, 1999). In humans, eight members of the IAP family have been identified, including NAIP, XIAP, c-IAP1, c-IAP2, Ts-XIAP, ML-IAP, Apollon and survivin (Pižem and Cör, 2003). Among molecules of the IAP family, survivin is the smallest member. It is unique in having a single BIR located in NH₂-terminal half, and consists of a three standard antiparallel β sheet (residues 15-89) surrounded by four small α-helices, and an extended COOH-terminal α-helix and dimeric structure predicted to form a coiled coil (Figure 1.3) (Verdecia et al., 2000). The BIR domain is thought to be critical for antiapoptotic function, whereas the coiled domain interacts with tubulin structures.

![Figure 1.3](image)

**Figure 1.3** Overall structure of human survivin. Ribbon representation of the survivin dimer: one monomer is blue, the other is purple (Verdecia et al., 2000).
In vitro, survivin crystallizes as a dimer, and the interaction is thought to be mediated through two hydrophobic regions, one in its extreme NH₂ end, amino acids 6-10, and the second in the linker region, amino acids 89-102, between the BIR domain and the COOH terminal helix.

Survivin’s most striking feature is its 65Å long C terminal helix α6, comprising residues 100-140. Both hydrogen bonding and hydrophobic contacts between the BIR domain and residues in the first few turns of α6 stabilize and fix the direction of this helical rod. The two α6 helices of the dimer form an approximate 110° angle while maintaining an interhelical distance of 111Å (Figure 1.4).

![Figure 1.4](image) A close view of the BIR domain in dimeric structure of survivin showing the angle formed between the α6 helices of the dimer and the interhelical distance between them of 111Å (Verdecia et al., 2000).

The survivin molecule contains three separate and chemically distinct surfaces, including acidic and basic regions on the BIR domain and a hydrophobic helical surface on α6. This arrangement is consistent with functionally relevant protein-protein interaction surfaces (Verdecia et al., 2000)
A single copy survivin gene located on chromosome 17q25 (Li et al., 1999) gives rise to four splice variants of human survivin (survivin-2α, survivin-2β, survivin-δ- Ex3 and survivin-3β) (Mahotka et al., 2002, Badran et al., 2004; Caldas et al., 2005a; Noton et al., 2006). The full length of survivin gene consists of a three- intron and four- exon structure. Survivin-2β is generated by insertion of an alternative exon-2, whereas survivin-δ- Ex3 arises from the removal of exon-3, and survivin-3β results from the introduction of a novel exon-3. The sequence alterations produced from the splice variants result in marked changes in the corresponding protein structure and cause differences in their ability to inhibit apoptosis. While survivin and survivin-δ- Ex3 are both anti-apoptotic, survivin-2β has lost its anti-apoptotic potential due to the insertion of exon-2 which interrupts the essential BIR domain (Johnson and Howerth, 2004). No function has yet been described for survivin-3β (Caldas et al., 2005b). There is also different subcellular localization of survivin and its splice variants. Survivin and survivin- 2β are predominantly cytoplasmic whereas survivin-δ- Ex3 is primarily nuclear. These different isoforms of survivin and their varied locations in the cell may represent a regulatory balance between apoptosis and inhibition of apoptosis (Mahotka et al., 2002).

1.3.2 Survivin expression in normal tissues and human tumours

One of the most significant features of survivin is its differential distribution in cancer compared with normal tissues (Ambrosini et al., 1997). Its expression has been extensively studied in normal and neoplastic tissues by western blotting, in situ hybridization, RT- PCR and immunochemistry (Pižem and Cör, 2003). Survivin is strongly expressed in embryonic and foetal organs but is undetectable in most differentiated normal tissues. Adult normal cell types that express survivin include thymocytes, CD34+ bone marrow derived stem cells, endothelial cells, basal epithelial cells of colonic mucosa and epithelial cells of normal uterine cervix (Frost et al., 2002; Altieri, 2003a). Of human thymocyte subsets, double positive (CD4+/CD8+) thymocytes express the highest levels of survivin, demonstrating upregulation in this subset of T lymphocytes (Kobayashi et al., 2002). There is down-regulation of survivin, however,
in mature single-positive (CD4⁺ or CD8⁺) T cells, and peripheral T cells are negative for survivin. Survivin is also normally expressed in CD34⁺ haematopoietic stem cells, which raises the concern that survivin targeted therapy for cancer cells could disturb normal haematopoiesis (Fukuda et al., 2001). CD34⁺ cells expressed survivin in all phases of the cell cycle, whereas cancer cells specifically expressed survivin in a G2/M specific manner. Similarly, survivin upregulation in CD34+ cells was not as dramatic as in leukaemic cells, implying dysregulation of survivin in leukaemia. It is possible that CD34⁺ cells, under the regulation of haematopoietic growth factors, require only small amounts of survivin to undergo mitosis rather than apoptosis. Survivin expression can also be detected in normal colonic tissue and in premalignant and malignant lesions of the colon (Gianani et al., 2001). This indicates that survivin cannot be used as a specific marker for colon cancer. In addition, there is a strong nuclear expression of survivin in mucosal surface epithelial cells of the stomach. Weak signals have also been detected in placenta and proliferative and secretory endometrium (Tetsuhisa and Nobuhiko, 2001).

In contrast to normal tissues, survivin expression is significantly upregulated in cancer. In genome wide searches, survivin has been identified as the fourth “transcriptome” expressed in cancers of colon, lung, breast, brain and melanoma, but low or undetectable in the same normal organs (Velculescu et al., 1999; Altieri, 2001). It was also demonstrated in tumours of stomach, oesophagus, liver, bladder, uterus, ovaries, large cell non Hodgkin’s lymphoma, leukaemias, neuroblastoma, gliomas, soft tissue sarcomas and non melanoma skin cancers (Altieri, 2001; Zaffaroni and Daidone, 2002). Survivin overexpression has been shown in preneoplastic lesions of the skin, pancreas, uterine cervix and colonic adenomas, suggesting that survivin upregulation occurs early during tumourgenesis (Satoh et al., 2001; Kim et al., 2002).

Several studies showed that patients whose tumour cells expressed survivin have a decreased overall survival, an increased rate of disease recurrence and resistance to therapy (Kawasaki et al., 1998; Monzo et al., 1999; Swana et al., 1999; Kato et al., 2001). In the majority of solid tumours investigated for survivin expression (including breast, lung, colorectal, gastric, liver, bladder and kidney cancers, neuroblastoma and
soft tissue sarcoma), high levels of the protein were predictive of tumour progression, either in terms of disease free survival or overall survival, thus providing prognostically relevant information (Altieri, 2003b). A significantly shorter survival was observed in large B-cell lymphoma as well as mantle cell lymphoma patients with high survivin expression (Zaffaroni et al., 2005). High survivin expression in tumours correlates with a more aggressive and invasive clinical phenotype. This in return might lead to a poorer prognosis and a decreased responsiveness to chemotherapeutic agents (Johnson and Howerth, 2004). Similar to the poor prognosis observed with its overexpression in carcinomas, high survivin expression in sarcomas is likewise a negative prognostic parameter. In a study involving patients with large B-cell lymphoma, survivin was shown to be an independent factor for poor prognosis in this cancer (Adida et al., 2000).

1.3.3 Survivin function and apoptosis pathway

Apoptosis is a genetic program of cellular suicide, and inhibition of apoptosis is an important mechanism involved in cancer formation by extending the life span of cells (LaCasse et al., 1998). It plays an important physiological role in several processes, ranging from embryonic development to maintenance of adult tissue homeostasis. This genetic programme of cell death is characterized by unique biochemical and morphological features that distinguish it from necrosis (Hengartner, 2000) including: cytoskeletal disruption, shrinkage, the development of membrane leakage, and degradation of nuclear chromatin (Strasser et al., 2000). Apoptosis differs from necrosis in two of important ways. Firstly, unlike apoptosis, necrosis is accidental cell death, induced by cell injury. Secondly, necrotic cells induce inflammatory responses and damage to the surrounding tissue as a result of the rupture of their cell membranes and the local release of macromolecules. Defects in the physiological pathways for apoptosis contribute to many diseases including cancer (Reed, 2001). Moreover, impairment of apoptotic cell death might negatively affect the response of cancer cells to chemotherapy and irradiation and lead to treatment resistance (Borst et al., 2001).
Survivin is an identified member of the family of inhibitor of apoptosis proteins. Its over-expression in most human cancers suggests a general role of apoptosis inhibition in tumour progression (Mirza et al., 2002). It is known as a bifunctional protein that suppresses apoptosis and regulates cell division. It acts as an inhibitor of apoptosis by directly binding caspases (Kawamura et al., 2003). Caspases are a family of cysteine proteases that become activated by proteolysis and cleave multiple cellular substrates (Zaffaroni et al., 2005). They are the key mediator molecules of apoptosis, and they are either initiator caspases (caspase 8 and 9) or effector caspases (caspase 3 and 7).

Several IAP’s in humans and Drosophila have been shown to directly bind and inhibit caspases via their BIR domains (Deveraux and Reed, 1999). For example, XIAP binding and inhibition of caspase-9 is through its third BIR domain (BIR3), whereas caspase-3 and 7 are suppressed via a region within the molecule between BIR1 and BIR2 (Sun et al., 1999). However, the precise mechanism by which survivin suppresses apoptosis is still incompletely understood. Although direct suppression of caspase-3 by survivin has been studied by some investigators, survivin lacks structural components present in other IAPs that allow their direct binding to caspase-3 (Riedl et al., 2001). There is also speculation that survivin binds to caspase-9. Another possibility is that survivin requires the cofactor hepatitis B X-interacting protein to bind to procaspase-9 to prevent apoptosis via the intrinsic pathway (Marusawa et al., 2003). Finally, survivin binds to Smac/ DIABLO, which is a proapoptotic protein that binds IAPs and prevents them from inhibiting caspases (Verhagen et al., 2000).

Two major pathways of apoptosis have been identified. The “extrinsic” pathway is triggered by the binding of ligands to extracellular membrane receptors (death receptors) which belong to the tumour necrosis factor family and leads to activation of caspase-8 (Kim et al., 2001). The “intrinsic” apoptotic pathway is triggered by multiple death signals, either intracellular (unrepaired DNA damage) or environmental function. It involves mitochondria which respond to pro-apoptotic signals by an increased permeability in their outer membrane leading to the release of cytochrome c and SMAC/ DIABLO. These proteins facilitate the assembly of a multi-protein caspase activating
complex (apoptosome) leading to activation of caspase-9 and initiation of protease cascade (Cryns and Yuan, 1999). This pathway is primarily regulated by proteins of the Bcl-2 family which is able to differentially affect mitochondrial homeostasis and cytochrome c release, therefore modulating the intrinsic pathway of apoptosis (Adams and Cory, 1998). Both intrinsic and extrinsic pathways converge to activate effector caspases. Finally, effector caspases cleave cellular proteins that are involved in DNA repair, cytoskeletal organization and nuclear integrity. This is the basis for morphological changes of apoptotic cells (nuclear fragmentation, condensation of the cytoplasm, detachment from the neighbouring cells, apoptotic bodies fragmentation) and their clearance by phagocytosis (Pižem and Cör, 2001) (Figure 1.5).

Figure 1.5 Schematic representation of the two major apoptotic pathways and the site of survivin antiapoptotic action: Intrinsic (mitochondrial) and extrinsic (the death receptor-mediated) pathways which meet into a common downstream pathway of effector caspase activation. Survivin most probably blocks directly or indirectly caspase 9 activation (adapted from Pižem and Cör, 2001).
1.3.4 The role of survivin in cell division

Evidence indicating a role of survivin in cancer cell division was developed from studies with survivin antisense or dominant negative mutant approach, as that resulted in cells with centrosome deregulation, multipolar mitotic spindles and multinucleated or giant nuclei (Li et al., 1999).

Survivin localization to the mitotic apparatus indicates its role in cell division. Targeting survivin has resulted in remarkable mitotic progression, leading to failed cytokinesis and multinucleation (Altieri, 2003a). It is essential during embryonic development as its homozygous deletion in mice leads to catastrophic defects of microtubule assembly, with absence of mitotic spindles, complete failure of cell division and multinucleation with 100% lethality at day ~ 4-5 (Reed, 2001). The apparent requirements for survivin in normal cell division suggests that overexpression of survivin in tumours could affect normal cell cycle control. Therefore, survivin functions in an essential and evolutionarily conserved step in late- stage cell division, that is cytokinesis (Adams et al., 2001). Into support of this, survivin was shown to form a complex with molecules thought to regulate cytokinesis, including Aurora B kinase and INCENP on kinetochores and the anaphase central spindles (Wheatley et al., 2001). Recent data demonstrated that this model is probably unsatisfactory. However, inhibition of survivin expression in vivo by survivin antisense mislocates Aurora B from chromosome to a diffused pattern in the cell which reflects the role of survivin (Chen et al., 2003).

Upon expression at mitosis, survivin localizes to various components of the mitotic apparatus, including centrosomes, microtubules of the metaphase and anaphase spindle and the loose ends of the mitotic apparatus (Figure 1.6).

A broader role of survivin in cell division became clear in antibody microinjection studies, which revealed a multiple phenotype of prolonged metaphase capture, occasionally followed by apoptosis, formation of multipolar mitotic spindles and defects in chromosome attachments (Kallio et al., 2001; Giodini et al., 2002).
1.3.5 Survivin as a diagnostic and prognostic marker of cancer

Its differential expression in cancer, compared to most normal tissues, makes survivin a candidate for a molecular marker of cancer. The percentage of survivin positive patients within the same tumour series is variable, ranging 30-100% (Altieri, 2001). This might reflect genetic heterogeneity of individual tumours.

There is good evidence that survivin is a powerful negative prognostic marker in most tumours studied. This fact might allow a simple immunohistochemical detection of survivin in tumour specimens, which might provide a quick prognostic indicator for identifying patients at risk of recurrent disease and those who would benefit from more
aggressive follow up and alternative treatment protocols (Altieri, 2001). Survivin can be detected in biological fluids of cancer patients, as a result of the detaching of the tumour cells from the primary sites. Survivin protein proved to be detectable in urine from patients with primary or recurring bladder carcinomas but not in those from disease free patients or healthy people (Smyth et al., 2001). Its presence in urine has been used to diagnose bladder cancer and to differentiate neoplastic lesions from inflammatory conditions with a sensitivity of 100% and specificity of 95% (Johnson and Howerth, 2004).

As survivin is undetectable in most normal tissues, cancer patients might recognize it as non-self protein, and mount an immune response to it. According to that, measuring the immune response to survivin in cancer patients might provide a useful diagnostic tool. Antibody reactivity against survivin has been observed in sera from lung and colorectal cancer patients but not in healthy volunteers. Whether this could be applied as a broad screening tool is still unknown (Rohayem et al., 2000). Moreover, the presence of survivin has been also detected in a variety of pre-neoplastic lesions including polyps of colon, breast adenomas and Bowen’s disease, suggesting that re-expression of survivin may occur early during malignant transformation (Altieri, 2001). Besides, Rosato et al. (2006) showed that survivin expression and pattern of distribution provide a prognostic marker for esophageal squamous tumors. Another study (Kami et al., 2004) suggested that survivin expression in pancreatic cancer tissues could be a useful prognostic marker in pancreatic cancer patients.

In the majority of neoplasms investigated for survivin expression (including breast, lung, colorectal, gastric, liver, bladder and kidney cancers, neuroblastoma, glioma, soft tissue sarcomas and haematological malignancies), high levels of the IAP protein were predictive of tumour progression providing prognostically relevant information (Altieri and Marchisio, 1999; Altieri, 2001).
1.3.6 Survivin as a therapeutic target for tumours

Survivin is an attractive target because it is selectively expressed in cancer, and is potentially required for the viability of cancer cells. A survivin based anticancer therapy would be expected to carry limited toxicity for normal cells and to be effective in removing the general cell viability system provided by survivin over-expression (Altieri, 2003b). Various approaches to targeting survivin have been tested in vitro and in vivo. Experiments using phosphorylation defective survivin mutant Thr34 → Ala revealed that phosphorylation of threonine at position 34 is required for cancer viability and might contribute to survivin stability at mitosis.

Survivin synthesis (translation from survivin mRNA) can be blocked by using antisense technology and ribozymes. With the use of these approaches, loss of survivin expression can be sufficient to trigger apoptosis, to enhance chemotherapy and radiotherapy and to dysregulate cell proliferation in tumour but not in normal cells.

Immunotherapy also appears to be a promising approach to treating survivin- positive tumours. Spontaneous CTL response to survivin, in a major histocompatibility complex class I restricted manner, has been detected in patients with B-CLL, melanoma and breast cancer (Anderson et al., 2001a,b). In addition, in vitro cytolytic T-cell induction against a survivin epitope results in cytolytic T-cell activity against a wide variety of human tumours, including renal cell carcinomas, breast cancer, colon cancer, multiple myeloma, and leukaemias (Schmidt et al., 2003). According to that, survivin appears to be a universal tumour antigen and immunotherapy a possible approach to treating survivin positive tumours.
1.4 Tumour immunology

The theory of immune surveillance, which is used to describe the immune system’s role in cancer, was first conceptualized in 1909 by Paul Ehrlich (Herberman, 2000). He proposed that the immune system surveys the body looking for cancerous cells, recognizes them as foreign and eliminates them. Burnet and Thomas years later further developed this concept and suggested that cell mediated immunity had evolved to destroy cancer cells (Goldsby et al., 2000), and the nature of tumour recognition by the immune system was considered as fundamental in Burnet and Thomas interpretations’ of immune surveillance. Although Burnet proposed the self/nonself discrimination hypothesis, and Thomas modified the T cell role in homograft rejection to clarify their important function against cancer, there was not much support for any of these hypotheses until the 1990’s (Smyth et al., 2001).

Lymphocytes were originally thought to form the basis of a "cancer immunosurveillance" process that protects against primary tumour development. However, this suggestion and the Burnet and Thomas concepts were challenged to a large extent when no differences in tumour development were found between athymic nude mice and syngeneic wild type mice. Nevertheless, this view was changed according to the observations that nude mice do not totally lack T cells and that both of interferon gamma (IFN-γ), perforin and lymphocytes played role in the prevention of tumour growth (Smyth and Trapani, 2001). Data have provided evidence that the immune system influences the development of some spontaneous epithelial malignancies. For example, the infiltration of tumours with lymphoid cells, the spontaneous regression of tumours, the increase in tumour incidence in immunosuppressed and old-age individuals when the efficiency of the immune system declines support the immune surveillance idea and count towards the evidence (Roitt et al., 1998). Greater relative risk ratios have, however, been seen for a broad number of tumours with no apparent viral aetioloxy in humans. Approximately fourfold increase in the incidence of de novo malignant melanoma after organ transplantation have been reported (Sheil, 1986). A review of data collected by the Cincinnati Transplant Tumour Registry from 1968 to 1995 found a twofold greater risk in transplant patients for
developing melanoma over that of the general population (Penn, 1996). The same study also showed that only 0.3-0.4% of melanomas occur in the general pediatric population whereas the occurrence in pediatric transplant patients was 4% (Penn, 1996). In Australia and New Zealand, the tracking of 925 patients who received renal transplants from 1965 to 1998 showed increased risk ratios for development of colon, pancreatic, lung, and endocrine tumours as well as malignant melanomas. Another assessment of 5,692 renal transplant patients from 1964 to 1982 in Finland, Denmark, Norway and Sweden showed higher standardised cancer incidence ratios for colon, lung, bladder, kidney, ureter and endocrine tumours as well as malignant melanomas as compared with the general population (Birkeland et al., 1995). Thus, individuals with severe deficits of immunity indeed have a higher probability of developing a variety of cancers with no known viral aetiology. In addition to the supporting epidemiological data described above, there is accumulating evidence showing a positive correlation between the presence of lymphocytes in a tumour and increased patient survival. In Clark et al. (1989) and Clemente et al. (1996) studies, 500 patients with primary melanoma were sorted in three categories for tumour lymphocyte infiltration during the vertical growth phase of cutaneous melanoma. Comparing their survival statistics showed that patients in the brisk tumour infiltrating lymphocyte (TIL) response category survived one and one-half to three times longer than patients in the absent TIL response group, whilst patients in the nonbrisk response group had intermediate survival times. Also, the large numbers of lymphocytes found in solid tumours suggest a role of the cellular immune system in recognizing the tumour cells and fighting them (Garcia-Lora et al., 2003). In addition, a study has shown that more than 50% of a mouse strain that completely lacks functional lymphocytes developed tumours when they were injected with a chemical carcinogen compared with 19% of the wild type (Garcia-Lora et al., 2003). Based on these concepts, the main idea of the immune surveillance theory is that tumours arise if the immune system is impaired or if the tumour cells escape immune surveillance either by down regulating their expression of tumour antigens or by impairment in the immune response to these cells (e.g. in the way the antigen is taken up, processed and presented) (Goldsby et al., 2000; Davis, 2000).
However, Shankaran et al. (2001) have shown that the immune system may protect hosts against tumor development and facilitates outgrowth of tumors with reduced immunogenicity, that are capable of escaping immune recognition and destruction. These findings prompted the development of the cancer immunoediting hypothesis, which is a dynamic process composed of three phases: elimination, equilibrium and escape (Figure 1.7) (Dunn et al., 2004). After transformation of cells in a normal layer into cancerous cells, attack by various different cell types of the immune system may lead to elimination of the cancerous cells. Invasive growth of the tumour causes minor disruptions within the surrounding tissue that induce inflammatory signals leading to the recruitment of cells of the innate immune system (NKT, NK, T cells and macrophages (Mφ)) into the site and to produce IFN-γ (Smyth et al., 2001). When the elimination process is successful in deleting the developing tumour, it represents the complete editing process without progression to the subsequent phases. If it is unsuccessful, the immune system and the cancer can reach an equilibrium in which immune cells keep the cancer in check but cannot remove it completely. In this process, lymphocytes and INF-γ apply selection pressure on the tumour cells. Many of the original escape variants of the tumour cells are destroyed, but new variants arise carrying different mutations that provide them with increased resistance to immune attack. It is likely that equilibrium is the longest of the three processes and may occur over a period of many years. In the escape process, mutated cancer cells that have acquired insensitivity to detection and/ or elimination begin to grow and expand in uncontrolled manner causing a malignant disease.
Figure 1.7 The three E’s of the cancer immunoediting. The process of the cancer immunoediting arises in three phases: elimination, equilibrium and escape. The first phase, the elimination phase consists of the recognition of transformed cell by the innate and the adaptive immune system, leading to the killing of these cells. If some tumour cells are not killed in the elimination phase, the process can progress to the second phase, the equilibrium phase. In this phase the tumour persists but is prevented from expanding by immune pressure. The third phase, escape, begins when the balance between the immune response and the tumour tilt towards tumour growth (Dunn et al., 2004).

The concept of tumour immune surveillance has been considered with the development of understanding the immune system. As a result, it has been found that an effective anti-tumour response is determined by: a) the immunogenicity of the tumour, the extent to which the tumour expresses antigens which can serve as targets for the activated cytotoxic T lymphocytes (CTL) and b) the sensitivity of a tumour cell to lysis by non-specific effector mechanisms such as NK cells (van der Pompe et al., 1998). Effective immune surveillance depends on several characteristics of the immune cells (Whiteside and Herberman, 1995):
They should be distributed all over the body and have the ability to extravasate and migrate to different sites.

They should respond to the appropriate stimulatory signals and factors.

They should possess killing and lytic machinery, and switch them on when it is needed to destroy the cancer cells.

They should express receptors that are involved in the recognition of the abnormal cells and be able to protect the nearby tissue cells.

They should respond to regulatory mechanisms and block their function when it is not required.

NK cells seem to meet all these criteria and their major role in the immune surveillance against cancer will be clarified in the coming content.

Humans have different strategies to control or delay the tumour growth, formation and metastases. For example, both of the innate and the adaptive immune systems play a role in anti-tumour activity. This suggest that immunosurveillance may be a heterogenous process requiring the actions of different immune effectors depending on the tumour's cell type of origin, mechanism of transformation, anatomical localization and mechanism of immunologic recognition.

_Innate tumour immune surveillance:_

The prevention of tumour initiation and metastasis is controlled by a complex network of immune cells. The role of innate immunity in immune surveillance to cancer has received much attention, although the major focus has been on cytotoxic T lymphocytes and their role in vaccines (Smyth et al., 2002). NK cells are an important component of the innate immune system. They are distinguishable from T and B lymphocytes by surface phenotype, cytokine profile and the ability to mediate spontaneous cytotoxicity against pathogens and tumour cells. NK cells form the first line of defense against these target cells as they kill them without the need for previous sensitization (Whiteside and Herberman, 1995; Peakman and Vergani, 1997).
The presence of NK cells and the increase in their levels as well as their activation in cancer patients determine the individual's capability in controlling the growing tumour cells (Whiteside and Herberman, 1995). Evidence has also shown that NK cells are important in acting against metastases as patients with metastatic disease often had abnormalities in NK cell function and/or numbers (Whiteside and Herberman, 1995). In addition, there have been many studies which have shown the role of NK cells in eliminating tumour cells. Their role was first shown in their ability to destroy tumour cells in mice (Riccardi et al., 1980; Gorelik et al., 1984) and rats (Barlozzari et al., 1983). Then after it was shown that NK cells spontaneously kill MHC class I deficient tumour cells in vitro and in vivo (Ljunggren et al., 1985; Karre et al., 1986). Moreover, the presence and activation of NK cells might determine the outcome of the disease in addition to the control and elimination of tumour cell spread (Whiteside and Herberman, 1995).

NK cells have several properties which allow them to function effectively in the immune surveillance of cancer. They upregulate their cytolitic, proliferative and anti-tumour function as well as increase their secretion of a number of cytokines such as: interleukin-2 (IL-2), IL-12, IL-15 and IFN-γ. These cytokines cause NK cell activation to kill target cells. For example, IFN-γ increases NK cell adherence to target cells and increases their role in lysis while IL-2 expands NK cells and enhances their cytotoxic activity. IFN-γ also activates the cytotoxic CD8+ T cell response and provides signals to CD4+ T cells and activates them leading to a Th1 state. Besides, these cytokines activate phagocytes such as macrophages and increase the expression of adhesion and other molecules towards an effective cellular immune response as well as providing signals to T cells that result in a T helper 1 (Th1) response (Kishi et al., 2002). In addition, NK cells are able to kill tumour cells by different pathways and are capable of extravasation and localization in tissues (Smyth et al., 2001). They are also able to differentiate between malignant and normal cells by receptor systems such as natural killer receptor-P1 (NKR-P1), which recognizes the carbohydrates on the target cells and another receptor family which recognizes MHC class I molecules expressed on all nucleated normal cells and switches off the cytotoxic activity (Whiteside and Herberman, 1995). NK cell functions
are regulated by inhibitory and activating receptors. Inhibitory receptors bind class I MHC molecules and activating receptors bind to viral or stress induced ligands (McCann et al., 2002). Every NK cell has at least one inhibitory receptor that recognizes a self MHC class I molecule and when they bind together, NK cytotoxicity and cytokine effector functions are blocked (Alexandroff et al., 1998). Therefore, normal nucleated cells which express MHC class I molecules are not attacked by self NK cells, whereas cells that have down regulated MHC class I expression such as transformed cells are triggered and destroyed by NK cells which sense the loss of MHC class I molecules (Smyth et al., 2002; Moretta et al., 2002; Diefenbach and Raulet, 2002).

It appears that NK cells participate either directly or indirectly in multiple developmental, regulatory and communication networks of the immune system. They are viewed as remarkably efficient effector cells which are not only equipped for killing but are also capable of rapid responses to exogenous and endogenous signals. The ability of NK cells to produce hematopoietic cell growth factors, interferons, interleukins, tumor necrosis factors, transforming growth factor, coupled with their ability to increase migration to tissue sites, is responsible for the importance of the NK cell as a mediator or effector of the intercellular communication network. They produce these effector molecules in order to control tumour growth and to transmit important information to the remaining immune cell network to act against it. NK cells can be activated by tumour cell- or DC- surface molecules, such as CD70, CD80 or CD86. These molecules interact with CD27 and CD28 on the surface of NK cells. NK cell activation following DC-NK interaction can result in DC maturation. These DCs can facilitate the generation of CD8+ T cell responses through their ability to internalize and present T cells with tumour specific antigens, which were derived from NK- cell mediated tumour cell lysis (Smyth et al., 2002). Upon activation, NK cells also express and upregulate the receptors for a variety of chemotactic factors, cytokines, growth factors, and hormones, which allows them to remain responsive to signals generated not only within the immune system but also elsewhere in the body. NK cells could also have an effect on the development of B cell responses and on the regulation of B cell production of anti-tumour antibodies. Although it is generally accepted that the innate immune system can regualte secondary
adaptive immune responses, little is known about the mechanisms that link NK function to B cell responses (Jensen et al., 2004). Snapper et al. (1993) have reported that in mice, activated NK cells can induce large B cells to produce IgM antibody if they have been primed with a T cell independent antigen. The effect of NK cells has been explained by the production of cytokines (e.g. TNF-α and IFN-γ) that act upon activated B cells in most studies. However, Gray and Horwitz (1995) reported that in addition to the production of these factors, human NK cells can induce T cell independent antibody production by a two step process. The first step consists of surface contact between NK cells and resting B cells that enables these B cells to become cytokine responsive. The second step is the secretion of appropriate cytokines by activated NK cells. Thus, when NK cells are activated, they are capable to induce B cells to produce IgM and IgG. The figure below illustrates NK cells role in immunosurveillance:

![Figure 1.7](image)

**Figure 1.7** NK cells and immune responses to tumour cells. The figure shows the potential role of NK cells in tumour immune surveillance and in the network of immune cells that respond to tumours (Smyth et al., 2002)

NKT cells, a subset of T cells that share some characteristics with NK cells and coexpress some of their markers, have also shown a positive role in influencing tumour
rejection and in immunoregulating CTL and NK anti-tumour activity. Their ability to produce high levels of Th1 and Th2 cytokines modulates their function and cross links between the innate and acquired immune systems (Smyth and Godfrey, 2000; Godfrey et al., 2000). In addition to the infiltration of lymphocytes in human tumours, macrophages, granulocytes and mast cells can also take place and play a role in the immune response to tumours (Roitt et al., 1998). In apparent contrast with the above studies, NK T cells promote potent tumor rejection in response to exogenous factors such as IL-12 (Cui et al., 1997; Smyth et al., 2000) and galactosylceramide (GalCer). GalCer–induced NK T cell–dependent antitumor activity is dependent on IFN-γ production. Terabe et al. (2000) reported that NK T cells were responsible for incomplete tumor regression by IL-13–mediated inhibition of tumor-specific CTL, suggesting that NK T cells may normally inhibit tumor immunity, perhaps reflecting their immunosuppressive role in autoimmune disease. In contrast, Smyth et al. (2001) reported that NK T cell deficient mice were more susceptible to methylcholanthrene (MCA)-induced fibrosarcoma, suggesting an aggressive role for NK T cells in preventing growth of these tumors.

**Adaptive tumour immune surveillance:**

The acquired CTL mechanisms which contribute to anti-tumour immunity have been supported with the discovery of the pathway of antigen presentation to T lymphocytes and the identification of tumour antigens recognized by T lymphocytes (Garcia-Lora et al., 2003). The increased occurrence of tumours in immunosuppressed patients as well as the development of malignancies in T cell immunodeficient nude mice have also supported CTL-mediated activity in protection against tumours (Smyth et al., 2002). The acquired immune surveillance performance was suggested in a study which showed that T cell immunodeficient nude mice developed tumours faster and with a higher incidence than the congenic normal mice (Svane et al., 1999). In addition, tumour immunity has been found to be mediated by CD8+ T cell in a number of animal models. CD8+ T cells role started after the tumour inoculation on day 6, peaked on day 9 and then declined with the tumour growth (Fuchs and Matzinger, 1996). Data in a recent study done by Galon and his group (Galon et al., 2006) also support the hypothesis that the adaptive
immune response influences the behaviour of human tumours. They investigated the relationship between the type, density, and location of immune cells within cohorts of human colorectal cancers and the clinical outcome of the patients. They found a positive correlation between the presence of cytotoxic and memory T cells and a low incidence of tumour recurrence.

There are three processes that must take place for tumour elimination by CTLs. First, the cancer must be recognized by the immune system, second, lymphocyte activation and infiltration to the tumour site must occur and finally the cancer cells must be capable of being killed by the different killing mechanisms. The immune system can recognize tumour antigens by the presentation of these antigens in the form of peptides associated with self MHC class I or class II molecules to T cell receptors (TcR). MHC (Major histocompatibility complex) class I molecules are essential for the antigen presentation to CTLs, and any influence on the expression of MHC class I molecules on the tumour surface will have an effect on the tumour recognition by T lymphocytes (O’Callaghan, 2000). The immune response is then initiated by the interaction between antigen presenting cells (APC) and T cells (Pardoll, 2001). This interaction influences a cascade of events which lead to cell proliferation, cytokine production and target cell lysis (Little and Parham, 1999; Garcia-Lora et al., 2003).

CD4+ T helper1 (Th1) cells recognise tumour-specific antigens in association with MHCII molecules on professional APC and receive signals from costimulatory molecules. Th1 cells in turn become activated and produce significant cytokines such as IL-2 and IFN-γ. As a result, CD4+ T cells help the differentiation of CD8+ T cells and cause their activation and proliferation. CD8+ cells are now able to kill target cells and are called cytotoxic or killer T cells (Wood, 2001) (Figure 1.8).

The nature of the APCs is an important factor that determines immune responsiveness. According to this idea, most tumours are poor APCs because they lack the correct costimulatory molecules to activate T cells. This suggestion may lead to the assumption that the better the APC function of a tumour is, the easier it is controlled by the immune
system (Pardoll, 1996). Memory CD4⁺ T cells and CD8⁺ T cells have a crucial role in maintaining protective immunity and the memory response may also rely on tumour derived molecular actions and signals operating in the innate immunity stage (Smyth et al., 2001).

**Figure 1.8** Probable mechanism of immune recognition and killing of tumour cells. Tumour antigens are taken up and presented by APCs with appropriate costimulatory-signals. Cytotoxic T cells specific for tumour antigens are recruited and activated by release of cytokines such as: IL-2 by antigen specific Th1 cells. (Peakman and Vergani, 1997).

T lymphocytes recognize peptide antigens presented by class I and class II molecules encoded by the major histocompatibility complex (MHC). Classical antigen-presentation studies showed that MHC class I molecules present peptides derived from proteins synthesized within the cell, whereas MHC class II molecules present exogenous proteins captured from the environment. However, dendritic cells have a specialized capacity to process exogenous antigens into the MHC class I pathway. DC uptake proteins from cells and these proteins are processed into peptides once they are inside DCs. In the presence of inflammatory “danger” signals provided by necrosis or infection for example, DC mature acquiring co-stimulatory molecules belonging to the B7 (CD80/CD86) superfamily on their surface. Mature DC home to lymph nodes where processed
peptides bound to MHC class I or II on DCs interact with CD8⁺ T cells and CD4⁺ T cells, respectively through TcR. This interaction is termed cross presentation and it is necessary for immunity against most tumours and against viruses that do not infect APC. Costimulation by the binding of CD28 on T cells with its B7 superfamily ligands and other costimulatory signals on DCs, transmits an activating stimulus to T cells turning them to effector cells: CD8⁺ CTLs and CD4⁺ helper cells. Effector cells migrate to the tissues and specifically attack tumour antigen bearing cells. CD8⁺ CTLs do the killing and CD4⁺ helper cells provide augmenting cytokines such as IL-2 (Heath et al., 2004) (Figure 1.9).

Figure 1.9 Cross presentation by DCs to CTLs. DCs have a central role in the initiation and control of T cell-mediated immunity. Immature DCs in tissues endocytose soluble antigens, microbes or apoptotic cells and receive microbial or inflammatory maturation signals. Maturing DCs migrate to lymph nodes via lymphatic vessels and present peptides derived from protein degradation on MHC class I and class II molecules to CD8 and CD4 T cells, respectively. However, DCs also have the ability to present exogenous antigens via the MHC class I pathway which is called the cross presentation process (Heath et al., 2004). Cytotoxic CD8⁺ T cells are able to kill the tumour cells by two distinct mechanisms including: Granule exocytosis (calcium dependent cytotoxicity) pathway and Fas-dependent cytotoxicity (calcium independent) pathway (Winkler et al., 1997; Pena et al., 1997; Wood, 2001).
1. **Granule exocytosis:**

In this mechanism, CTL secrete cytotoxic granules into the intracellular space between the target cells and CTL. These released granules contain soluble proteins, of which perforin and granzymes are considered the most important ones. Perforin is released as a monomer; however, these monomers bind to the target membrane and polymerize within the target cell membrane in the presence of calcium to form transmembrane channels (Pena et al., 1997). This leads to the formation of cylindrical pores with an internal diameter of 5-20 nm (Goldsby et al., 2000) which might be enough, on one hand, to lyse some of the target cells. On the other hand, many of the target cells require the presence of other substances. Granzymes, a collection of serine esterases (enzymes), are thought to enter target cells through perforin pores and cleave the target cell’s proteins. This in turn induces apoptosis pathway activation and DNA degradation and the target cell undergoes programmed cell death (Roitt et al., 1998; Wood, 2001). A feature of cell death for apoptosis is the involvement of the caspase family of cysteine proteases. Caspases are available in the cell in inactive form, procaspases. Cleavage of these procaspases produces an active initiator caspase, which cleaves other procaspases yielding to the activation of their proteolytic activity. The caspase cascade results in the apoptotic death of the target in the end (Goldsby et al., 2000). Once the scenario of delivering the killing blow by T cells is complete, cytotoxic T cells become able to kill other target cells expressing the specific Ag/ class I molecule (Wood, 2001) (Figure 1.9).

2. **Fas mediated cytotoxicity:**

While perforin and granzymes play important roles in the induction of cell death, some CTL lines have been shown to lack perforin and granzymes and the cytotoxicity is otherwise mediated by Fas (Lieberman, 2003). Fas, which is a member of the TNF-receptor family, is a transmembrane protein and a death molecule (Goldsby et al., 2000). It is not expressed in all cells so, in turn, this mechanism is only effective against cells that express Fas such as immature thymocytes, activated T cells, nonlymphoid cells, NK cells and tumour cells (Arase et al., 1995; Wood, 2001). The interaction between Fas and its ligand triggers a cascade of subcellular events which lead to apoptosis of Fas-expressing target cells. Binding of the TcR of CD8+ cytotoxic T cells to its specific
Ag/MHC class I causes expression of Fas-ligand (Fas-L). Fas-L is expressed on mature CD4+ T cells and CD8+ cells after activation (Roitt et al., 1998). A Fas-expressing target cell can be lysed upon interaction with Fas-L expressed on T cells (Wood, 2001). Ligation of Fas on a target cell by Fas ligand on the CTL causes the activation of initiator caspase. This takes place after the association of Fas with a protein known as FADD (Fas- associated protein with death domain). As a result, caspases are activated, initiating a caspase cascade, which in the end causes apoptosis of the target cell (Goldsby et al., 2000) (Figure 1.10).

![Diagram](image)

**Figure 1.10** Proposed model of target-cell apoptosis stimulated by CTLs. The introduction of granzymes by the granule exocytosis pathway (left) or the engagement of Fas by Fas ligand (right) activates a caspase cascade that results in the death of the target cell (Goldsby et al., 2000).

Although the immune system is able to recognize and kill tumour cells through lymphoid effectors, cancer patients still have insufficient anti-tumour response (Paola et al., 2003). Tumours probably develop when malignant cells are allowed to escape from these immune surveillance actions. Tumour cells are able to grow, invade and metastasize in the host in spite of the active immune responses in the healthy immune system. This may be a result of a failure to express tumour specific antigens, total or partial loss of surface human leukocyte antigen (HLA) molecules (Garcia-Lora et al.,)
the development of anergy among reactive T cells (Peakman and Vergani, 1997), abnormalities in signal transduction molecules associated with TcR (Paola et al., 2003), antigenic modulation and lymphocyte homing, alteration of the apoptosis program, expression of inhibitory cytokines or immunological ignorance (Garcia-Lora et al., 2003).

1.5 Tumour immunotherapy

Until the end of the 19th century the possibility that a tumour could be rejected merely by the body’s immune system was no more than a vision. After more than 100 years of preclinical and clinical research in the field, the vision of cancer immunotherapy became real and has entered clinical practice. Immunotherapy for cancer is essentially the stimulation of the immune system via a variety of agents such as vaccines, infusion of T cells, or cytokines. These agents act through one of several mechanisms:

- By stimulating the antitumor response, either by increasing the number of effector cells or by producing one or more soluble mediators such as lymphokines
- By decreasing suppressor mechanisms
- By altering tumor cells to increase their immunogenicity and make them more susceptible to immunologic defenses

Immunotherapy is generally thought of as conferring either passive or active immunity. *Passive immunity* supplies the immune response with preformed effectors—e.g. antibodies or cytotoxic T cells—rather than activating the immune system directly.

*Active immunity* elicits **specific** or **non-specific** antitumour reactions by stimulating the patients’ immune system. This type of immunotherapy may be ideal where the immune system is primed to recognize the tumor as foreign and might therefore respond. This approach has not been successful in patients with widespread disease, as their immune systems are unable to mount a sufficient response. In the past several years, efforts have focused on using active immune therapies in patients with minimal disease. However, it has been seen that the immune system can be quite functional.
despite advanced stage cancer when the patient has been treated to give a maximal response (Bocchia et al., 2000). **Non-specific** immune responses are mediated by cells of the macrophage and NK cell lineages and/or by soluble factors such as inflammatory cytokines. Non-specific immunostimulation with bacteria or cytokines have both been applied for treatment of cancer. Non-specific treatment with bacteria extracts started in the early 1890s when Dr Coley noticed that certain cancer patients experienced regression of their tumours when they suffered from bacterial infections. He then injected live bacteria into a patient with advanced cancer, and subsequently went to develop a safe and more effective mixture of bacteria for treatment of cancer patients (Coley, 1991). Since this observation, the field has evolved a better understanding of the mechanisms of tumour rejection. However, the limited efficacy and significant toxicity of this approach has led to the exploration of the immunostimulatory potential of bacterial components or genetically modified bacteria. Among the first, non-specific immunostimulation with CpG oligodeoxy-nucleotides (ODN) has attracted particular attention (Krieg, 2003). Bacillus Calmette-Guerin (BCG) immunotherapy has been shown to be effective in preventing recurrence and progression in bladder cancer. The study by Decobert et al. (2008) showed that patients who received at least 3 maintenance BCG cycles had a significantly reduced risk of tumour recurrence by approximately 80%. Regarding cytokines they not only control resistance against infectious pathogens but based on the cytokine and tumour type, can either inhibit or enhance tumour growth. IFN-γ licensed for treatment of chronic myeloid leukaemia, hairy cell leukaemia and follicular lymphoma. IL-2 licensed in metastatic renal cell cancer but toxic side effects limit use.

The antigen **specific** immune system is based on T cells and antibody producing B cells (Zinkernagel and Hengartner, 2001). Adoptive transfers of antibodies or T cells, as well as cancer vaccines, represent tools for cancer immunotherapy. Figure 1.11 summarises the immunotherapeutic strategies and how the immune defense is mediated through antigen specific and non-specific immune mechanisms.
Figure 1.11 Mediators of the immune defense and immunotherapeutic strategies (Huber and Wölfel, 2004).

Haematologic malignancies offer unique characteristics that make them ideal targets for vaccine based therapy. The generation of effective immunotherapeutic strategies is facilitated by the ease of tumour accessibility, the ability to achieve a minimal residual state with current treatments, the APC properties of many of these tumours of lymphoid origin, and the ability of myeloid cells to differentiate in vitro to functional DCs. These features, as well as the non cross-reactive nature of immunotherapy and chemotherapy have brought to reality the combination of tumour vaccination with current treatment used in the clinical field (Borrello and Sotomayor, 2002).

In general, tumour vaccine strategies can be divided into two categories: (1) antigen-specific strategies, in which the tumour antigens have been identified and be isolated to develop a vaccine, (2) cellular or non antigen specific, in which the tumour specific antigens are unknown but presumed to exist within the material used to generate the vaccine (Borello and Sotomayor, 2002). Rosenberg (2001) classified vaccines based on either cancer cells or on the genetic identification of cancer antigens. The first type is derived from: whole cancer cells (both autologous and allogeneic preparations); gene modified cancer cells (genes encoding cytokines or costimulatory molecules); cancer cell extracts (lysates, membranes and heat shock proteins) and cancer cells fused to APCs. Vaccines based on the genetic identification of cancer antigens include: cancer
antigens, synthetic peptides, “naked” cDNA (e.g. plasmids), recombinant viruses (e.g. adenovirus) and recombinant bacteria (Bacille Calmette-Guerin (BCG) and Listeria) (Figure 1.12).

Figure 1.12 Cross-presentation of tumor antigens derived from cancer vaccines. Several immunologic manipulations lead to a common pathway of cross presentation of proteins derived from tumor antigens. (A) Gene-modified tumor vaccines; (B) whole-cell tumor vaccines; (C) Bacillus Calmette-Guerin (BCG); (D) peptide epitope immunization, recombinant viral vector immunization, and heat shock proteins; (E) naked DNA immunization; (F) immunocytokines; and (G) allogeneic MHC intratumoral plasmid injection, all lead to release of tumor antigens, which are picked up by host APCs. These APCs, the most powerful of which are the dendritic cells, circulate through the afferent lymphatic vessels to the T-cell areas of lymph nodes. There they cross-present the tumor antigen to T lymphocytes (Ribas et al., 2003).

A critical requirement for the generation of effective antitumour immune responses is the ability to effectively process and present the tumour specific or associated antigens in the appropriate MHC context to the T cells. DCs represent the most potent APCs capable of initiating effective T cell responses (Banchereau, 1998). Many features appear to be responsible for the unique antigen presenting capabilities of DCs. They express 50 fold higher levels of MHC molecules than macrophages, thus providing
more peptide/ MHC ligand for T-cell receptor engagement. DCs also express extremely high levels of co-stimulatory and adhesion molecules critical for T cell activation. According to their origin and surface markers, DCs are classified into myeloid derived DCs (MDC) and plasmacytoid DC (PDCs). Functionally, DCs can be divided into an immature form, capable of capturing antigens, and a mature form that presents antigens (Giannopoulos and Schmitt, 2006). Defects in quantity and quality of DCs have been reported in many cancers including CLL. MDCs were described as phenotypically immature and functionally deficient in patients with B-CLL, lacking the maturation antigen CD83 and the costimulatory molecule CD80, and being unable to induce a significant proliferative response in the allo-mixed lymphocyte reaction (MLR) (Orsini et al., 2003).

The enhanced ability to prime T cell responses, coupled with the recent development of techniques for obtaining large numbers of human DCs, has therefore opened the possibility of using DCs for therapeutic vaccination. Two general strategies have been used to obtain human DCs for clinical studies:

1. Direct purification of immature DC precursors from peripheral blood, and (2) the in vitro differentiation of DCs from peripheral blood CD14+ monocytes or CD34+ haematopoietic stem cells. The first approach requires the isolation of DC precursors that represent 0.5% of PBMC. These cells are isolated in vitro from a T cell/ monocyte depleted cytokine free culture. These culture conditions usually yield 5 x 10^6 DCs from a single leukapheresis (Freudenthal and Steinman, 1990; Markowics and Engleman, 1990). The other in vitro culture from CD14+ monocytes or CD34+ stem cells occurs in two steps. Immature DCs are initially generated in the presence of GM-CSF and IL-4 with a 4 or 8 days culture. Additional culture with TNF-α, LPS, GM-CSF and IL-4 results in a stable maturation of these DCs (Figure 1.13). These cells express high levels of MHC, adhesion, and costimulatory molecules and are capable of processing and presenting antigens to T cells. With this method, 5x10^6 DCs can be generated from 50-60 ml of blood (Sallusto and Lanzavecchia, 1994; Morse et al., 1997).
Immunotherapy of B-CLL using dendritic cells. Tumour antigen-loaded DCs, which are considered as the professional APC, is one of the most promising approaches to the immunotherapy of cancer. Immature DCs can be generated from CD14+ monocytes, loaded with tumour antigen and induced to mature using cytokines. The ability of DCs to induce autologous T cell responses can be monitored using in vitro assays (Suresh et al., 2005).

The description of culture procedures to generate large quantities of dendritic cells starting from haematopoietic precursors or peripheral blood monocytes has allowed extensive testing in promising preclinical models and trials. Different antigen loading procedures have been used for dendritic cell antigen presentation. For well-characterized antigens, synthetic HLA-binding peptide epitopes or the complete DNA sequence in a viral vector can be used to load the dendritic cell vaccines (Ribas et al., 2002). In addition, dendritic cells can be loaded with defined antigens to take advantage of antigen uptake surface receptors, such as Fc receptors to take up immune complexes carrying a tumor antigen (Rafiq et al., 2002). Several methods of loading dendritic cells with tumor antigens, tumor lysates or apoptotic bodies containing tumor antigens have also been tested. Whole sequences from unique cancer-derived proteins, such as idiotypes from the variable region of immunoglobulins produced in myelomas and B-cell lymphomas, can also be incubated with dendritic cells to allow their endogenous processing and MHC class I and II presentation (Hsu et al., 1996). mRNA can be isolated from tumor cells and inserted into dendritic cells, which would allow the dendritic cells to produce the
same proteins as the tumor cells and allow presentation of antigens (Boczkowski et al., 1996). Finally, dendritic cell–tumor cell hybrids constructed using techniques similar to those used to generate hybridomas allow the endogenous processing and presentation of the proteins produced by the tumor cells with the dendritic cell’s antigen presenting machinery (Kugler et al., 2000) although this technique requires additional validation. Table 1.7 illustrates some of DC immunotherapy approaches in clinical trials in various cancers. The purpose of these approaches is to stimulate antigen- specific T cell responses to antigens expressed by the patient’s tumour cells.

<table>
<thead>
<tr>
<th>Type of vaccine</th>
<th>Type of cancer</th>
<th>Vaccine composition</th>
</tr>
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<tbody>
<tr>
<td>DC/ APC</td>
<td>Melanoma</td>
<td>DC+ apoptotic bodies</td>
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<tr>
<td></td>
<td></td>
<td>DC+ allogeneic tumour lysates</td>
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<tr>
<td></td>
<td></td>
<td>DC+ irradiated tumour cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC+ MART+gp100 peptide</td>
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<tr>
<td>Non Small Cell Lung Cancer (NSCLC)</td>
<td></td>
<td>DC+ autologous tumour lysate</td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>DC+ p53 peptide</td>
</tr>
<tr>
<td></td>
<td>Ovary</td>
<td>DC+ Her2/neu peptide</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>DC+ allogeneic tumour lysate</td>
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<tr>
<td></td>
<td>Hepatocellular</td>
<td>DC+ allogeneic tumour lysate</td>
</tr>
</tbody>
</table>

Table 1.7 Some clinical trials of DC vaccination which were tested for a variety of human cancers (Ribas et al., 2003).

T cells play an important role in cancer immunotherapy and they are either cytotoxic (CD8\(^+\)) or helper (CD4\(^+\)) T cells. Unlike antibodies, which react to intact proteins only, the CD8\(^+\) T cells react to peptide antigens presented at the surface of a cell by MHC class I molecules. Peptide antigens are proteins that have been digested by the cell, and presented as protein fragments or peptides displayed in the MHC. MHC class I molecules present 8-11 amino acid long peptides derived from intracellular proteins digested by the proteasome complex and transported via the endoplasmic reticulum to
the cell surface to be recognized by CD8\(^+\) T cells (Fig 1.14). MHC class II molecules have a more restricted distribution, and are mainly expressed on the surface of professional antigen-presenting cells (APCs), such as dendritic cells, macrophages, and activated B cells (Fig 1.15). The peptides presented by MHC class II are longer, usually 10 to 34 amino acids, and derive from exogenous proteins endocytosed into endosome-lysosome compartments.

**Figure 1.14** MHC class I presentation. Intracellular proteins undergo degradation by the proteasome complex, are brought into the endoplasmic reticulum (ER) where they bind to MHC class I molecules and traffic through the Golgi apparatus in their way to the cell surface. On the cell surface they are recognized by CD8\(^+\) T lymphocytes (Ribas et al., 2003).
Figure 1.15 MHC class II presentation. Exogenous proteins enter the endosomes/lysosome vesicles in antigen presenting cells after internalization by phagocytosis or endocytosis. Here they are degraded into peptides by proteases. These peptides bind to MHC class II molecules in MHC class II compartments (MIIC), from where they are transferred to the cell surface by transport vesicles, where they are recognized by CD4+ T lymphocytes (Ribas et al., 2003).

By understanding how T cells interact with the immune system and how antigens are recognized, a better assessment of the immune response in cancer patients was obtained. Most studies analyzing T cell response to TAAs have emphasized CD8+ T cells thus far. However, CD4+ T cells may play a crucial role in both the induction and activation of TAA-specific memory CD8+ T cells toward cytotoxic effector T cells (Gao et al., 2002).

Several immunotherapeutical approaches in B-CLL have been evaluated in vitro (Figure 1.16). Some of those approaches are described below:
Generation of a T-cell mediated response targeted at the TAA/leukaemia associated antigens (LAA) might represent a novel therapeutic approach to patients with B-CLL. Vaccination by TAA/LAA-derived peptides might allow exact monitoring of T-cell responses to these particular antigens (Giannopoulos and Schmitt, 2006). Goddard et al. (2001) showed that T cells from patients with B-CLL, stimulated by lysate-pulsed autologous DCs induced specific cytotoxicity to autologous B-cell targets in a MHC class I restricted manner. The same group in 2003 (Goddard et al., 2003) demonstrated that hybrid cells of mature dendritic cells and B-CLL cells generated higher levels of specific cytotoxicity against CLL targets than lysate-pulsed DCs, activating both MHC class I and II type of responses. Another approach was tested by Kokhaei et al. (2003, 2004) in which they showed that production of oxidized tumour cells, transfection of co-stimulatory molecules, or fusing CLL cell with professional APCs enhanced T cell proliferation and immune responses. Apoptotic tumour cells, DCs pulsed with tumour cell RNA or CLL-DC hybrid cells all also demonstrated the ability to stimulate tumour specific T cell immune responses in vitro (Kokhaei et al., 2003; 2004). Palena et al. (2005) showed a successful increase in costimulatory capacity of the CLL cell by transfection with the TRICOM, CLL cells infected with the modified vaccine virus Ankara (MVA) vector encoding costimulatory molecules B7-1, ICAM-1 and LFA-3. Immunostimulatory effects by cytidine-guanosine oligo-deoxynucleotides (CpG-ODNs) also lead to an increase of expression surface markers CD40, CD58, CD80, CD86, CD54 and MHC class I, on B-CLL cells (Decker et al., 2000). Transfection of CD40L or OX40L resulted in an upregulation of costimulatory molecules that improved recognition of CLL cells by CTLs (Dannull et al., 2005), and that vaccination of tumour bearing mice using DCs transfected with OX40L mRNA resulted in significant enhancement of therapeutic anti-tumour immunity.
Even though several groups have demonstrated B-CLL specific immune responses \textit{in vitro}, only limited clinical trials have been carried out in B-CLL, which demonstrated anti-CLL immunity following vaccination with CLL cells transfected with an adenoviral vector expressing CD40L (CD154). The first clinical trial was conducted with Ad-CD154 in 11 patients. One time injection of Ad-CD154-trasduced B-CLL cells induced an upregulation of immune accessory molecules on tumour cells, high blood levels of IL-12 and IFN-\(\gamma\) and an increase of blood T-cells. A reduction in leukaemia cell count and lymph node size was also demonstrated without signs of autoimmunity (Kato \textit{et al.}, 1998; Wierda \textit{et al.}, 2000). In contrast to these active immunizations, T cells transduced with antigen receptors and donor leukocyte infusions (DLI) represent adoptive (passive) immunotherapies. Recent advances of gene transduction technologies enabled improvement of immunotherapeutic strategies based on genetic modification of malignant cells or adoptive T cells. At the moment it remains unclear if the novel immunotherapeutic strategies will be able to play a similar role in the treatment of B cell malignancies than the already established antibody-based immunotherapy.
The future of immunotherapy holds several challenges. We need to discover antigenic formulations that target multiple antigens. Monovalent vaccines, which target a single protein, may not supply the long-term immunity necessary to prevent relapse. Single-antigen vaccines may have a role in advanced disease, if the targeted antigen is critical to tumor growth. Clinically useful tumor vaccines will have to immunize against multiple immunogenic proteins, targeting the important proteins involved in malignant transformation. To determine a therapeutically effective vaccine with antitumor protection is also needed. New advances in the formulation of cancer vaccines brought by a more precise knowledge of the requirements for the generation of cellular immune responses to tumor antigens, together with the ability to monitor these responses will likely provide powerful vaccines in the near future.

1.6 Aims and objectives

B-CLL is the most common adult B-cell malignancy in USA and the western world. The clinical course is heterogeneous, some patients with aggressive form of the disease progressing rapidly with early death while others exhibit a more stable, non progressing type of the disease lasting many years. Despite progress in B-CLL treatment, relapse occurs and disease still remains incurable. The clinical management of the disease is therefore challenging and considerable efforts have been directed towards novel therapeutic strategies to reduce minimal residual disease and the relapse state. The identification of tumour associated antigens recognized by CTL opened the way for the development of more effective immunotherapeutic strategies in patients with cancer. Survivin, a member of the family of inhibitor of apoptosis proteins, is expressed in most human cancers of epithelial and haematopoietic origin. The fact that survivin expression is restricted to malignant tissues makes survivin an interesting target for the development of immunotherapeutic strategies. Various approaches to targeting survivin for immunotherapy of B-CLL have identified cytotoxic T cell responses against survivin peptides and HLA class I restricted cytolytic T cells’ existence in patients with B-CLL. However, more studies are still needed to be carried out to clarify this point and to determine the role of survivin as a potential target for immunotherapy of B-CLL. Therefore, the aim of this research was:
1- To investigate the potential of survivin as a target for immunotherapy of B-CLL.

The first step in order to achieve this aim was:

a. To identify survivin expression by B-CLL cells, and the effect of in vitro activation on its expression, in both unstimulated B-CLL cells and following coculture with CD40L L cells, CD32 L cells (as a control), immuno-stimulatory CPG-oligo nucleotides (ODN) and with both CD40L L cells and CpG together, to induce cellular activation. Flow cytometry, western blotting and RT-PCR techniques were used to look into this.

b. To investigate in vitro induction of autologous survivin specific and/or tumour-specific CTL from patients with B-CLL. This was to prove the presentation of T-cell epitopes by B-CLL cells by:
   1. Induction of autologous tumour specific response in vitro, using the patients own tumour cells or survivin pulsed T2 cells (HLA-A2 restricted).
   2. Attempt to generate a survivin specific CTL line from a normal healthy individual by repeated stimulation of peripheral blood T cells by peptide-pulsed autologous DCs, and then to address whether survivin specific T cells could kill HLA-A2+ target B-CLL cells using this CTL cell line.

2- As there was not a systematic study for the expression of tumour associated antigens in B-CLL, and as new target tumour antigens are required that might represent potential targets for an effective antitumour immune response in CLL and that might also give a broader basis for polyvalent immunization strategies, the second aim of this study was: to identify other tumour associated antigens expressed in a panel B-CLL cells that may be targets for immunotherapy of B-CLL, and whether their expression is upregulated post-activation.
2. Expression of Survivin in B-CLL
2.1 Introduction

B-CLL is characterized by the accumulation of a clonal population of CD5⁺ B-cells in blood, bone marrow and lymphoid tissues (Guipaud et al., 2003). While alkylating agents and/or nucleoside analogs have been used for palliation of symptoms and manifestations of the disease, new therapies are currently under investigation aiming to achieve a curative therapy for B-CLL (Kay et al., 2002). Monoclonal antibodies against antigens on B-CLL and stem cell transplantation have been used as ways of B-CLL treatment. However, there are a number of tumour antigens which are being tested for the immunotherapy of different types of cancers, including B-CLL. Identification of tumour antigens, cytotoxic T lymphocyte (CTL) epitopes and human leukocyte antigen (HLA) restriction are required for the generation of peptide based cancer vaccines.

Several articles were published about using survivin as a target for the immunotherapy of B-CLL (Schmitz et al., 2000; Schmidt et al., 2003; Pouniotis et al., 2004). Survivin, a 16.5 KDa protein, is highly expressed during normal fetal development but is undetectable in normal, differentiated adult tissues. Its differential expression in tumour cells compared to most normal tissues, and direct involvement in the oncogenic process, makes survivin a candidate for a molecular marker of B-CLL and an attractive target for its immunotherapy.

In this chapter, survivin expression was tested in the peripheral blood mononuclear cells (PBMC) and CD19⁺ separated B cells from diagnosed B-CLL patients. Its expression in those malignant haemopoietic cells was assessed by flow cytometry, western blot and RT-PCR. The role of activating B-CLL cells by CD40 ligation, CpG oligodeoxynucleotide (ODN) or a combination of both, on survivin expression was also examined to determine whether its expression was induced after activation with any of the ways used.
2.2 Materials and Methods

2.2.1 Patients and clinical samples

Patients with B-CLL who attended the Haematology Department at Leicester Royal Infirmary, who fulfilled the diagnostic criteria for B-CLL (Oscier et al., 2004) and who had not been treated with any therapeutic drugs, have kindly donated their blood for this research. Prior to donation of blood, written informed consent was obtained, and the study had the approval of the Local Research Ethics Committee. Whole blood samples, 30-40 ml in tubes containing preservative-free heparin at 10U/ml blood as anticoagulant, were obtained from those patients, to be used in different experiments. Table 2.1 summarizes the main clinical data of all the B-CLL patients used throughout the research.

<table>
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<th>Patients</th>
<th>Patients identifier</th>
<th>Sex</th>
<th>Age</th>
<th>White cell count (x10^9/l)</th>
<th>CD5^+ CD19^+ (%)</th>
<th>Lymphocytes count (x10^9/l)</th>
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Table 2.1 Clinical data of the B-CLL patients studied in the research.
2.2.2 Cells and cell lines

Human cell lines, HeLa (epithelial carcinoma cell line) and U266 (multiple myeloma cell line), were cultured in growth medium and were used as positive controls for the PCR and western blotting. Both cell lines were maintained at 37°C in 5% CO₂ humidified incubator before being used for the experiments. PBMC were obtained from venous blood from normal volunteers, separated as described below in 2.2.3, and were used as negative controls for expression of survivin.

2.2.3 Cell separation and freezing

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood which was diluted with an equal volume of washing medium (Roswell Park Memorial Institute (RPMI) 1640 medium, 2mM L-glutamine, 100U/ml penicillin) (Sigma, UK). 20-25ml of the diluted blood were layered over 15ml Lymphoprep (Axis-Shield Diagnostic, Norway). After density gradient centrifugation at 1450 rpm (brake off) for 20 minutes, PBMC were isolated from the interface, washed twice in washing medium and resuspended in growth medium (RPMI 1640 + 10% fetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin) (Sigma, UK). Cells were then counted using the fast counting chamber slide (ISL, UK). The sample was introduced into the application area of the chamber which consists of 10 slots of 4x4 grids. The concentration was given by:

\[
\text{Counts/ml} = \frac{\text{total counts}}{\text{Number of 4x4 grids counted}} \times 10^4
\]

After that, PBMC were either diluted in an appropriate volume of growth medium for direct use, or to isolate CD19⁺ cells from them. The remaining PBMC (2-5 x10⁷ cells/vial) were frozen in aliquots as detailed below.

CD19⁺ cell isolation: B-CLL lymphocytes were purified by positive selection using anti-CD19 MicroBeads according to the manufacturer’s instructions (MACS, Miltenyi Biotech, UK). CD19 Microbeads are developed for the selection of human B cells based on the expression of the CD19 antigen. 20μl of CD19 Microbeads and 80μl of washing buffer were added per 10⁷ total cells. Cells were then incubated for 15 minutes at 4-8°C,
washed twice and resuspended in 500μl of Minimax buffer (PBS/ 0.5%BSA). Then, the cell suspension was loaded onto a LS Column which was placed in the magnetic field of a MACS separator (MACS, Miltenyi Biotech, UK). The unlabeled cells ran through the column and this fraction was depleted of CD19+ cells. After removal of the column from the magnetic field, the magnetically retained CD19+ cells were eluted in Minimax buffer with the plunger supplied, and this was the positively selected cell fraction. B-CLL cell purity was assessed by FACS analysis by using PC5 conjugated anti-CD20 monoclonal antibody (Beckman Coulter, UK) and was routinely > 95%.

**Cell freezing:** Cells (2-5x 10^7) were centrifuged at 1250 rpm for 5 minutes, and the supernatant was discarded. 1ml of freezing medium (10% DMSO and 90% FBS) (Sigma, UK) was added to cells, mixed by gentle pipetting and transferred to a chilled cryovial. Cryovials were transferred immediately to -80°C freezer, and moved to a liquid nitrogen tank in vapour phase after 24 hours.

### 2.2.4 RNA extraction

Total cellular RNA was isolated from 10^7 B-CLL cells using RNeasy minikit (Qiagen, UK) according to the manufacturer’s protocol. The CD19+ separated B-CLL cells were centrifuged for 5 minutes at 1250 rpm. The supernatant was discarded and the cell pellet was loosened by flicking the tube. Buffer RLT (350 μl) was added to the cell pellet for lysis after mixing with β₂- Mercaptoethanol (β₂- ME) at 10 μl β₂- ME/ 1ml buffer RLT. Samples were homogenized by passing the lysate through a 23 guage needle fitted to a 1ml syringe, and 350 μl of 70% ethanol was added. 700 μl of the sample was applied to RNAse mini column and centrifuged at 10000 rpm for 15 seconds. After this wash, 700 μl of Buffer RW1 was added to the column and centrifuged at 10000 rpm for 15 seconds. The RNAse mini column was moved to a new 2ml tube and 500 μl of Buffer RPE was pipetted into it and centrifuged at 10000 rpm for 15 seconds. Another wash was done with 500 μl of Buffer RPE at 10000 rpm for 2 minutes. The column was then transferred into a 1.5 ml collection Eppendorf tube and 30 μl of RNAse free water was pipetted directly onto the middle of the RNAse silica gel membrane and centrifuged at the same speed for 1 minute. The final solution in the tube was the total RNA, and it
was either reverse transcribed into a single stranded cDNA or kept at -80˚C until required.

RNA concentrations were determined by measuring the absorbance at 260nm ($A_{260}$) using a spectrophotometer (Pharmacia Biotech, UK). An absorbance of 1 unit at 260 nm corresponds to 40 μg of RNA per ml ($A_{260}$ = 1 = 40 μg/ ml).

The calculation involved in RNA quantitation is shown below:

Volume of RNA sample = 100 μl  
Dilution= 5 μl of RNA sample+ 95 μl of RNase free water (1/20 dilution)  
Measure absorbance of diluted sample in a 1ml cuvette (RNase- free) = $A_{260}$  
Concentration of RNA sample (μg/ ml) = 40x $A_{260}$ x dilution factor  
Total yield (μg) = concentration x volume of sample in milliliters

### 2.2.5 Reverse transcriptase- polymerase chain reaction (RT- PCR)

RNA was reverse transcribed to cDNA using cDNA Superscript™ First- Strand Synthesis System (Invitrogen, UK). 5μg of total RNA with 1μl of Oligo(dT)$_{12-18}$ (0.5μg/ μl) as a primer and 1μl of 10mM of deoxynucleotides mix (dNTP) were incubated at 65°C for 5min, then placed on ice for at least 1min. A reaction mixture containing 2μl of 10x RT buffer (200mM Tris- HCl, pH 8.4, 500mM KCl), 4μl of 25mM MgCl$_2$, 2μl of 0.1M DTT and 1μl of RNaseOUT™, was prepared. 9μl of this reaction mixture were added to RNA/ primer mixture and incubated at 42°C for 2min. 1μl of 50U reverse transcriptase (RT) (Invitrogen, UK) was then added to each tube and incubated at 42°C for 50min. The RT reactions were then terminated by incubating at 70°C for 15min followed by rapid cooling on ice.

5μl of the cDNA product was then supplemented with 2.5μl of 10x PCR buffer (Invitrogen, Paisley, UK), 1μl of a 10mM dNTP mix (Invitrogen, UK), 1.5μl of 10mM solution of primers, 0.2μl of 5U/ μl Taq polymerase (Invitrogen, UK), 0.75μl of 50mM MgCl$_2$ and water to a final volume of 25μl. The sequence of the primer pairs used for amplifying survivin was as follows (Schmidt et al., 2003):
Forward primer 5′-CACCGCATCTCTACATTCAA- 3′
Reverse primer 5′- CACTTTCTTCGCAGTTTCCT- 3′

Cycling conditions were as follows:
Initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, elongation at 72 °C for 30 seconds and final extension at 72°C for 7 minutes; a total of 35 cycles was performed (T1 Plus Thermocycler, Biometra, Germany). The cDNA amplified segment for survivin was 275bp.

As an internal PCR control, the housekeeping gene human ß- actin was amplified using the following primers (SuperScript™ III first- strand synthesis system for RT-PCR, Invitrogen):
Forward primer 5′- GCTCGTCGTCGACAACGGCTC- 3′
Reverse primer 5′- CAAACATGATCTGGGTCATCTTCTC- 3′

The PCR temperature profiles were as follows: 2 minutes pretreatment at 94°C, then 30 seconds at 94°C, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. A total of 35 cycles was performed (T1 Plus Thermocycler, Biometra, Germany). The control primers produce a 353bp RT-PCR product.

Following RT-PCR, the PCR products (12μl) were separated by 2% agarose gel electrophoresis (SeaKem LE Agarose, FMC Bioproducts, USA) in the presence of 0.5 μg/ml ethidium bromide (Sigma, UK). Each sample of DNA was mixed with 3μl of loading buffer (0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanole FF, 40% (w/v) sucrose in water) and loaded into the wells of the gel. 100bp DNA ladder (New England Biolabs, UK) was loaded into one well of the gel to allow estimation of size of PCR products. Electrophoresis was performed by applying a constant voltage of 100V for 50 minutes in 1xTAE buffer. After electrophoresis, the PCR product was visualized with an UV transilluminator (UVP, USA).
2.2.6 Flow cytometry

2.2.6.1 Survivin Expression

B-CLL cells were analyzed for the expression of survivin by using a Phycoerythrin (PE)-conjugated anti-human survivin monoclonal antibody (R&D Systems, MN). Intracellular staining was performed using the fixation and permeabilization kit (Dako Cytomation, UK) following the manufacturer’s instructions. Cells (10^5-10^6 cells/tube) were harvested and washed twice in cold PBS/0.5% BSA by centrifuging at 1250 rpm for 5 minutes. They were then fixed in Reagent A (5% paraformaldehyde) for 10 minutes at room temperature to minimize leakage of proteins out of the cell. Following a wash in PBS/0.5% BSA, cells were permeabilized by Reagent B (0.1% saponin) to create holes in the cell membrane, facilitating Ab entry into the cells. After fixation and permeabilization, 10 μl of anti-survivin Ab was added and incubated for 20 minutes in the dark at room temperature. After that cells were washed twice with PBS/0.5% BSA by centrifugation at 1500 rpm for 5 minutes and finally suspended in 500 μl of 0.5% paraformaldehyde. The same steps of intracellular staining were also done but using 10 μl of IgG1 isotype control PE-conjugated monoclonal antibody (Beckman Coulter Instrumentation Laboratory, UK) as a negative control. The fixed cells were stored at 4°C in the dark until they were analysed by flow cytometric analysis using FACSCalibur flow cytometer and CellQuest software (Becton- Dickinson Ltd, Cowley, UK).

2.2.6.2 Flow cytometry for surface marker expression

Immunophenotyping was performed by flow cytometry to investigate upregulation of surface markers following activation of B-CLL cells. Surface staining of B-CLL cells was done using the following monoclonal antibodies (mAbs) from Beckman Coulter, UK: PE-labeled anti CD54 (ICAM-1), fluorescein isothiocyanate (FITC) labeled CD80 (B7.1), PE-labeled CD86 (B7.2), PE and FITC labeled IgG1 isotype controls. 10 μl of the appropriate mAb were added to cells activated by different ways (See section 2.2.8), and incubated for 15 minutes protected from light at room temperature. Cells were washed twice in PBS/0.5% BSA and fixed by 0.5% paraformaldehyde. Analysis of expression of cell surface markers was performed by FACSCalibur flow cytometry and Cellquest software (Becton- Dickinson Ltd, UK).
2.2.7 Western Blotting

Whole cell protein extracts were prepared from 2x10^6 B-CLL cells using Tri-reagent (200μl/10^6 cell) (Sigma, UK), which is a mixture of guanidine thiocyanate and phenol, and effectively dissolves protein. After standing samples in Tri-reagent for five minutes at room temperature, 0.2ml of chloroform per 1ml of Tri-reagent was added. The sample was shaken vigorously and left at room temperature for 2-15 minutes. After centrifuging at 13,000 rpm, the mixture separated into 3 phases as shown in the picture below:

![Diagram of centrifuged sample phases]

The aqueous phase was carefully removed and discarded before 300μl of 100% ethanol/1ml of Tri-reagent was added to precipitate the DNA from the interphase and organic phase. Samples were then mixed by inversion and centrifuging at 5240 rpm for 5 minutes at 4°C. To precipitate proteins from the phenol-ethanol supernatant, 1.5ml of isopropanol per 1ml of Tri-reagent was added. Samples were then allowed to stand for 10 minutes at room temperature and centrifuged at 13,000 rpm for 10 minutes at 4°C. After discarding the supernatant, samples were washed three times in 0.3M guanidine hydrochloride/ 95% ethanol solution, using 2ml per 1ml of Tri-reagent used in sample preparation. During each wash, samples were stored in wash solution for 20 minutes at room temperature and centrifuged at 10,000 rpm for 5 minutes at 4°C. After three washes, 1ml of 100% ethanol was added to the protein pellet, which was vortexed and allowed to stand for 20 minutes at room temperature. The pellet was then washed by centrifuging at 10,000 rpm for 5 minutes at 4°C. Finally, it was dissolved in 50μl of 1% Sodium Dodecyl Sulphate (SDS) by resuspending. The protein solution was then used immediately for western blotting or stored at -20°C until use.
The concentration of protein was determined using the Bio-Rad Protein Assay kit (Bio-Rad, UK). The kit consists of Reagent A (an alkaline copper tartrate solution), Reagent B (a dilute Folin Reagent) and Reagent S. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Three to five dilutions of a protein standard from 0.2mg/ml to 1.5mg/ml protein were prepared. 5μl of samples and standards were pipetted into 96-well plate in triplicate and 25μl of working Reagent A (20μl of Reagent S/ 1ml of Reagent A) were added. After adding 200μl of Reagent B to each well and mixing gently for 5 minutes, the absorbance was read at 750 nm. Due to the amino acids tyrosine and tryptophan and to a lesser extent, cystine, cysteine and histidine, a characteristic blue colour developed. Each protein elicited greater or less blue colour formation depending on the protein concentration. Amounts of each sample protein were then determined by comparing the results to a standard curve produced by serial dilutions of known amounts of protein. After determining the concentration of protein, 10-15μg were used in the western blot.

Protein samples were diluted 1:1 with Laemmli Buffer which ensures optimal band resolution when preparing proteins for sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Low molecular weight size standard marker, ready to load (Bio-rad Catalog no.161-0374, UK) and cell lysates (10-15μg / lane) were boiled at 90°C for 3-5 minutes and separated by 12% polyacrylamide gels (Bio-Rad, UK) on SDS-PAGE. The gel was run in 1x SDS running buffer for one hour. The current was set to 20mA/cm gel for the stacking gel, and was increased to 30mA/ gel when the proteins entered the resolving gel. After electrophoresis, a blotting cassette assembling the blotting ‘sandwich’ was made up to the order shown next:
A pre-soaked fibre pad in blotting buffer was placed on one side with a piece of pre-soaked filter paper on top. The gel was then put on the center of the filter paper and a nitrocellulose membrane (Amersham Biosciences, UK) on top of it. A second piece of filter paper was placed over the membrane and any air bubbles were removed by gently rolling a test-tube over the sandwich. Another fibre pad was placed on the top and the cassette was closed. The cassette was placed in the blotter tank full of 1x blotting buffer (25mM Tris, 192 mM Glycine and 0.1 % (w/v) SDS) with the gel towards the anode. The blot was run at 250 mA for one and a half hours. When blotting was complete, the membrane was blocked in 5% skimmed milk in 0.1% Tween 20 in PBS for one hour.

After washing the membrane three times for 10 minutes, immunoblottings were performed using mAb against survivin (mouse 1:250 mAb 1 μg/ml; R&G Systems, MN) overnight with shaking. The membrane was then rinsed briefly with two changes of wash buffer (0.1% (v/v) Tween 20 in PBS) and washed for 15 minutes at room temperature with shaking. Five washes for 5 minutes were then done before Horseradish peroxidase (HRP) - antigoat IgG (1:2000, Dako Cytomation) was used as a secondary Ab. The same washing steps were performed before the membrane was soaked briefly for a minute in the detection reagents (equal volume of detection reagent A and detection reagent B). The excess detection reagent was drained off by holding the membrane with forceps and touching the edge against a tissue. The plot, protein side
down, was placed on to a fresh piece of Saran Wrap, wrapped up and gently smoothed out any air bubbles. The wrapped blot, protein side up, was placed on an X-ray film cassette and the film was set on the top of the membrane. Bands were visualized by electrogenerated chemiluminescence (ECL) staining (Amersham Biosciences, UK) as described below. Luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. In chemiluminescence the excitation is effected by a chemical reaction. ECL is based on the emission of light during the horseradish peroxidase (HRP)- and hydrogen peroxide-catalyzed oxidation of luminol. The emitted light is detected on X-ray film (Figure 2.1). The film was exposed for 10-20 minutes. The band size expected for survivin was 16.5 KDa. The membrane was blocked after that in 5% skimmed milk in 0.1% Tween 20 in PBS for one hour and incubated with anti β-actin Ab (1:2000, Dako cytomation) as a standard for the protein level.

![Figure 2.1 Principles of ECL western blotting (ECL western blotting detection reagents and analysis system instructions manual, Amersham Biosciences, UK, 2002).](image)

To accurately quantify protein expression, the blots were scanned using a GS-710 Calibrated Imaging Densitometer (BioRad, UK). The measured amount of survivin protein was divided by the amount of protein from β-actin standard measured in the same sample to normalize for possible variation in the amount and quality of protein between different samples. This normalization permits accurate comparison of survivin expression between different samples, provided that the expression of the reference (β-actin) used in the normalization was very similar across all the samples.
2.2.8 Activation of B-CLL Cells

2.2.8.1 CD40 ligation

B-CLL cells were stimulated by co-culture with fibroblasts that had been stably transfected with a plasmid encoding human CD40L, or CD32 as a control (gift of Dr John Pound, University of Birmingham, UK). The expression of CD40L on the L cells was checked regularly by monoclonal antibody staining and flow cytometry. CD40L- L cells and CD32 L cells were pretreated with mitomycin C, 100μg/ml for an hour, and precultured overnight at 5x10^5/ well in 6-well plates (Nunc Brand Products, UK) in RPMI 1640 medium (Sigma, UK) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin. During the pre-incubation, the L cells adhered to the base of the wells. Thawed B-CLL cells (typically >80% vital cells) were resuspended in the above described medium, and 1 ml of 2x10^6 cells/ well were added to the adherent L cells. After 3 days, B-CLL cells were gently removed by resuspension using a Pasteur pipette for subsequent use or analysis.

2.2.8.2 CpG-ODN activation

Nuclease-resistant phosphorothioate-modified ODN were provided by Coley Pharmaceutical Group Inc (Wellesley, MA). Specific sequences were as follows:

- CpG ODN 2006 5’ TCG TCG TTT TGT CGT TTT GTC GTT -3’
- CpG ODN 10103 5’ TCG TCG TTT TTC GGT CGT TTT -3’

ODN were added at a final concentration of 3μg/ml, which had been found in preliminary experiments to give optimal stimulation.

Cells were cultured with CD32 L cells, CD40L L cells, CpG –ODN or a combination of CD40L L cells and CpG-ODN, or left untreated. B-CLL activated cells by the four different ways, plus untreated B-CLL cells, were then used for total cellular RNA isolation for RT-PCR, protein extraction for western blotting, or flow cytometric analysis.
2.2.9 Mixed lymphocyte reaction (MLR)

B-CLL cells activated by the different ways mentioned above, were used as allogeneic stimulator cells in a 5 day T cell proliferation assay. The activated B-CLL cells were pretreated with 100 µg/ml mitomycin C (Sigma) for one hour to inhibit cell proliferation. Responder T cells were obtained from peripheral blood mononuclear cells (PBMC) that were isolated from heparinized blood of healthy donors unrelated to the B-CLL patients, and separated by density gradient centrifugation over Lymphoprep (Axis-Shield diagnostic, Norway) (See 2.2.3 Cell separation section). $10^5$ cultured PBMC cells/ well were used in triplicate cultures in growth medium, and were stimulated with Mitomycin-C treated B-CLL cells in a ratio of 1:1 in a 96- well U bottom culture plate. After 5 days at $37\degree C$, 5% CO$_2$, cells were pulsed with 1 µCi $^3$H- thymidine/ well (Amersham, UK) and harvested after incubation (8hours, $37\degree C$, 5% CO$_2$) with a Tomtec cell harvester onto glass- fibre filters. Incorporated radioactivity was measured (as counts per minute [c.p.m]) in a liquid scintillation counter (1450 Microbeta Plus liquid scintillation counter, Wallac, Finland).
2.3 Results

2.3.1 Expression of survivin in B-CLL cells

2.3.1.1 Identification of survivin expression in B-CLL cells by RT-PCR

To identify the expression of survivin in PBMC from patients with B-CLL, RNA was prepared from PBMC, cDNA was then synthesized and RT-PCR was performed. Survivin expression was undetectable in the majority of B-CLL samples, but weak expression was detected in 3 out of 11 samples tested: lane 2, 5 and 7 (Figure 2.2). As a positive control, cDNA from the myeloma cell line U266 was used, which reproducibly produced a PCR product of the predicted size (275 bp). cDNA of healthy donor PBMC was used as a negative control (not shown) and β-actin was the house keeping gene which gave a band of the correct size for all samples (353 bp).

A.

<table>
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![PCR gel with bands of 275 and 353 bp](image)

275bp

B.

β-actin

![β-actin gel](image)

353bp

**Figure 2.2** Survivin expression in B-CLL samples by RT-PCR. A. Expression of survivin was analyzed in 11 PBMC samples from B-CLL patients. U266 was the positive control (lane 12). B. β-actin was used as a house keeping gene to confirm the quality of the cDNA.
2.3.1.2 Identification of survivin expression in B-CLL cells by flow cytometry

The intracellular expression of survivin protein in B-CLL cells was assessed by flow cytometry using the PE-conjugated anti-human survivin monoclonal antibody (R&D Systems, MN). B-CLL cells were purified from patients’ peripheral blood by positive selection using anti CD19+ microbeads (MACS, Miltenyi Biotech, UK). 6 samples were stained for intracellular survivin using the fixation and permeabilization kit. U266 cell line was used as a positive control for survivin staining, and PBMC from a normal donor was used as a negative control (Figure 2.3). The ∆ mean fluorescence intensity (ΔMFI) was obtained by dividing the MFI of survivin stained cells by the MFI of the isotype stained cells. In contrast to normal B-cells, survivin expression was detected in three out of the six B-CLL samples tested (Figure 2.4). However, the levels of survivin expressed by resting B-CLL cells were variable in the samples tested. Low levels of survivin expression were detected in one sample out of the six samples (CLL-1) with ΔMFI= 2.7 compared to a higher level seen on CLL-6 and CLL-11 (ΔMFI= 7.6 and 7.39 respectively). The other three samples showed negative expression of survivin as their ΔMFI was less than that of the negative control (normal PBMC).

![Figure 2.3](image)

**Figure 2.3** Survivin expression in U266 cell line (left) and normal PBMC (right). The histogram on the plot on the left shows the strongly survivin positive control (U266) and the plot on the right shows negative survivin expression in normal PBMC.
Figure 2.4 Survivin expression in B-CLL cells by flow cytometry. Representative flow cytometry histograms depicting the expression of survivin (dark blue histogram) overlayed on the isotype control (pink histogram).
2.3.1.3 Identification of survivin expression in B-CLL cells by Western Blotting

To investigate the protein expression of survivin in the same six B-CLL samples, western blotting was performed. CD19\(^+\) cells were separated from patients’ PBMC and lysed with Tri-reagent to extract protein. Protein samples were then analysed by running them on SDS-PAGE gel, blotting them, probing with anti-survivin mAb (R&D Systems, MN) and secondary Ab, and finally developing the film.

Survivin was not expressed in any of the 6 resting B-CLL cells tested by western blotting (Figure 2.5). Monoclonal antibody against survivin detected a 16.5kDa protein in the positive control samples (HeLa and U266). \(\beta\)-actin was used as a control and was positive in all cases.

![Survivin expression in B-CLL by western blotting. A. PBMC from B-CLL patients were lysed and analysed by SDS-PAGE and western blotting using anti-survivin monoclonal antibody. B. Anti \(\beta\)-actin antibody was used as a control.](image)

From the previous results and by correlating them together using the three techniques (RT-PCR, flow cytometry and western blotting), survivin was weakly/ not expressed in resting B-CLL cells by RT-PCR and western blotting. However, flow cytometry showed apparent expression of survivin in three of the six B-CLL samples tested.
2.3.2 Activation of B-CLL cells by different means

Resting B-CLL cells are poorly immunogenic and to direct immunity against CLL, highly immunogenic tumour cells or professional APC loaded with the tumour antigen are essential (von Bergwelt-Baildon et al., 2004). B-CLL cells express major histocompatibility complex (MHC) class I and II molecules and several tumour associated antigens (TAA). Although most B-CLL cells are ineffective antigen presenting cells (APCs) with a low expression of costimulatory molecules, stimulation with CD40 ligand (CD40L) restores their poor antigen presenting capacity (Mayr et al., 2005b). The interaction of CD40 with its physiological ligand CD40L (CD154) also stimulates B-cells, dendritic cells and monocytes to proliferate, differentiate, and upregulate costimulatory molecules (von Bergwelt-Baildon et al., 2004). CpG-ODN also induces the activation of APCs such as dendritic cells and B-cells through the activation of MAP kinase and NF-kB pathways. As a consequence, an increase in the expression of costimulatory molecules occurs (Decker et al., 2000). In this part, the effect of activation of B-CLL with CD40L L cells, CD32 L cells as a control for the CD40L cells, CpG-ODN, and a combination of CD40 ligation and CpG-ODN was examined. Untreated CLL cells were used as a control. Preliminary experiments were done to see the optimal time for activation for each activating stimulus. This was achieved by staining 3 different B-CLL cells for activation markers using anti-CD54, anti-CD80 and anti-CD86 conjugated monoclonal antibodies (Beckman Coulter, UK) and flow cytometry. The time intervals tested were day 0, day 1, day 3 and day 5. Day 0 reflects the cells before activation (controls). The figures below (Figure 2.6 A, B and C) demonstrate each way of activation used, and show that day three gave the highest response and upregulation of the three different cell surface markers for each activating condition tested. Data is presented for one B-CLL sample only, but is representative of all three B-CLL samples tested for the time course of response to each way of activation used. The effect of CD32L cells on the expression of the same activation markers on B-CLL cells was also assessed, but no increase in the expression levels of CD54, CD80 and CD86 was observed (data not shown). Based on these results, three days culture was considered the optimal period used for activating B-CLL cells.
**Figure 2.6** Time course of activation for B-CLL sample stimulated by three different ways: A. CD40 ligation, B. CpG-ODN and C. combination of both CD40 ligation and CpG-ODN. Following culture for the time periods indicated, the cells were stained with anti CD54, anti CD80 and anti-CD86 conjugated monoclonal antibodies, and analysed using flow cytometry. (Note: different scales in Y axis for A, B and C).
2.3.2.1 Expression of surface markers by unactivated and activated B-CLL cells

Purified B-CLL cells were cultured with CD40L expressing L cells, immunostimulatory CpG-ODN, a combination of CD40L and CpG-ODN, or CD32 expressing L cells or medium alone as controls. After 3 days culture period, the expression of surface molecules CD54, CD80 and CD86 was examined by flow cytometry. The results of a typical flow cytometry analysis are shown below for only one B-CLL sample (Figure 2.7) and Table 2.2 summarizes the results for all 6 samples tested. Resting (unactivated) B-CLL cells expressed moderate levels of CD54 and relatively low levels of CD80 and CD86. Culture of B-CLL cells with CD32 L cells had no effect compared with resting B-CLL cells. The stimulation of B-CLL cells by CD40 ligation, CpG-ODN or a combination of the two up-regulated the expression of all the cell surface markers tested. Stimulation by CD40 ligation resulted in a stronger up-regulation of surface molecules as compared with either stimulation of CpG-ODN or a combination of CD40L and CpG-ODN, as shown in Table 2.2 for all 6 samples tested. CpG-ODN activation resulted in lower upregulation of the surface markers, whilst the combination of CD40 ligation and CpG-ODN showed higher levels of expression of the three activation markers than using the CpG-ODN alone. The results were consistent, as the experiment was repeated three times for each sample per condition.
Table 2.2 Expression of activation markers in unactivated and activated B-CLL cells by: A. CD40 ligation, B. CpG-ODN and C. CD40 ligation and CpG-ODN. CD32 L cells were used as unactivated control cells for CD40L and for both stimulators together, whereas resting B-CLL cells were used as unactivated controls for cells activated with CpG-ODN. Data represent the mean fluorescence intensity of triplicates+S.E.M. *P<0.05, **P<0.005, ***P<0.001.

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Figure 2.7 Phenotypic analysis of surface markers (CD54, CD80 and CD86) in unactivated and activated B-CLL cells by three different ways: CD40 ligation, CpG-ODN, or a combination of CD40 ligation and CpG-ODN. The open histogram shows the profiles obtained with isotype control mAb. The pink histogram shows cells stained with mAbs for the specific surface markers for CD32 activated cells as controls in the plots of cells activated with CD40 ligation or combination of CD40 ligation and CpG-ODN (A and C), or for B-CLL cells in the absence of CpG-ODN (B). The purple histogram shows cells stained with specific mAbs following activation of CLL cells by each of the three methods.
2.3.2.2 Induction of allogeneic mixed lymphocyte reactions (AMLR) by B-CLL cells

In order to investigate the immunostimulatory capacity of B-CLL cells and the effect of different activating stimuli on this, their ability to induce primary allogeneic T-cell proliferation in vitro was tested. This was done by evaluating the proliferative responses of T cells from normal healthy donors to stimulation with allogeneic B-CLL cells, either resting (untreated) or activated by the same conditions of activation as used before. $10^5$ cultured T cells/well were used in triplicate cultures and stimulated with stimulator cells in a ratio of 1:1. Data for allogeneic T cell stimulation by 5 B-CLL samples are presented in Figure 2.8, and are representative of two independent experiments using separate donors of responder T cells. The results correlated with the FACs analysis, as stimulation by co-culture with CD40 ligated B-CLL cells consistently induced the highest proliferative response of allogeneic T cells. Resting B-CLL cells induced little T cell proliferation in AMLR. CpG-ODN pretreatment of the B-CLL cells did not significantly enhance their ability to stimulate in AMLR. Co-culture with CD32 L cells consistently resulted in a slight increase in T cell stimulation although the differences were not significantly different to those induced by resting B-CLL cells. Both CD40 ligation and CD40 ligation plus CpG-ODN significantly enhanced the ability of B-CLL cells to stimulate T cell proliferation in AMLR, with CD40 ligation alone showing a slightly stronger activation than CD40 ligation plus CpG-ODN.
Figure 2.8 Stimulation of allogeneic T cells by 5 different B-CLL samples, following activation of the CLL cells by CD40 ligation, co-culture with CD32 L cells, treatment with CpG-ODN, or CpG-ODN plus CD40 ligation. AMLR was determined by $^3$H Thymidine incorporation, and stars indicate significantly different responses compared with corresponding non-activated CLL stimulator cells (*P<0.05, **P<0.01, ***P<0.001).

2.3.3 Effect of cellular activation on survivin expression by B-CLL cells

As the PCR and western blot data on unactivated B-CLL cells (Fig 2.2 and 2.5) suggested that these cells expressed little or no survivin. We therefore wished to investigate whether activation of the B-CLL cells induced expression of survivin.

2.3.3.1 Detection of survivin expression by RT-PCR

As the immunohistochemical staining for the costimulatory molecules showed the highest upregulation in CD40-activated cells, cells were only extracted for RT-PCR under this condition, and compared with resting (non-activated) B-CLL cells by survivin-specific RT-PCR. After three days of co-culturing B-CLL cells with CD40L L cells, RNA was extracted and reverse transcribed to cDNA. RT-PCR was then performed and the PCR products were separated by 2% agarose gel electrophoresis. Data from 11 B-CLL samples are presented in Figure 2.9. As shown previously (Fig 2.2), unactivated B-CLL cells showed little or no expression of survivin mRNA. Following CD40 ligation, 9 out of 11
samples showed clear upregulation of survivin expression with one other showing weak expression. All of the samples (both pre- and post activation) were positive for β-actin. U266 was included as a positive control for survivin expression.

**Figure 2.9** RT-PCR detection of survivin from unactivated and CD40L activated B-CLL. A. Survivin specific PCR of unactivated B-CLL cells. B. β-actin specific PCR of unactivated B-CLL cells. C. Survivin specific PCR of the same B-CLL cells following CD40 ligation. D. β-actin specific PCR of CD40L activated B-CLL cells (A and B shown previously in Figure 2.2).

### 2.3.3.2 Detection of survivin protein expression by flow cytometry

Intracellular staining with anti-survivin mAb of B-CLL cells from 6 patients following three days culture in medium alone, with CD40L L cells, with CD32 L cells, with CpG-ODN or a combination of CD40 ligation and CpG-ODN was performed to check the expression of survivin protein, and the effect of those activating stimuli on it. Using flow cytometry, low levels of survivin were detected in 3 out of the 6 samples tested prior to activation (resting B-CLL cells) as was shown in Figure 2.3. Culture of B-CLL cells with CD32 L cells showed low levels of survivin expression and were considered as the unactivated cells for cells stimulated with CD40 ligation and CD40 ligation plus CpG-ODN. Table 2.3 illustrates the effect of CD40 ligation, CpG-ODN and CD40 ligation+ CpG-ODN as activators on survivin expression in 6 B-CLL samples. Data from 3 of those
6 patients are presented in Figure 2.10. The experiment was done in triplicates and data points represent the MFI of three parallel samples plus/minus S.E.M. Data were analysed using paired t-test using GraphPad prism 4 software 4.03 and they were considered significant when \( p \) value was less than 0.05, and very significant when \( ***p \) value was less than 0.001. Stimulation by CpG-ODN alone resulted in significant down-regulation of survivin expression in four out of the 6 activated cells. CD40 ligation of B-CLL cells upregulated survivin expression in one out of the 6 samples tested while down-regulation was seen in other sample (CLL-12). A similar effect was observed in CD40 ligation+ CpG-ODN activated cells in 3 out of 6 cases in which the expression was upregulated in CLL6 and 11, and down-regulated in CLL12. Overall, the degree of upregulation of survivin expression was not much different when comparing stimulation by CD40 ligation with the combination of CD40 ligation plus CpG-ODN.

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</tbody>
</table>

Table 2.3 Expression of survivin in unactivated and activated B-CLL cells by CD40 ligation, CpG-ODN and CD40 ligation+ CpG-ODN. CD32 L cells were the unactivated cells for CD40 ligated cells and CD40 ligated cells+CpG-ODN whereas resting B-CLL were the unactivated cells for CpG-ODN activated cells. The table depicts the mean florescence intensity (MFI) of survivin expression before and after using each activating stimulus, where the values in bold type show significant results for survivin expression after activation. Data represent the MFI± S.E.M of triplicates. \( *p < 0.05 \), \( **p < 0.005 \), \( ***p < 0.001 \).
Figure 2.10 Effect of activation of B-CLL cells from three patients (CLL-2, CLL-4 and CLL-12) on survivin expression by flow cytometry. The blue line represents the isotype control mAb, the purple histogram represents the activated cells and the pink histogram represents CD32 L cells (A and B) or resting B-CLL (C) as controls for the activated cells. Panel (A) represents CD40L activated CLL cells; (B) CD40L plus CpG-ODN activated CLL cells; and (C) CpG-ODN activated CLL cells.
2.3.3.3 Detection of survivin protein expression by western blotting

Protein was extracted from unactivated B-CLL cells and from B-CLL cells which had been cultured with CD32 L cells, CD40L L cells, CpG-ODN, and combination of CD40L L cells and CpG-ODN. They were then lysed and analyzed by SDS-PAGE and western blotting using anti-survivin mAb. The data from 3 B-CLL samples (representative of 6 samples tested) are presented in Figure 2.11, and the results were consistent in all six samples. As previously described in Figure 2.5, resting B-CLL cells were negative for survivin expression. Culture with CD32 L cells resulted in a slight upregulation of survivin expression. Culture with CD40L L cells or a combination of CD40 ligation and CpG-ODN resulted in a marked upregulation of survivin in all B-CLL cells tested, with CD40 ligation on its own or in combination with CpG-ODN giving the strongest induction of survivin expression by western blotting.

A. Survivin

<table>
<thead>
<tr>
<th>CLL-4</th>
<th>CLL-11</th>
<th>CLL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
<td>1 2 3 4 5</td>
</tr>
</tbody>
</table>

B. β-actin

Figure 2.11 Effect of activation on survivin expression by western blotting. A. expression of survivin B. expression of β-actin in same samples. For each sample, the numbers below indicate the activation condition relating to the lane on the blot: 1. CD40L+ CpG, 2. CD40L, 3. CpG, 4. CD32L, 5. CLL cells alone.
Figure 2.12 summarises the data from all 6 samples tested, following semi quantitativ e analysis of the blots by densitometer and normalisation of survivin expression against β-actin expression. Quantification of survivin expression levels in B-CLL cells activated with a combination of CD40 ligation and CpG-ODN showed a strong upregulation of survivin in all samples, compared with resting B-CLL cells which showed no/weak expression of survivin as seen in CLL-6 and CLL-12. CD40L activated B-CLL cells were the next highest survivin expressing cells. Activation of B-CLL cells with CpG-ODN induced low level of survivin expression in 2 out of the 6 samples tested (CLL-2 and CLL-4) whereas CLL-12 showed a much higher expression of survivin. Culture of B-CLL cells with CD32L cells (unactivated CLL) also induced upregulation of survivin expression in all six samples tested (Figure 2.11 and 2.12) with a higher expression than that in CD40 ligated cells in CLL2.

Figure 2.12 A summary of the effect of activation by CD32, CD40 ligation, CpG-ODN or a combination of both on the expression of survivin by western blotting.
2.4 Discussion

To establish a specific cancer immunotherapy, a target cancer antigen should not be expressed in normal tissues but in cancer cells, and should be an important molecule for progression and survival of cancer cells. Survivin is a tumour associated antigen and a member of the IAP family. It is overexpressed in most human cancers of epithelial and haemopoietic origin, but not in normal tissues, in contrast to other members of the IAP family which are widely expressed in human tissues (Ambrosini et al., 1997; Schmidt et al., 2003). Regarding survivin expression in lymphocytic leukaemia cells, previous studies have revealed an overexpression in some ALL cases (Moriai et al., 2001; Paydas et al., 2003) and in B-CLL cases (Nakagawa et al., 2004), while other groups indicated that survivin was undetectable in the majority of B-CLL samples (Munzert et al., 2002; Zeis et al., 2003). Data in Figure 2.2 and 2.5 match the last group’s findings, as survivin was not expressed in most patients’ samples used (8 of 11, 73%) by RT-PCR in my study. In the remaining 3 patients (CLL 2, 5 and 7), survivin cDNA was amplified in the right size, but expression appeared weak compared with U266 myeloma cells. Survivin expression was also undetectable in all 6 samples tested by western blotting. This evidence was not completely confirmed at the protein level using flow cytometry, as results showed apparent expression in 3 out of 6 of the same samples used in western blotting. In this respect, our data is in contrast to Nakagawa et al. (2004) findings in which the immunohistochemical staining revealed a very high frequency of survivin expression in CLL cells. Although the reasons behind this discrepancy in results are not clear, it could be due to the anti-survivin antibody used or due to the differences in the methods applied for preparing cells prior running the experiments.

According to the data overall, my results suggest that survivin was not expressed in resting B-CLLs by all the applied methods. These data were consistent with Granziero and her group (2001), as survivin expression was undetectable in most patients (25 of 30) by RT-PCR and this was also confirmed by flow cytometry and western blotting in their study.
Granziero et al. (2001) demonstrated that survivin expression in B-CLL cells was induced in vitro by CD40 stimulation, and that survivin was the only IAP family member whose expression is modulated by CD40L. Besides, in previous studies, CD40L activation had a unique potential to activate B-CLL cells regarding the expression of adhesion and costimulatory molecules, such that they become highly immunogenic and able to induce an antigen specific immune response superior to that of unstimulated CLL cells (Kato et al., 1998; Wendtner et al., 2002; Biagi et al., 2003; 2005b). Prior studies have also demonstrated that CpG-ODN triggered a strong and consistent upregulation of CD40, CD54, CD80, CD85, CD86, MHC class I and surface molecules in B-CLL cells (Yellin et al., 1994; Dazzi et al., 1995; Pamer et al., 1998; Jahrsdorfer et al., 2005). All these molecules have been described to be of major importance for professional APC function (Yellin et al., 1994; Dazzi et al., 1995; Pamer et al., 1998, Jahrsdorfer et al., 2005). Based on these studies, several ways of activation of B-CLL cells, by CD40 ligation, CpG-ODN or combination of both, were used to check the activation effect on B-CLL cells and on survivin expression by them.

To determine the optimal length of stimulation by CD40L, CpG-ODN or combination of both, for full activation of B-CLL cells, time course experiments were performed. B-CLL cells were cultured in the absence and presence of the different stimulators for one, three and five days. An upregulation of CD54, CD80 and CD86 was detected in the three time intervals tested. However, expression of the adhesion and costimulatory molecules reached its maximum on day 3 (Figure 2.6) by each way of activation used. Therefore, 3 days was the optimum period used for activating cells. These results are consistent with Buhmann et al. (1999) by showing that stimulation of B-CLL cells by CD40L resulted in a significant upregulation of both adhesion and costimulatory molecules in a time dependent manner, which peaked at three days.

Data presented in Figure 2.7 and Table 2.2 confirm the findings of the previous studies (Yellin et al., 1994; Dazzi et al., 1995; Pamer et al., 1998, Jahrsdorfer et al., 2005) on the effects of CD40 ligation and CpG-ODN activation on CLL cells. All of the three ways of activation resulted in an upregulation of expression of the adhesion and costimulatory
molecules on the!surface of B-CLL cells. However, CD40L activated CLL cells induced the highest upregulation of the activation markers in all 6 samples tested compared to CpG-ODN activated cells or cells which were activated by both stimulators together.

Despite B-CLL cells express high levels of MHC class I and II molecules, they are ineffective APCs. Indeed, they are unable to stimulate allogeneic T cells in a mixed lymphocyte reaction (MLR) (Yellin et al., 1994; Van den Hove et al., 1997). We, therefore, investigated whether activated B-CLL cells provide a proliferative stimulus to allogeneic T cells using MLR. T cell proliferation was assessed by incorporation of [3H] Thymidine. The results shown in Figure 2.8 represent five different B-CLL cases and are representative of two different allogeneic normal T cell donors. CpG activation of B-CLL cells did not significantly enhance their ability to stimulate T cell proliferation in allogeneic MLR. Stimulation by co-culture of CD40L and CpG-ODN induced a higher response of allogeneic T cells compared to cells activated with CpG-ODN alone. However, the highest stimulatory capacity was with CD40L activated cells as stimulators. These results fit broadly with the FACS analysis of the immunophenotyping of the surface markers, as a marked difference was seen between unactivated and CD40L activated cells. However, CpG-ODN also induced upregulation of the costimulators CD80 and CD86, although to a lesser degree than CD40 ligation, so it is surprising that CpG-ODN treatment of B-CLL cells had no effect on their ability to stimulate allogeneic T cells in MLR. In work similar to ours, Buhmann et al. (1999) investigated whether CD40L activated B-CLL stimulated the proliferation of allogeneic T cells, and found that these activated cells gave the highest response. Decker et al. (2000) also evaluated whether CpG-ODN activated B-CLL could act as stimulator cells in an allogeneic MLR. They also examined the stimulatory capacity of B-CLL cells that had been cultured with CD40L in the presence or absence of CpG-ODN. They showed that CD40L activated B-CLL enhanced a higher increase of thymidine incorporation than cells activated with CpG-ODN or CD40 ligation plus CpG-ODN. Krackhardt et al. (2002a) investigated the proliferative potential of allogeneic and autologous T cells stimulated with CD40L activated CLL cells. They found that proliferation of allogeneic T cells was enhanced compared to autologous T cells in all experiments performed in response to CD40L activated CLL cells. However, Schattner et
al. (1998) data contrast with our results and of those mentioned above, as they did not observe any significant increase in thymidine incorporation in allo- T cells stimulated by CD40 ligated B-CLL cells. The literature therefore contains contradictory reports on the effects of CD40L or CpG-ODN activation of B-CLL cells on their ability to act as stimulator cells in allogeneic MLR.

From the results described earlier, it was clear that CD40L activation triggered the strongest upregulation of the activation markers tested, and increased the proliferation of allogeneic T cells in the AMLR by $[^3]$H] Thymidine incorporation. According to these results, CD40L activation was tested on survivin expression by RT-PCR, flow cytometry and western blotting, as well as the other ways of activation. Data demonstrated that the stimulation with CD40L, CpG-ODN or both stimulators together was able to induce survivin expression in B-CLL cells. Upregulation of survivin expression was first observed by RT-PCR (Figure 2.9) and then confirmed at protein level by FACS analysis (Figure 2.10) and western blotting (Figure 2.11). However, in the present study, survivin expression patterns were not uniform in B-CLL samples post-activation by different ways. Some cases exhibited very strong expression while others little or no increase in the level of expression. Detection of survivin expression by RT-PCR was significantly altered by CD40 stimulation (Figure 2.9). RT-PCR analysis revealed that survivin was detected post activation by CD40 ligation in 10 of the 11 samples tested. As large amount of cells were required for RNA extraction and reverse transcription to cDNA, and as CD40L activated B-CLL cells showed the highest upregulation in the expression of activation markers, CD40 ligation was the only way used to activate B-CLL cells and check their expression of survivin by RT-PCR. Increased expression of survivin in CD40L stimulated B-CLL cells by RT-PCR has been reported previously (Granziero et al., 2001) and our data match these results. Granziero et al. (2001) also confirmed their findings by flow cytometry in which they showed that survivin expression was upregulated in all the samples tested after 48 to 96 hours of CD40 engagement in all CD40L responders.
Our immunohistochemical staining results (Table 2.3) also revealed a moderate upregulation in survivin expression in one out of the six samples tested of cells activated by CD40 ligation and in two out of the six activated samples by CD40 ligation plus CpG-ODN. Significant down-regulation in survivin expression was seen in four out of six activated cells using CpG-ODN. In general, the mean intensities of survivin in CD40L and CD40L+CpG activated B-CLL cells were higher than those of resting B-CLL cells in most of the samples tested (four out of six of the samples tested).

Following western blotting, proteins were quantified by using Calibrated Imaging Densitometer and the amount of survivin was normalized against β-actin. No/weak expression of survivin was observed in response to CpG-ODN by western blotting. Three out of the six samples activated by CpG-ODN showed weak upregulation in survivin expression (CLL4 and CLL6) with a higher upregulation in CLL-12. In contrast to those results, an enhanced expression of survivin was detected in all CD40L and CD40L+CpG-ODN activated B-CLL cells. CD40L activated cells gave a strong induction of survivin expression, but activation using CD40 ligation and CpG-ODN resulted in the highest intensity and strongest expression of survivin in all the samples tested using western blotting. Overall, the data (Figure 2.12) indicates that CD40 ligation strongly induced survivin expression in B-CLL cells, whilst treatment with CpG-ODN alone resulted in a weak induction of survivin expression.

Taking results together using the different methods, they were in broad agreement with each other for B-CLL 1, 4, 6 and 11 as survivin expression was upregulated in CD40L activated CLL cells. On the other hand, CLL-2 showed no upregulation by RT-PCR or flow cytometry but did by western blotting. CLL-12 also showed some discrepancy as positive survivin expression was detected by RT-PCR (data not shown) and western blotting, but not by flow cytometry.
Even if the number of patients studied was small, it is possible that the response to different stimulators used might be related to clinical parameters. It could potentially be correlated, for example, with stage or duration of disease, disease activity. It also could be related to the signaling pathways of these stimulators. However, we did not have sufficient clinical information on the patients to undertake such an analysis. Studies on CD40 signal transduction have resulted in a complex picture of the different mediators and pathways involved. Although CD40 has no kinase domain, CD40 ligation activates several systems (van Kooten and Banchereau, 2000). The most detectable step after CD40 activation is the activation of protein kinases. Many studies (Berberich et al., 1996; Grammer et al., 1998; Purkerson et al., 1998) have concentrated on the involvement of serine/threonine kinases: stress-activated protein kinase/c-jun amino-terminal kinase (SAPK/JNK), p38, MAPK and extracellular signal-regulated mitogen-activated protein kinase (ERK). Coupling of the CD40 receptor to different signaling pathways has been better understood by the identification of a new family of associated proteins TRAF (TNF-R associated factor). Six different members of the TRAF family have been identified. The first member associated with CD40 has been TRAF3 (Cheng et al., 1995). TRAF2 also associates with CD40. Induction of NF-κB activation after CD40 cross linking can be attributed to TRAF2 signaling. Two other TRAF proteins, TRAF5 and TRAF6, were also demonstrated to associate with the CD40 receptor (Ishida et al., 1996a, b). Kashiwada et al. (1998) demonstrated that CD40 activates ERK both by ras-dependent pathway and a ras-independent pathway involving TRAF6. A number of receptors, such as those of the cytokine receptor superfamily, lack obvious catalytic domains in their cytoplasmic regions. However, ligand binding to these receptors initiates a protein tyrosine phosphorylation cascade and intracellular signaling by activating members of Jak family (Hanissian and Geha, 1997). Cytokine and growth factor receptors that signal via Jak kinases contain in their intracellular domain motifs referred to as box 1 and box 2 (Murakami et al., 1991). Box 1 is critical for stable association of Jak kinase with cytokine receptors where as box 2 may play a crucial role in kinase activation (Ihle et al., 1995). The activated Jaks phosphorylate both themselves and other substrates, including members of a family of signal transducers and activators of transcription termed STATs which then dimerize.
cytokine genes containing IFN-γ (Ihle et al., 1995). Figure 2.13 shows the signaling pathways of CD40-CD40L interaction.

Figure 2.13 Signaling pathways coupled to the CD40 intracellular region (van Kooten and Banchereau, 2000).

The other signaling pathway in activated B-CLL cells by CpG-ODN is via TLR9. Upon recognition of CpG-rich sequences in the endosome, TLR9 initiates a TLR family signaling cascade that begins with the recruitment of the adaptor protein MyD88 via the Toll/interleukin (IL) 1 receptor domain (Ahmad-Nejad et al., 2002). MyD88 then recruits IL-1 receptor–associated kinase (IRAK) 4 (Wesche et al., 1997). When phosphorylated by IRAK-4 interacts with TRAF6 (TNF receptor–associated factor) 6 (Li et al., 2002) and disengages from the receptor. A complex consisting of TRAF6, TAK1 (TGFβ-activated kinase 1), and TAB (TAK1 binding protein) 1 and 2 goes on to activate the IkB kinase complex, resulting in nuclear factor (NF) κB translocation to the nucleus (Regnier et al., 1997; Jiang et al., 2003). CpG-induced NF-κB activation initiates the upregulation of costimulatory molecules and the secretion of proinflammatory cytokines, such as TNFα and IL-6 (Figure 2.14).
Figure 2.14 Signaling pathway for the activation by CpG-ODN. The recognition of CpG-ODN begins through a receptor localized at the plasma membrane (PM). Upon the endocytosis of CpG-ODN to the cells, TLR9 signaling pathway will be activated. CpG-ODN induced activation event occurs at the endosome when internalized CpG-DNA binds TLR9 and initiates the MyD88-dependent cascade leading to the activation of MAPK and NF-κB pathways (Assaf, 2008).

Treatments approaches using CD40 activated B-CLL cells have shown promising preliminary results but there are still several issues that need to be addressed (von Bergwelt-Baildon et al., 2004):

1- Efficient transfection with human CD40L under clinical conditions
2- Role of the transfected versus the non transfected CLL cells
3- Activation via soluble CD40L or CD40 mAb
4- The therapeutic value of these approaches

Jahrsdörfer et al. (2001) suggested that CpG-ODN has potential as an effective immunotherapeutic agent in a variety of B cell malignancies. Possible beneficial effects include:
1- Increased expression of antigens targeted by therapeutic antibodies
2- Activation of effector cells responsible for antibody dependent cell mediated cytotoxicity (ADCC).
3- Improved immunogenicity of malignant B cells
4- Enhanced development of an antigen specific immune response by CpG-ODN.

To explore these possibilities further, continued investigation of the effects of CpG-ODN at the molecular level and on tumour cell phenotype, proliferation and apoptosis is needed.

My studies suggest that CD40 ligation is likely to have a greater therapeutic effect in B-CLL than CpG-ODN. In conclusion, there was no/little expression of survivin in resting B-CLL cells which was increased following activation of the cells by CD40 ligation, CpG-ODN or both stimulators together. CD40 ligation resulted in greater B-CLL cell activation and survivin expression than the other two ways of stimulation. Activation using this way also resulted in upregulation of the major T cell costimulatory ligand molecules, CD80 and CD86 and in enhanced activity of B-CLL cells to stimulate allogeneic T cell proliferation.

In view of these data, our results suggest the possibility of using CD40L activated CLL cells to generate autologous tumour specific T cell responses. The strong and consistent upregulation of costimulatory molecules by CD40 ligation, and its effect on survivin expression also suggest survivin as an attractive target for such immunotherapeutic strategies in B-CLL.
3. Expression of Tumour Associated Antigens in B-CLL
3.1 Introduction

Tumour associated antigens (TAA) are proteins expressed by tumours but not by normal somatic cells, that the immune system is capable of recognizing as foreign. It is well established that T lymphocytes are able to specifically recognize tumour cells, which is the principle of antigen specific immunotherapy. Most, if not all, tumours express antigens, which are presented by MHC molecules, which then can be recognized and attacked by CTL.

Since the early 1990s, a large number of TAA have been identified (reviewed by Nicholaou et al., 2006) which has made it possible to assess them as targets for cancer immunotherapy in a variety of different types of cancers. However, there has been no systematic study of the expression of TAA in B-CLL.

In this chapter, I investigated the expression of a number of known TAA by B-CLL cells. As the previous chapter showed that CD40L stimulation induced the upregulation of survivin expression, the influence of CD40 ligation on the expression of those TAA in B-CLL cells was also examined.

In total, expression of seven additional TAA (Table 3.1) was investigated in 20 B-CLL samples by RT-PCR, using previously published primer pairs that yield target antigen-specific PCR amplicons.
3.2 Materials and Methods

3.2.1 Samples derived from B-CLL patients

After informed consent, whole blood was obtained from patients satisfying the diagnostic criteria for B-CLL (Table 2.1) (Oscier et al., 2004). PBMC were isolated as previously described in 2.2.3 and cryopreserved in aliquots (2-5x10^7 cells) in liquid nitrogen in freezing medium (10% DMSO and 90% FBS) until needed.

3.2.2 Cell lines and cell cultures

The following cell lines were used as positive controls for the PCR: U266 (human multiple myeloma cell line) for MAGE-A1, MAGE-A3 and NY-ESO-1, HMy-2 (human lymphoblastoid cell line) for PRAME, KG-1 (human myeloid leukaemia cell line) for MUC-1, and HL60 (human myeloid leukaemia cell line) for Proteinase-3 and WT-1. All cell lines used as positive controls for the PCR were maintained in culture in an appropriate amount of growth medium (GM) (RPMI 1640 + 10% fetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin) (Sigma, UK) at 37°C, 5% CO₂ humidified atmosphere.

3.2.3 CD40L stimulation of B-CLL cells

PBMC from B-CLL patients were used as unactivated B-CLL cells for RNA extraction, and also for CD40L- induced activation by co-culturing them with CD40L L cells for 3 days (as previously described in 2.2.8) before RNA extraction and analysis by RT-PCR.

3.2.4 RNA extraction and RT-PCR

Total cellular RNA (10^7 cells) was extracted as described in 2.2.4 from unactivated B-CLL cells on day 0, and from CD40L activated cells after 3 days culture. The obtained RNA was then reverse transcribed into cDNA using 50U reverse transcriptase (Invitrogen, UK) and amplified by RT-PCR following the same steps explained in 2.2.5. Table 3.1 summarizes the antigens tested, the primer sequences and the thermal cycling conditions used. β-actin using the same primers sequences and RT-PCR conditions, was used as a housekeeping internal PCR control, as described in 2.2.5.
<table>
<thead>
<tr>
<th>TAA</th>
<th>Primers</th>
<th>RT-PCR Cycling Conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGE-A1</td>
<td>5’-CGGCCGAAGGAACCTGACCCAG-3’&lt;br&gt;5’-GCTGGAACCTCCTACTGGTGCC-3’</td>
<td>94°C- 4min 94°C- 1min</td>
<td>(van Baren et al., 1999)</td>
</tr>
<tr>
<td>MAGE-A3</td>
<td>5’-TGAGAGACCAGAGGCCCCC-3’&lt;br&gt;5’-GGAGCTATCAGGAGCGCTGC-3’</td>
<td>72°C- 2min 72°C- 15min 30 cycles</td>
<td></td>
</tr>
<tr>
<td>Proteinase-3</td>
<td>5’-ACCTCAGTTCAGTCGCA-3’&lt;br&gt;5’-GAAATGCAAATGTATG-3’</td>
<td>94°C- 4min 94°C- 1min 52°C- 1min 72°C- 2min 72°C- 10min</td>
<td>(Greiner et al., 2004)</td>
</tr>
<tr>
<td>WT-1</td>
<td>5’-ATGAGATCCATGGGCAGCA-3’&lt;br&gt;5’-CCTGGGACACTGAACGGTCCCCGA-3’</td>
<td>94°C- 4min 94°C- 1min 64°C- 1min 72°C- 10min</td>
<td>(Greiner et al., 2004)</td>
</tr>
<tr>
<td>MUC-1</td>
<td>5’-CTGTGGGACATCTGGGTACC-3’&lt;br&gt;5’-GGTACCTCCTCTCCTACCTCCTC-3’</td>
<td>94°C- 5min 94°C- 15sec 64°C- 30sec 72°C- 30sec 72°C- 15min</td>
<td>(Brossart et al., 2001)</td>
</tr>
<tr>
<td>PRAME</td>
<td>5’-CTGTACTCATTTCCAGAGCCAGA-3’&lt;br&gt;5’-TATTTAGGAGGTCTTTCAAGGGTT-3’</td>
<td>94°C- 4min 94°C- 1min 67°C- 1min 72°C- 15min</td>
<td>(van Baren et al., 1999)</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>5’-CCCACCGCTTCCCCGTG-3’&lt;br&gt;5’-CTGGCCACTCGTGCTGGGA-3’</td>
<td>94°C- 4min 94°C- 1min 60°C- 1min 72°C- 15min 72°C- 10min</td>
<td>(van Baren et al., 1999)</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-GCTGTCGTCGACAAAGGCTC-3’&lt;br&gt;5’-CAAAATGATCGGTCATCTTCT-3’</td>
<td>94°C- 2min 94°C- 30 sec 60°C- 30 sec 72°C- 30 sec 72°C- 7min</td>
<td>SuperScript™III first-strand synthesis system for RT-PCR, Invitrogen</td>
</tr>
</tbody>
</table>

Table 3.1 Tumour associated antigens tested, the primer sequences and the conditions used for RT-PCR to check the expression of these TAA by unactivated and CD40L activated B-CLL cells.
3.3 Results

To analyze the expression of different TAA in B-CLL cells before and after CD40L activation, RT-PCR was performed. As CD40 ligation showed the highest upregulation in the activation markers by B-CLL cells, compared to CpG on its own and both CD40L and CpG together, and strong upregulation of survivin expression, its effect on TAA expression was checked to see whether expression of other tumour associated antigens was upregulated by cellular activation.

3.3.1 Expression of tumour associated antigens in unactivated B-CLL cells

RNA was extracted from 20 B-CLL samples (Table 2.1) and reverse transcribed to cDNA. cDNA obtained was tested for integrity by amplification of β-actin transcripts in a 35-cycle RT-PCR reaction. RT-PCR was then done for the different TAA under the conditions indicated in Table 3.1. Each RT-PCR experiment was repeated three times using the same primers and cDNA samples from the same samples but different RNA extractions, together with appropriate controls. Intensities of PCR products were found to yield only faint amplification signals in some samples. Based on visible band on gel following electrophoresis, cases were scored as positive if they were consistently positive (+) and as negative (-) when they were consistently negative. If results were inconsistent, they were scored as (+/-).

RT-PCR analyses showed no expression of any of the TAA tested in the unactivated B-CLL samples of the 20 patients with the exception of MUC-1 and NY-ESO-1, which were positive in 3 out of the 20 samples (B-CLL1, B-CLL3 and B-CLL8), and in 6 out of the 20 samples (B-CLL1, B-CLL2, B-CLL3, B-CLL4, B-CLL10 and B-CLL11) respectively, as seen in Table 3.2 and Figure 3.1. None of the sample tested expressed MAGE-A1, MAGE-A3, PRAME, Proteinase-3 and WT-1. All of the cDNA samples tested were positive for β-actin expression, and appropriate positive control cDNA were included in each PCR, and were consistently positive.
3.3.2 Expression of tumour associated antigens in CD40L activated B-CLL

RT-PCR was done for CD40L activated B-CLL to assess if the TAA expression is induced by CD40 ligation of the cells, as was found for survivin expression. The data are presented in Table 3.2, no bands were seen following RT-PCR of CD40 ligated B-CLL samples for any of the following TAA: MAGE-A1, MAGE-A3, Proteinase-3 and WT-1. However, there was an apparent upregulation of expression of PRAME in 8 out of the 20 samples tested (6 of them were highly positive and the other two were weak positives). For NY-ESO-1, the same six samples that were positive pre-activation were also positive after CD40L activation, but with apparent down-regulation in the level of expression in two of them (B-CLL3 and B-CLL7). However, an apparent up-regulation of NY-ESO-1 expression was seen in one of the samples (B-CLL5). For MUC-1 expression, three samples (B-CLL1, B-CLL3 and B-CLL8) were very weakly positive in the unactivated B-CLL cells with a very faint band B-CLL4. Expression for MUC-1 was seen post-activation in the same samples, except that B-CLL4 showing weak expression before activation appeared negative following CD40 ligation. A very faint band was also seen in lane 7 after CD40 ligation (Figure 3.1). Putting these results together, 8 out of the 20 samples tested showed upregulation in PRAME expression following CD40 ligation, with only one sample out of the twenty each showing weak upregulation for NY-ESO-1 or MUC-1 expression.

β-actin

A.

B.
NY-ESO-1
A.
B.

MUC-1
A.
B.

MAGE-A1
A.
B.

MAGE-A3
A.
B.
Figure 3.1 Expression of TAA in 20 B-CLL samples before and after CD40L activation. For each antigen tested, row A represents unactivated B-CLL cells and row B represents CD40 ligated B-CLL cells from the same patients. Lanes 1-20 represent B-CLL samples as listed in Table 3.2, whilst lane 21 represents an appropriate positive control for the TAA being tested (U266 for MAGE-A1, MAGE-A3 and NY-ESO-1; KG-1 for MUC-1; HMY-2 for PRAME; HL60 for Proteinase-3 and WT-1).

Table 3.2 below summarizes the results of the TAA expression in all the 20 B-CLL samples tested, before and after CD40L stimulation.
Table 3.2 Expression of the 7 different TAA tested in PBMC from 20 B-CLL patients before and after CD40L stimulation. The symbols represent the following: += antigen expressed, -= No antigen expression, +/- gave inconsistent results.

<table>
<thead>
<tr>
<th>Samples</th>
<th>MAGE-A1</th>
<th>MAGE-A3</th>
<th>MUC-1</th>
<th>NY-ESO-1</th>
<th>PRAME</th>
<th>Proteinase-3</th>
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3.4 Discussion

Treatment of cancer by inducing a tumour specific immune response has been the focus of many recent studies and might provide an efficient therapeutic concept in the near future (Hsu et al., 1996; Thurner et al., 1999). An important requirement for an effective cancer immunotherapy is the identification of tumour specific or tumour associated antigens for recognition by CTL. By using serologic screening of recombinant expression libraries (SEREX) for solid tumours, expression cloning using spontaneous immune responses from cancer patients as screening probes, as well as by tumour reactive T lymphocytes, a variety of TAAs was defined (Sahin et al., 1995; 1997; Old et al., 1998; Scanlan et al., 1998; Jäger et al., 2001; Niemeyer et al., 2003; Giannopoulos and Schmitt, 2006). It now needs to be demonstrated that haematological tumours also present different immunogenic targets for T-cell recognition. For B-CLL, only a few TAAs have been identified. Based on that, the aim of this chapter was to check the expression of 7 different TAA by B-CLL cells, and to investigate whether CD40 ligation would enhance their expression, as a first step in identifying their potential role as targets for the immunotherapy of B-CLL.

Cancer/testis antigens (CTA) are an expanding family of immunogenic proteins expressed in human neoplasms. Members of the MAGE (Van der Bruggen et al., 1991), BAGE (Boel et al., 1995), GAGE (van den Eynde et al., 1995) and SSX family (Türeci et al., 1996; 1998; Güre et al., 1997) as well as NY-ESO-1 (Chen et al., 1997) and HOM- Tes-14/SCP-1 (Türeci et al., 1998), belong to this class. MAGE-A1 was initially isolated from a human melanoma cell line as a gene encoding an antigenic peptide presented to an autologous CTL by HLA-A1 molecules (van der Bruggen et al., 1991). This gene is 1 of 12 closely homologous members of the MAGE-A family, all located near the telomeric end of the long arm of chromosome X (De Plaen et al., 1994). Antigens encoded by MAGE type genes may provide safe targets for specific immunotherapy, because of their broad representation in different types of cancer and restricted expression in normal tissues with the exception of testis and in some cases placental trophoblast cells (Van der Bruggen, 1991; van Baren et al., 1999). MAGE-A1
and MAGE-A3, but also MAGE-A2, -A4, -A6 and -A12 are widely expressed in cancers such as melanoma, lung carcinoma, head and neck tumors, oesophagus carcinoma and bladder carcinoma, but their expression in leukaemia cells has not been well studied (De Plaen, 1994). However, Shichijo and his group (1995) found that MAGE-A1, -2, -3, -4, -6 were expressed in 17 of 34 (50%) samples of T cell leukaemia, and in 7 of 16 (44%) cases of B cell leukaemia but no expression was detected in 23 myelomonocytic leukaemia cases. On the other hand, Chambost et al. (2001) reported no MAGE-A1 gene expression in 72 AML, 43 ALL from B or T lineages (29 pre-B-ALL, 2 Burkitt, 12 T-ALL) or 10 B-CLL samples. Also, Giannopoulos et al. (2006) showed no expression of MAGE family antigens in PBMC from 43 B-CLL patients. As seen in table 3.2, our results of MAGE-A1 and MAGE-A3 expression fit with the findings of the last two groups, as no expression was detected in any of the samples tested. Tanaka et al. (1997) reported the in vitro induction of specific CTL by using the HLA-A2-restricted MAGE-A3 peptide from the PBMC of a healthy donor. CTL responses were induced from unseparated PBMC by stimulation with freshly isolated, peptide-pulsed PBMC as antigen-presenting cells. The induced CTL were able to recognize and lyse not only HLA-A2 target cells pulsed with the peptide but also HLA-A2 tumor cells expressing MAGE-3, in an HLA-class-I-restricted manner. Akiyama et al. (2004) investigated the specific CTL responses to 5 HLA-A2-restricted peptides derived from gp100, tyrosinase, MAGE-A1, MAGE-A2 and MAGE-A3. A CTL induction culture was performed using peripheral blood lymphocytes and cultured DC pulsed with HLA-A2-restricted melanoma peptide cocktail. The CTLs derived from melanoma patients were able to kill HLA-A2+ melanoma cells. Zhang et al. (2007) also estimated the specific CD8+ T cell immune response to MAGE-A3 peptide in the peripheral blood of human hepatocellular carcinoma patients (HCC) in order to evaluate its immunotherapeutic potential in these patients. After expansion in vitro, the functional specific CD8+ T cells were detected in 30.8% (8/26) of HLA-A2+ MAGE-A3+ HCC patients. The effector CD8+ T cells were able to release cytotoxic molecules of granzyme B and perforin after restimulation with natural HLA-A2+ MAGE-A3+ HCC cell lines in the samples tested.
NY-ESO-1 is a highly immunogenic CT antigen, inducing simultaneous cellular and humoral immune responses in a high percentage of patients with advanced NY-ESO-1 expressing tumours (Stockert et al., 1998; Jäger et al., 1998). NY-ESO-1 antigen was cloned from an oesophageal cancer by serological expression cloning method, an approach based on the screening of recombinant tumour cDNA libraries for specific interactions with autologous serum antibodies (Sahin et al., 1995; Chen et al., 1997). Expression of NY-ESO-1 protein has been shown in multiple cancer types such as breast cancer, head and neck, melanoma and multiple myeloma. It is also expressed in spermatogonia, oogonia and placenta (Jungbluth et al., 2001; Simpson et al., 2005). Although NY-ESO-1 mRNA expression has been detected at low levels in some other normal tissues, it is unclear whether there is significance in the absence of detectable protein (Sugita et al., 2004). NY-ESO-1 was not detected by RT-PCR in 36 leukaemia specimen tested (19 AML and 17 T-ALL samples) (Niemeyer et al., 2003), but there is not any study covers its expression in B-CLL. In my study, NY-ESO-1 was expressed in 6 of 20 of the B-CLL cells before activation. Upon CD40L stimulation, the expression frequency was the same in 4 of those 6 positive samples, whilst down regulation was seen in the remaining two samples. Upregulation of NY-ESO-1 was seen in one out of the 20 CD40L activated B-CLL cells tested. Jäger et al. (2000) detected NY-ESO-1 specific CD8+ T cell responses in more than 90% of NY-ESO-1 antibody positive patients, whereas NY-ESO-1 antibody negative patients showed no detectable NY-ESO-1 specific T cell reactivity. Thus NY-ESO-1 represents a potentially important TAA in cancer immunotherapy.

PRAME (Preferentially expressed antigen of melanoma) is a tumour antigen that was first defined in a melanoma patient, and encodes a protein consisting of 509 amino acids. Its function is unknown (Ikeda et al., 1997). It is not expressed in normal tissues except for testis, placenta, endometrium, ovary and adrenals in very low levels (Novellino et al., 2004). It is expressed in a high percentage (91-97%) of melanoma, as well as in a variety of other tumours, including 70-78% of lung carcinomas, 40% of renal cell cancer and 30% of head and neck cancers (Greiner et al., 2000; Paydas et al., 2005). Oberthuer and his group (2004) have also detected a remarkable frequency of PRAME expression.
in 93% of primary neuroblastoma and 100% of patients with advanced disease. PRAME has been studied in a relatively limited number of cases of haematopoietic neoplasias (Matsushita et al., 2001; 2003). Its expression has been found in 35-64% of the cases with AML (van Baren et al., 1998; Matshushita et al., 2001; Proto-Siqueira et al., 2003), 40-60% of ALL (Tajeddine et al., 2005), and high expression has been described in CML, lymphoma and multiple myeloma (Matshushita et al., 2003). Giannopoulos et al. study in 2006 showed low expression frequencies in 43 B-CLL patients, whereas van Baren et al. (1998) showed no PRAME expression in the 5 B-CLL samples tested. In Proto-Siqueria’s study (2003) with the highest number of cases with chronic lymphoproliferative disorders, PRAME was detected in 6 of 38 cases with B-CLL. PRAME antigen was not expressed in any of the 20 unactivated samples used in my study. However, following CD40 ligation, I found PRAME expression in 6 out of 20 B-CLL samples, with weak expression in a further 2 samples. Studies evaluating the PRAME expression in B-CLL are limited and the expression of PRAME in B-CLL after CD40L activation has not been addressed in literature. However, since the expression of this antigen has not been consistently observed in normal PBMC by Greiner et al. (2000) or Proto-Siqueira et al. (2003), its findings in the patients with chronic lymphoproliferative disorders or other tumours is most probably linked to the disease. The molecular mechanism responsible for the expression of the antigen in malignant tissues seems to be the widespread change in gene methylation patterns observed in tumours. I was unable to investigate whether PRAME expression in B-CLL correlated with any other clinical factors, such as tumour stage, however, the association of PRAME with higher tumour stages and advanced disease of myelomas was shown in the study of van Baren et al. (1999). Similar to this observation, Paydas et al. (2007) showed that in the B-CLL cases tested, PRAME was negative at the early stages of the disease, but it was found to be expressed in accelerated phase of the disease. Kessler et al. (2001) showed that leukaemic cells were able to process the PRAME protein, to present its peptide on their cell surface by HLA-A2 and to be lysed by cytotoxic cells. Matsuhita and his group (2001) also found that a PRAME positive leukaemic cell line and leukaemic cells were susceptible to lysis by PRAME specific CTLs established from a patient with melanoma. Another study by Griffioen et al. (2006) screened HLA-A2 healthy individuals and
advanced melanoma patients for T cells directed against four identified HLA-A2 binding PRAME epitopes by ELISpot and tetramer staining. T cells were able to recognise and lyse tumour cells expressing high levels of PRAME supporting that PRAME could be a target for the immunotherapy of not only melanoma, but also in cases of B-CLL patients for this TAA.

The function of the MAGE, BAGE, GAGE, LAGE-1, NY-ESO-1 and PRAME proteins is unknown, and their involvement in tumour progression remains unproven. The expression of these genes has been demonstrated to be linked to the demethylation of the promoter regions, because experimental demethylation leads to the upregulation of CT antigen expression (Ayyoub et al., 2004; Claus et al., 2005) that activates other genes involved in tumour progression (van Baren et al., 1999). The expression of individual members of a given CT family appears to be coregulated. For example, MAGE-1 expression was found to be restricted to a fraction of MAGE-3 positive tumours. Boon and van der Bruggen (1996) has described that, almost consistently, melanomas that express MAGE-1 also express MAGE-2 or MAGE-3. However, this was not the case in Sahin et al. study (1997), where members of SSX and MAGE family appeared to be activated independently in breast cancer. In general, the overall proportion of CT antigen negative tumours remains high, and this stresses the importance of identifying additional tumour antigens to enlarge the pool of potential targets for cancer vaccination.

The Wilms’ tumour-1 (WT-1) antigen gene was first isolated as a gene responsible for a pediatric renal cancer, Wilms’ tumour (Call et al., 1990; Gessler et al., 1990). This gene encodes a zinc finger transcription factor involved in cell proliferation and differentiation, in apoptosis and in organ development and was categorized as a tumour suppressor gene (Menke et al., 1998). WT-1 is known to be a genetic marker for a wide variety of haematological malignancies. WT-1 has been reported to be expressed at high level in acute leukaemias, (67%) in AML (Greiner et al., 2004) and also expressed in ALL, and in blast crisis of CML (Matsushita et al., 2001; Azuma et al., 2002). Furthermore, it was found recently that 80% of solid tumour cell lines examined, including lung, gastric, colon, and breast cancer cell lines, overexpressed the wild type
WT-1 gene (Oji et al., 1999). A recent study found no expression of WT-1 in PBMC from B-CLL patients (Giannopoulos et al., 2006). These data are in keeping with the results of my study, in which I did not detect WT-1 expression in PBMC from any of the 20 B-CLL patients tested. Ohminami et al. (2000) confirmed WT-1 expression in most cases of acute leukaemia, and showed that a WT-1 T2 peptide-specific CTL line lysed leukaemic cell lines and freshly isolated leukaemia cells but not normal cell lines in an HLA-A2 restricted manner. Oka et al. (2007) were able to generate human CTLs specifically to lyse WT1-expressing tumor cells with HLA class I restriction. They also demonstrated that mice immunized with the WT1 peptide rejected challenges by WT1-expressing cancer cells, and survived with no signs of auto-aggression to normal organs that expressed WT1. Furthermore, they detected IgM and IgG WT1 antibodies in patients with haematopoietic malignancies (AML and CML), indicating that the WT1 protein was highly immunogenic, and cellular immune responses were elicited in these patients. Azuma et al. (2002) identified a novel WT-1 derived epitope and established a specific WT-1 CTL line which was able to lyse HLA-A2 WT-1 positive leukaemic cells, but not normal or HLA-A2 leukaemic cells. Taken together, these data indicate the potential of WT-1 as an antigen for immunotherapy of WT-1 leukaemias, but that this antigen is not commonly expressed in B-CLL.

Proteinase-3 (PR3), also known as myeloblastin, neutrophil PR3 and Wegener’s autoantigen, is a granule serine protease present in monocytes and in the azurophilic granules and the secretory vesicles of neutrophils. However, some PR3 is present in the outer cell membrane of resting neutrophils (Witko-Sarsat et al., 1999; Schreiber et al., 2003). PR3 degrades a variety of extracellular matrix proteins, including elastin, fibronectin, type IV collagen and laminin and inactivates p65 NF-κB (Preston et al., 2002). PR3, which exists in a soluble and a membrane-bound form, is the major autoantigen in Wegener’s granulomatosis, the most common autoimmune systematic vasculitis in adults (Frosch et al., 2004; Novick et al., 2006). PR3 is also an important protein in myeloid leukaemia, and has a whole array of physiological and possibly pathophysiological functions (van der Geld et al., 2001), as it is overexpressed in a variety of acute and chronic myeloid leukaemias (Gabay et al., 1986). In addition, it may
be involved in the process of the leukaemic transformation or the maintenance of the leukaemia phenotype, because inhibition of PR3 expression by antisense oligodeoxynucleotides inhibits cell proliferation and induces differentiation in the HL60 leukaemia cell line (Bories et al., 1989; Molldrem et al., 1996). PR3 was detected in AML and CML samples tested in Dengler et al. (1995) study, whereas cases of ALL and CLL as well as other malignant lymphomas were consistently negative, indicating that PR3 maybe used as a specific marker for the discrimination between myeloid and lymphoid leukaemias. Our results match this study as PR3 was not expressed in any of the B-CLL samples tested. In the study of Molldrem et al. (1996), several peptides derived from PR3 which bind to HLA-A2 motifs were identified. They used these peptides to stimulate PR3 specific CD8 T cells which were able to lyse AML and CML blasts in an MHC class I, HLA-A2 restricted manner. Scheibenbogen and his group (2002) reported similar findings, as CD8 T cell responses against HLA-A2 binding epitopes were detected in patients with AML. However, they were not able to identify whether PR3 specific T cells lysed the leukaemic blasts. The data suggest that the usefulness of PR3 as a target for tumour immunotherapy is likely to be limited to myeloid lineage leukaemias.

The epithelial mucin MUC-1 is a highly glycosylated type I transmembrane glycoprotein that is overexpressed on the surface of many epithelial tumours such as breast, pancreatic and ovarian carcinomas in an underglycosylated form (Barratt-Boyes et al., 1996), as well as on some B-cell lymphomas and multiple myeloma (Takahashi et al., 1994). In the study of Brossart et al. (2001), MUC-1 expression was detected in 92% of multiple myeloma samples, 67% of samples from patient with AML and 19% in B-CLL samples. MUC-1 was detected in 4 out of 20 of the samples tested in my study, which matches with Brossart and his group’s findings. This group was able to induce MUC-1 specific CTLs in vitro using peptide pulsed DCs from HLA-A2⁺ healthy donor. These CTLs lysed not only target cells pulsed with MUC-1 peptides but also tumour cell lines including multiple myeloma cells and primary AML blasts in an antigen-specific and HLA-restricted fashion. Wierecky et al. (2006) showed similar results, as they identified two HLA-A2 binding peptides of MUC1 and found that cytotoxic T cells generated after
pulsing DC with these peptides were able to induce lysis of tumor cells expressing MUC1.

No previous studies have investigated the effect of cellular activation of tumour cells on expression of these TAAs. CD40L activation was done to check whether it induces the upregulation of the expression for these TAA in B-CLL cells. There was not any expression detected after CD40L stimulation in any of the TAA tested apart from PRAME, which was detected in six samples out of the 20 samples tested, and NY-ESO-1 expression, which was induced by CD40L stimulation in one sample (whereas down regulation was seen in two of the samples tested).

The variability in results found in the studies mentioned above might reflect differences in the methods used for antigen detection and possibly differences in scoring criteria. Most studies are based on the detection of gene expression by RT-PCR or detection of protein by immunohistochemistry. Because RT-PCR is highly sensitive, it will often give higher frequencies of expression for a given tumour type than immunohistochemistry. Quantitative real time PCR (qRT-PCR) can also be used to confirm the identity of amplified DNA. It has the advantage of providing the quantitation for levels of gene expression, and is more reliable than measuring the band intensity (Vaughan et al., 2004). In addition to the variability resulting from different detection methods, the scoring system used and perhaps patient selection, it is likely that differences in tumour characteristics underlie the variability that was observed between reports. For example, tumour stage is well described as affecting antigen expression, particularly for MAGE family antigens (Barrow et al., 2006). Evaluation of expression of TAAs has proved to be technically challenging. Based on these circumstances, some investigators have scored tumours as being positive only when the result could be reproduced following repeated RNA extraction and specific RT-PCR from the same tumour specimen. This procedure was followed in my investigation of the TAA expression in B-CLL cells.
In the last few years, a number of new TAA were identified as potential targets for the immunotherapy of different cancers. Our aim was to check for the expression of these TAAs on B-CLL, before and after CD40L activation, but unfortunately the RT-PCR conditions were not provided in the references, and there was not enough time to optimize PCR conditions to give reliable data, although I have tried for two [anaplastic lymphoma kinase (ALK) and fibromodulin (FMOD)] of these antigens without success. Those TAA are described below, and more work is needed to have a clear idea of their expression and potential role in the immunotherapy of B-CLL.

ALK is a member of the insulin receptor superfamily, with high homology to leukocyte tyrosine kinase. It was identified in a proportion of anaplastic large cell lymphomas (ALCLs) (Shiota et al., 1995; Lamant et al., 2003), inflammatory myofibroblastic tumours (IMTs) (Cools et al., 2002; Hisaoka et al., 2003) and subset of large B-cell lymphomas (Gascoyne et al., 2003), but there is not any study about its expression in B-CLL. Passoni et al. (2002) identified two HLA-A2 restricted CTL epitopes, both located in ALK domain. They demonstrated that anti-ALK CTLs generated from peripheral blood lymphocytes of HLA matched healthy donors elicited an antigen specific, HLA-A2 restricted response, and were able to effectively kill tumour targets endogenously expressing ALK.

FMOD, a 59 KDa collagen binding protein, is one of the members of the leucine rich repeat protein family (Oldberg et al., 1989). It has the highest abundance observed in articular cartilage, tendon and ligament. FMOD was identified as the gene with highest fold difference in CLL expression compared with normal B lymphocytes in 3 independent gene expression profiling analyses (Klein et al., 2001; Jelinek et al., 2003; Vallet et al., 2003). The gene expression of B-CLL cells was compared with the expression of memory B cells, germinal centre B cells, naïve B cells and with peripheral blood B lymphocytes of age- matched healthy donors (Klein et al., 2001; Jelinek et al., 2003). The overexpression was checked by RT-PCR, and also confirmed by western blotting and flow cytometry at the protein level (Jelinek et al., 2003). FMOD was observed in all of 60 patients with B-CLL, whereas it was not detected in 13 CML
patients, 11 ALL patients and in 70 PBMCs of healthy donors tested (Mayr et al., 2005a). Mayr et al. (2005a) showed in their study that autologous FMOD-specific T lymphocytes derived from CLL patients not only recognized HLA-A2 binding FMOD peptides presented by T2 cells, but also FMOD- overexpressing autologous CLL cells in an HLA-A2’ restricted manner. These findings suggest that FMOD can serve as a TAA for the immunotherapy of B-CLL.

The human homolog of murine double-minute 2 (MDM2) oncoprotein, also known as HDM2, is a key regulator of cell growth and death and plays an important role in transformation of normal cells into tumour cells. The primary role of MDM2 under nonstressed conditions is to target the degradation of the tumour suppressor protein p53. However, in stress conditions, p53 is not affected by MDM2 and functions as a transcription factor that induces transcription of MDM2 as well as of genes involved in growth control or apoptosis. Therefore, MDM2 has both antiapoptotic and growth promoting activities (Momand et al., 2000; Vargas et al., 2003). Overexpression of MDM2 was observed in sarcomas, glioblastomas, leukaemias, non-Hodgkin’s lymphomas, while in B-CLL MDM2 was overexpressed in 64% to 73% of the cases (Oliner et al., 1992; Bueso-Ramos et al., 1993; Finnegan et al., 1994; Watanabe et al., 1994; Haidar et al., 1997). Mayr et al. (2006) detected MDM2 over-expression in 11 of 13 (85%) B-CLL cases tested. They have also tested MDM2 expression before and after CD40L activation, and MDM2 was not inducible by CD40L stimulation. They also showed that MDM2 overexpressing CLL cells present an MDM2- derived HLA-A2 binding epitope on their surface. Their study was the first report showing autologous functional MDM2- specific T cells in the human system that recognizes the MDM2 derived peptides and CLL cells, while normal B cells are not targeted.

SLLP1 is a cancer-testis antigen and a unique nonbacteriolytic, c-lysozyme-like protein isolated from human spermatozoa (Mandal et al., 2003). Wang et al. (2004) determined the expression of SLLP1 in haematologic tumour cells using RT-PCR, real time PCR and western blotting. The results showed that SLLP-1 was expressed in 2 out of 9 AML samples, 3 of 11 B-CLL, 4 of 14 CML patients, and 6 of 17 multiple
myeloma. In contrast, it was not expressed in corresponding specimens from any healthy donors. This group was the first to provide evidence of SLLP-1 expression in a proportion of patients with haematologic malignancies and a very restricted normal tissue expression. However, CTL responses to SLLP-1 have not been reported by this group or any other group either.

Ly9 (CD229) is a member of the immunoglobulin superfamily, representing signaling receptors. It was originally defined as a mouse cell surface glycoprotein and is expressed on thymocytes and mature T and B lymphocytes (Sandrin et al., 1996). Its structural features make it a member of the CD2 family (which includes CD2, CD48, CD58, CD84, CD244, NTB-A, CSI and CD150) (Davis et al., 1996; Romero et al., 2004). It is involved in cell adhesion and in the activation of lymphocytes and natural killer cells (Tangye et al., 2000). Using flow cytometry, Bund et al., (2006) detected CD229 expression at the protein level in all 18 B-CLL samples tested. They also demonstrated by IFN-γ assay that T cells were not only able to recognise HLA-A2 binding CD229 derived peptides presented by T2 cells, but also CD229 overexpressing autologous B-CLL cells in an MHC class I restricted manner.

Finally, the oncofetal antigen immature laminin receptor protein (OFA- iLRP) is a 37KDa protein that is expressed in fetal tissues and in many types of cancer including breast, lung, ovary prostate, renal cancer and lymphomas. It is not detectable on normal cells but it was detected in embryos/ early fetuses (Coggin et al., 1997; 1998). Siegel and her group (2003) detected OFA- iLRP expression on all AML and B-CLL samples tested by flow cytometry. They observed that both haematologic tumor cell lines and malignant cells from patients with AML and CLL endogenously expressing OFA-iLRP could be recognized and killed by OFA-iLRP–specific CTLs, whereas nonmalignant cell subpopulations derived from the haematopoietic system (CD34 progenitor cells, bone marrow cells, B lymphoblasts, and dendritic cells) were not affected. In the study of Siegel et al. (2006), specific OFA-iLRP peptide epitopes were identified using antigen specific CTLs that were generated in vitro by priming with peptide pulsed monocyte derived DCs. Both allogeneic and autologous CTLs recognised and killed OFA-iLE
expressing tumour cell lines and cells from patients with AML and CLL in an MHC-restricted fashion.

More studies are still needed to confirm the expression frequencies of each of these antigens in B-CLL cells. This information is crucial for the design of polyvalent vaccine strategies, because it is mandatory to know which antigens have to be chosen and to assess the number of patients that are suitable for a combined or sequential vaccination with two or more tumour antigens. Such a strategy could have the potential of reducing or even preventing the \textit{in vivo} selection of antigen loss tumour cell variants that might be generated by immunotherapies targeting only a single antigen (Jäger \textit{et al.}, 2005).

In conclusion, the aim of this chapter was to investigate a spectrum of TAA expression in B-CLL cells and the effect of CD40 ligation on their expression. I did not find evidence of expression of MAGE-A1, MAGE-A3, Proteinase-3 and WT-1 in any of the unactivated and activated B-CLL cells tested. However, an upregulation was seen in PRAME expression after activation in 8 samples, and in NY-ESO-1 expression in one sample. Based on that, more studies are needed for the identification of additional TAA with a more frequent expression in B-CLL. The increasing number of TAA and antigen derived T lymphocyte epitopes available with the diagnostic tools for expression analysis will provide rational basis for future cancer vaccination (Nestle \textit{et al.}, 1998; Rosenberg \textit{et al.}, 1998).
4. Induction of tumour specific T cell responses \textit{in vitro}
4.1 Introduction

It is well known that T lymphocytes are able to recognize tumour cells, which is the principle of antigen specific immunotherapy. Most, if not all, tumours express antigens, which are presented by MHC molecules, that CTLs can recognize and potentially attack. Spontaneous immune responses against human tumours have been reported in different types of cancer, especially in melanoma and renal cell carcinoma but also in other types of cancer, such as bladder carcinoma and breast cancer, indicating the specific interaction of the immune system with antigens presented by the tumour (Knuth et al., 1991; Wolfel et al., 1994). However, chronic lymphocytic leukaemia is an attractive target to examine immunotherapeutic studies on. Since B-CLL is a slowly progressive tumour, there is sufficient time to generate an immune response against CLL cells and to employ vaccination. Also B-CLL cells express both MHC class I and class II molecules, allowing direct presentation of antigen to both CD8\(^+\) and CD4\(^+\) T cells. Finally, B-CLL derives from a cell type (B lymphocytes) that have the potential to act as professional antigen presenting cells.

MHC class I restricted antigens recognized by CD8\(^+\) T lymphocytes \textit{in vitro} have been evaluated for specific immunologic effects in clinical cancer vaccine studies. Naturally occurring tumour reactive T cells were found in some B-CLL patients (Goddard et al., 2001; Gitelson et al., 2003), but their direct reaction against CLL cells was weak. On the other hand, marked progress has been achieved with regard to generation of specific immune responses by CTLs against CLL cells and to assess their response to therapy (Tsiodras et al., 2000; Hallek et al., 2005).

In this chapter, the induction of tumour specific T cell responses \textit{in vitro} was studied. This was achieved by:

1. Checking the ability of autologous CD40L activated B-CLL cells to induce CTL responses by ELISpot assay and \(^{51}\)Chromium release assay.
2. Induction of survivin peptide pulsed T2 cell stimulation of CTL in HLA-A2\(^+\) CLL patients compared with HLA-A2\(^-\) CLL.
4.2 Materials and Methods

As the experiments were designed in two categories, the methods below are described accordingly.

4.2.1 In vitro stimulation of autologous, tumour specific T cell responses in B-CLL patients

4.2.1.1 Autologous T cell responses to B-CLL cells

Unstimulated B-CLL cells and CD40L activated B-CLL cells were used as stimulators for the autologous tumour-specific T cell responses. CD40L activated CLL cells, following the same protocol mentioned in 2.2.8.1, were collected after three days coculture with CD40L L cells by the pasteur pipette, treated with mitomycin C (100μg/ml) for an hour, and washed twice with GM at 1450 rpm before resuspension at 10^5 cells/ml, to be used as stimulator cells. 3x10^5 autologous T cell enriched PBMC from B-CLL patients, following removal of B cells using anti-CD19 MicroBeads (MACS, MilteniBiotech, UK), as described in 2.2.3, were incubated in 3:1 ratio with stimulators for a week at 37ºC, 5% CO₂. Restimulation with unactivated and CD40L activated autologous CLL cells in the presence of IL-2 (20unit/ml) and IL-7 (10ng/ml) (Both from R&D systems, MN) was performed on days 7 and 14 for each culture tested. T cells on their own were used as a negative control, whereas HMY-2 stimulated T cells were the positive control. ELISpot assay was then performed after the second restimulation by adding the responders to stimulators in 3:1 ratio in triplicate wells in 96-well nitrocellulose filter plates and then by applying the steps mentioned below (section 4.2.1.2) until spots were developed and counted under a dissection microscope. ^51Cr release assay was also used to determine T cell mediated cytolyis of target cells, as described in section 4.2.1.3.

4.2.1.2 Enzyme Linked ImmunoSpot (ELISpot) Assay

The ELISpot assay allows the quantification of peptide-specific T cells at the single cell level by the detection of cytokine release. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation. The ELISpot assay was performed by using the human interferon-γ (IFN-γ) ELISpot plus kit (Mabtech AB,
Sweden). In the first step, the 96 well Multiscreen filter plates (MultiScreen MAHA, Millipore, USA) were coated with high affinity monoclonal antibody to the cytokine to be investigated (in this case, IFN-γ). The plate membrane was first pre-wet with 50µl of 70% ethanol per well and then washed 5 times with 200µl/ well sterile water. The coating monoclonal antibody (1-D1K; Mabtech, Sweden) was then diluted to 15µg/ml in sterile PBS. 100µl of the antibody solution was added per well and the plate was incubated overnight at 4-8ºC. After removing excess antibody by washing plate 5 times with 200 µl/ well sterile PBS, 200µl/ well of growth medium were added and left for ≥30 minutes at room temperature. After removing the media, 50µl of appropriate stimulator cells and 100µl of responder cells were pipetted into the wells. The microplate was then incubated at 37ºC, 5% CO₂ incubator for 48 hours. During this period, antigen specific responding cells secrete IFN-γ, which is captured by the anti-IFN-γ mAb in the immediate vicinity of the cells. After washing away cells and any unbound substances 5 times with PBS, 200µl/ well, a biotinylated polyclonal antibody (7-B6-1; Mabtech, Sweden) directed against IFN-γ was added to the wells. This detection antibody was diluted to 1µg/ml in PBS containing 0.5% fetal calf serum (PBS- 0.5% FCS) and 100µl/ well was added and incubated for two hours at room temperature. Following a wash to remove any unbound biotinylated antibody, 100µl/well of alkaline phosphatase conjugated to streptavidin (streptavidin- ALP) was added (1:1000) in PBS- 0.5% FCS. One hour later, unbound enzyme was removed by washing the plate as described above, and 100µl/ ml filtered substrate solution (BCIP/ NBT; Mabtech, Sweden) was added to each well. The reaction was terminated by washing extensively with tap water when dark purple coloured precipitate formed at the sites of cytokine localization and appeared as spots. Each spot represented an individual cytokine secreting cell, and those spots were counted under a dissection microscope. Comparison of the number of spots with the number of cells added gave the frequency of responding cells (spots/ 10^6 T cells). The plate was then stored in the dark at room temperature.
4.2.1.3 $^{51}$Chromium release assay

The $^{51}$chromium [$^{51}$Cr] release assay was used to test the cytolytic capacity of T cells from B-CLL samples against autologous tumour cells. The cytolysis was measured as the radioactivity counts per minute (cpm) in the supernatant which was released from disintegrating cells following co-culture with effector T cells. For this assay, effectors cells were prepared in the same way mentioned in section 4.2.1.1, where unstimulated CLL cells, CD40L activated CLL cells, and HMY-2 as a positive control, were incubated with autologous T cells for a week. After one week’s incubation, $^{51}$Cr release assay was performed. Target cells (unstimulated CLL cells, CD40L activated CLL cells and HMY-2) were labeled in 14ml tubes with 100µCi $^{51}$Cr (3.7 MBq) (GE Healthcare Ltd, UK) and incubated for an hour at 37ºC. During this period, effector cells were counted and resuspended at appropriate concentration for the assay of 3:1 (1.5x10$^5$ cells/ml), 10:1 (5x10$^5$ cells/ml) and 30:1 (1.5x10$^6$ cells/ml) effector: target cell ratios. After one hour’s incubation, target cells were washed three times with growth medium and seeded in 96- well rounded bottom microtiter plates at a concentration of 10$^5$ cells/ml in triplicate wells (50µl/well). Effector cells were added at various effector- target cell ratios (3:1, 10:1, 30:1) in a final volume of 150µl/ well. The plates were incubated for 4 hours at 37ºC in a humid atmosphere with 5% CO$_2$. After 4 hours, 50µl of the culture supernatant from each well were carefully harvested without disturbing the cell pellet, added to 5ml tubes and counted in a gamma counter (Packard, Cobra TM model B5005, Meriden). The percentage of specific lysis was calculated as:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Maximum chromium release was determined by the addition of 5% Triton-X (Sigma, UK), and spontaneous release was assessed in the presence of growth medium to the target cells.
4.2.2 *In vitro* stimulation of survivin peptide specific T cell responses in B-CLL patients

4.2.2.1 HLA-A2 binding assay

T2 cells, a TAP-deficient human B- T-lymphoblastoid cell line, were used to evaluate HLA-A2-specific survivin peptide binding and to assess the HLA-A2/ peptide complex stability. After T2 cells (3 × 10^6 cells/ml) were washed 3 times and transferred into Eppendorf tubes, cells were pulsed with one or other survivin peptides at 50μg/ml, plus 3μg/ml human β2-microglobulin (β2-M) (Sigma, UK). β2-M is a polypeptide that associates with the heavy chain of class I major histocompatibility complex (MHC) antigens on the cell surface. The interaction is dynamic and plays a critical role in the stability of the MHC antigens and their ability to present peptide antigens in CD8⁺ cells. A transient complex of MHC heavy chain and β2-M is known to be assembled into the TAP molecule (transporter associated with antigen processing). Binding of processed peptide releases the class I-β2-M complex to the cell surface. Absence of binding leads to degradation in the proteasome. Stabilisation of HLA-A2 β2-M- peptide complexes at the cell surface of T2 cells can be achieved by the addition to T2 cells of exogenous, HLA-A2 binding peptide and β2-M.

Two survivin peptides (Proimmune Limited, UK) were used: the first peptide sequence was ELTLGEFLKL (Schmidt *et al.*, 2003) and the second was LMLLGEFLKL (Granziero *et al.*, 2001). The survivin sequence was reviewed for peptides that could potentially bind to HLA-A*0201*, using a peptide- motif scoring system. The first peptide has already been defined as CTL epitope and it showed significant stabilization of HLA-A2 (Schmitz *et al.*, 2000; Anderson *et al.*, 2000) whereas the second peptide is a modified one of the peptide LTLGEFLKL, in which a better anchor residue (leucine or methionine) replaced threonine at position two. Both peptides are of similar high affinity. The HLA-A2 binding peptides derived for survivin were synthesized in an automated peptide synthesizer following the Fmoc/tBu strategy. After removal from the resin by treatment with trifluoroacetic acid/ phenol/ ethanedithiol/ thioanisole/ water (90: 3.75: 1.25: 2.5: 2.5 by volume) for an hour or 3 hours, arginine- containing peptides were precipitated from methyl-tert, butyl ether, washed once with methyl-tert, butyl
ether and twice with diethyl ether, and resuspended in water prior to lyophilisation. Synthesis products were analysed by reversed-phase high performance liquid chromatography (HPLC) and mass spectrometry (ProImmune Limited, UK).

The T2 cells were incubated with the 2 peptides over a range of concentration at 37°C, 5% CO₂ in humidified air for 4 hours. Following incubation, the cells were washed twice by centrifugation at 1450 rpm and stained with 50µl mouse anti-human HLA-A2.1 monoclonal antibody BB7.2 for 30 minutes at room temperature. After washing, the cells were incubated with 10µl goat anti-mouse IgG (FITC) secondary antibody for 30 minutes at room temperature. The cells were then washed twice with PBS by centrifugation at 1450 rpm and fixed prior to analysis by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson Ltd, UK). The mean fluorescence index (MFI) = (mean channel fluorescence of T2 cells pulsed with the peptide plus β2 microglobulin / mean channel fluorescence of T2 cells pulsed with β2 microglobulin only) was calculated to determine the up-regulation of HLA-A2.1 expression on T2 cells caused by peptide pulsing.

4.2.2.2 Stimulation of T cells from B-CLL patients with peptide pulsed T2 cells

T2 cells were washed twice by centrifugation at 1450 rpm before they were treated with Mitomycin C (100µg/ml) for an hour in 37°C, 5% CO₂ incubator. Cells were then washed 3 times with WM, resuspended at 3x10⁶/ml concentration and loaded with one or other survivin peptides at 50µg/ml plus 3µg/ml β2 microglobulin (Sigma, UK). During the 3 hours incubation of peptide pulsed T2 cells at 37°C in the presence of 5% CO₂, T cells were obtained from B-CLL cells using anti-CD19⁺ microbeads (section 2.2.3). After the incubation period was over, peptide pulsed T2 cells were washed twice and resuspended in GM to be used as stimulators. T cells (3x10⁶/ml) from B-CLL patients were cultured with T2 cells on their own, or peptide pulsed, as stimulators in GM for five days at 37°C in a humid atmosphere with 5% CO₂. ELISpot assay (section 4.2.1.2) was then performed using 1:1 responders to stimulators ratio, and the plate was incubated for 48 hours at 37°C, 5% CO₂ prior to estimation of number of IFN-γ secreting T cells.
4.3 Results

4.3.1 Effect of CD40L activation in autologous responses

4.3.1.1 Effect of CD40L activation in autologous responses by ELISpot

Because CD40 activation is an effective mechanism to increase APC function of malignant B-CLL cells, the ability of CD40L activated B-CLL cells to stimulate autologous T cell responses was investigated. As shown in 2.3.2.1, CD40 ligation caused the highest upregulation in the activation markers (section 2.3.2.1), induced the highest proliferative response of allogeneic T cells (section 2.3.2.2.) and also resulted in an increase of some TAA expression (section 3.3.2). Its effect was therefore checked by ELISpot to test autologous T cell responses in a total of four B-CLL patient PBMC samples. T cells were enriched by negative selection, using CD19+ beads to deplete B cells from PBMC of B-CLL patients, and the resulting T cell rich fractions were stimulated in the presence of autologous unactivated or CD40L-activated B-CLL cells in parallel. Unstimulated T cells were used as a negative control, and HMY-2 stimulated T cells were used as a positive (allogeneic) control. To determine the optimal ratio of T cells, a serial titration was performed (data not shown). Regardless of the T cells to tumor ratio, the frequency of tumor-reactive T cells was greater in CD40L activated cells compared to unactivated samples. The best ratio was achieved with T cells at $3 \times 10^5$ per well and stimulators at $10^5$ per well. Although reactivity to tumor cells was observed in CD40L activated samples at target cell concentrations $\geq 5 \times 10^5$ per well, the resulting ELISpots were too numerous to accurately count. $3 \times 10^5$ target cells stimulated with $10^5$ unactivated and CD40L activated cells were therefore used to evaluate T cell responses in these assays, which were repeated twice. The results shown in Figure 4.1 represent the data for autologous T cells responses on the four B-CLL samples tested.

A significant number of IFN-γ spots were detected in all conditions tested. However, activation of tumor cells with CD40L enhanced the sensitivity of the ELISpot assay by increasing the IFN-γ production by responding T cells as compared to unactivated tumor cells or control T cells on their own in all 4 patients that were tested (Figure 4.1). The difference observed between unactivated and activated B-CLL cells as stimulators in the
first sample (B-CLL 2) was not significant. On the other hand, the results represented significant differences (* $P < 0.05$, ** $P < 0.001$) of autologous T cells in the remaining three samples in response to CD40L activated cells compared to unactivated cells. There was also significant difference seen between unstimulated T cells and autologous T cells stimulated with CD40L activated B-CLL cells in B-CLL 8 ($P < 0.001$) but not for the other samples. Unstimulated T cells from B-CLL patient 10 exhibited higher responses than those stimulated with unactivated B-CLL cells ($P < 0.05$). Unstimulated T cells from the other samples, however, did not show any significant difference compared to autologous T cells responses to unactivated B-CLL cells (Figure 4.1).

**Figure 4.1** Autologous T cell IFN-γ responses of 4 different B-CLL patients in response to stimulation with unactivated and CD40L activated B-CLL cells. Autologous T cells were stimulated twice with unactivated or CD40L activated B-CLL cells at a responder: stimulator ratio of 3:1 and IFN-γ release was measured by ELISpot assay after two weeks of T cell culture. The white bar shows the control T cells on their own, whereas the blue bar represents T cells response to unactivated B-CLL cells, and the pink bar is for autologous T cells in response to stimulation with CD40L activated B-CLL cells. Stars indicate significantly different autologous T cell responses to either unactivated or CD40L activated B-CLL cells (* for $P < 0.05$, ** $P < 0.001$).
4.3.1.2 Effect of CD40L activation in autologous T cell responses by $^{51}$ Chromium Release Assay

To determine autologous T cell responses to unstimulated and CD40 ligated CLL cells, $^{51}$Cr release assay was performed. For target cells, unstimulated CLL cells, CD40L activated CLL cells and HMY-2 were labeled with 100μCi $^{51}$Cr at a concentration of $10^5$ cells/ml in triplicates. Effector cells were added in a ratio of 3:1, 10:1 and 30:1 and cocultured for 4 hours at 37ºC in a 5% CO$_2$ humidified atmosphere. After 4 hours the supernatants were harvested and the released $^{51}$Cr was measured in a γ- counter. Spontaneous and maximum releases were determined by incubation of target cells in the presence of either medium alone or 5% Triton X, respectively. Specific lysis was determined for each condition tested using the equation mentioned in 4.2.1.3.

T cells derived from patients with B-CLL were cultured alone (unstimulated T cells) or with T cell targets (unactivated B-CLL cells, CD40L activated CLL cells and HMY-2). The different effector cells were expanded for a week and then restimulated with unactivated and CD40L activated CLL cells for the $^{51}$Cr release assay. T cells responses to the different targets used were measured and resulted in less than 5%-0.5% specific lysis at E:T ratios of 30:1, 10:1 and 3:1 with 30:1 ratio showing the highest percentage of lysis. Cytotoxicity to unactivated CLL cells gave similar results to unstimulated T cells where autologous T cell responses were enhanced in activated cells with CD40 ligation ($P<0.05$). The results suggest either no or very weak autologous cytolytic T cell responses to B-CLL cells.

These findings are in line with autologous T cell responses to unactivated and CD40 ligated CLL detected by ELISpot. However, as the (allogeneic) cell line HMY-2 failed to induce a significant cytolytic T cell response, the data suggest that either the patients T cells were anergic to allogeneic stimulation, or that there were technical problems with the assay.
Figure 4.2 Cytotoxicity of B-CLL T cells by $^{51}$Cr release assay. Effector cells were raised against the target cells (HMY-2, unactivated and CD40L activated cells), and tested for cytolytic activity at E:T ratios of 30:1, 10:1 and 3:1, respectively. HMY-2 was used as targets for HMY-2 activated T cells; autologous CLL targets for unstimulated T cells and autologous CLL targets for unactivated CLL T cells and CD40L activated CLL T cells.

4.3.2 T2 peptide pulsed stimulation of CTL in HLA-A2$^+$ CLL patients

4.3.2.1 Upregulation of HLA-A2 on peptide pulsed T2 cell

To determine the binding ability of survivin peptides to HLA-A2 molecules, an in vitro cellular binding assay was performed using the TAP deficient cell line T2. T2 cells were incubated with 50μg/ml of each survivin peptides in serum free RPMI 1640 medium supplemented with 3μg/ml human β2 microglobulin for 4 hours at 37°C and 5% CO$_2$. Both survivin peptides were shown to bind HLA-A2 in the binding assay using T2 cells (Figure 4.3).
∆MFI was obtained by dividing the MFI of HLA-A2 expression on peptide pulsed T2 cells plus 3μg/ml of β2 microglobulin by the MFI of HLA-A2 expression on T2 cells plus 3μg/ml of β2 microglobulin in the absence of peptide. The ∆MFI of both peptide pulsed T2 cells was greater than 1, indicating the upregulation of HLA-A2 molecules due to the specific binding of peptide to the molecules on T2 cells.

### Figure 4.3

Specific binding of survivin peptides using T2 cells in HLA-A2 binding assay. T2 cells were pulsed with each of survivin peptides at 50μg/ml in the presence of 3μg/ml of β2 microglobulin and incubated for 4 hours, stained with mouse anti-human HLA-A2.1 monoclonal antibody BB7.2 and analysed using the flow cytometry. The open blue histogram shows T2 cells pulsed with β2 microglobulin only, whereas the purple histogram shows T2 cells pulsed with the peptide plus β2 microglobulin.

### 4.3.2.2 Stimulation of HLA-A2+ B-CLL cells with peptide pulsed T2 cells by ELISpot assays

In attempt to induce survivin specific CTL in T cells from HLA-A2+ B-CLL patients, to determine if autologous B-CLL cells are recognized in survivin- specific HLA-A2 restricted manner, and for further confirmation of the data obtained by HLA-A2 binding assay, ELISpot assay was performed on T cells that had been stimulated with T2 cells pulsed with survivin peptides or without peptides. Induced autologous T-cell response to T2 peptide pulsed cells varied among HLA-A2+ patients (Figure 4.4). The production of IFN-γ increased after T cells were stimulated with survivin peptide pulsed T2 cells compared to T2 cells in the absence of survivin peptides, in only one out of 5 patients
analyzed (B-CLL 23, $P<0.01$) in response to T2 peptide pulsed cells specific to survivin (LMLLGEFLKL) peptide as compared to T2 cells on their own (Figure 4.4). A lack of significant difference in the number of IFN-$\gamma$ spots in the remaining four samples indicates there was not a specific reactivity for peptide pulsed T2 cells compared with T2 cells without the peptides. Another IFN-$\gamma$ ELISpot assay was performed in parallel from representative HLA-A2$^+$ patients. The frequency of the survivin-reactive T cells to T2 peptide pulsed cells was not significantly different as compared to T2 cells alone in all four HLA-A2$^+$ patients (Figure 4.5). By comparing results together, short term culture of B-CLL cells (HLA-A2$^+$ n=5 or HLA-A2$^-$ n=4) with peptide pulsed T2 cells in ELISpot assay showed no difference in stimulation between HLA-A2$^+$ and HLA-A2$^-$ CLL patients, and only showed a significant peptide associated increase in IFN-$\gamma$ producing cells in one out of 5 HLA-A2$^+$ patients tested. ELISpot assay was repeated for three B-CLL patients (2 HLA-A2$^+$ and 1 HLA-A2$^-$) of which one of HLA-A2$^+$ samples showed significant differences to peptide pulsed T2 cells in the previous experiments (Figure 4.6). No significant response of B-CLL 23 T cells to T2 peptide pulsed was detected on this occasion, which raises the question as to whether previous result (Figure 4.4) was valid/ reproducible.

**Figure 4.4** T cells from 5 HLA-A2$^+$ B-CLL patients were stimulated using T2 pulsed with survivin peptides or T2 cells in the absence of peptide. IFN-$\gamma$ ELISpot assay was performed after two days incubation of responders: stimulators at a 1:1 ratio. The star represents significant difference where $P<0.01$. 

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Figure 4.5 T cells from 4 HLA-A2+ B-CLL patients were stimulated using T2 cells pulsed with survivin peptides or T2 cells in the absence of peptide. IFN-γ ELISpot assay was performed after two days incubation of responders: stimulators at a 1:1 ratio.

Figure 4.6 Repeated IFN-γ assay under the same conditions mentioned earlier for T cells from 3 B-CLL patients (2 HLA-A2+ and 1 HLA-A2- B-CLL patients) which were stimulated using T2 cells pulsed with survivin peptides or T2 cells in the absence of peptide and which showed peptide associated increase in Figure 4.4 and 4.5.
4.4 Discussion

There has been considerable progress over the past several years in identifying peptides derived from human tumour antigens recognized by CD8+ T cells (Boon and van der Bruggen, 1996; Rosenberg, 1999; Jäger et al., 2003). A growing list of peptides with the corresponding MHC class I restriction elements have been identified. Because peptides are relatively easy to prepare for clinical use, peptide-based cancer vaccines are being widely tested, either with peptides alone (Singh et al., 1999) or mixed with adjuvants (Singh et al., 1999), administered with a systemic cytokine such as GM-CSF (Jäger et al., 1996a) or IL-2 (Rodolfo et al., 1999) or presented by dendritic cells (Thurner et al., 1999) or other antigen presenting cells (Hu et al., 1996). Experience to date indicates that peptide vaccines can elicit specific CD8+ T cell responses. However, the use of different peptides, vaccine strategies, and methods to monitor CD8+ responses as well as variables involved in patient selection and responses, make it hard to assess which approach to peptide vaccination is most promising.

Vaccination strategies for patients with B-CLL using whole tumour lysate (Hus et al., 2005), irradiated cells or total RNA to induce anti-tumour immune responses are under current clinical investigation (Kokhaei et al., 2004). However, to reach a high concentration of T cell epitope peptide and to induce a clinically relevant T cell response, the definition of immunogenic TAAs and their T cell epitope in B-CLL is highly desirable. Moreover, only vaccination with antigenic peptides might allow a straightforward assessment of T cell responses to these particular peptides (Nagorsen et al., 2004). Based on that, the aim of this chapter was to investigate the nature of immune responses of T cells against B-CLL cells and to check the potential of CD40L stimulated CLL cells to activate T cells for tumour vaccination. To achieve this aim, ELISpot assays were performed in which, after 14 days of in vitro culture, a significantly higher number of IFN-γ-releasing T cells was obtained using CD40L activated B-CLL cells as APCs compared with unactivated B-CLL cells in 3 of 4 B-CLL patients tested. The effect of CD40L activation in T cell autologous responses was also checked by 51Cr release assay. Although 51Cr detection is easy and sensitive, its preparation is time consuming and it needs large numbers of effector T cells as well as the need of handling...
the radioactive material. An earlier study showed that allo-responsive CTLs were able to kill resting and CD40 stimulated B-CLL with equal efficiency (Chu et al., 2000). However, others found enhanced allogeneic CTL mediated killing of CD40 activated B-CLL cells compared with unstimulated B-CLL cells. In contrast, no autologous CTL mediated killing of both stimulated and unstimulated B-CLL was observed in the study of Krackhardt et al. (2002). The CTLs used in these studies were generated by priming CTLs with CD40 activated B-CLL cells. Our results contradict the last group findings, as there was specific lysis of unstimulated and CD40L stimulated cells ($P<0.05$) using $^{51}$Cr release assay. The inability of CD40L CLL cells to stimulate CD8+ T cells in the autologous system can be explained by several mechanisms (Cayabyab et al., 1994). First, CD40 expressed on B-CLL cells may costimulate CD4+ T cells rather than CD8+ T cells. Second, autologous peripheral blood lymphocytes might be less efficient in mounting a cytolytic response than T cells derived from the bone marrow (Cardoso et al., 1997). However, it is unlikely that autologous peripheral blood T lymphocytes are incapable of mounting a CTL response in B-CLL patients, because the generation of tumour cell specific CTLs from the peripheral blood of B-CLL patients has been demonstrated. Several other reasons might also be responsible for the low capacity to induce cytotoxic T cell responses against autologous CLL cells. Trojan et al. (2000) previously demonstrated that killing of CLL cells by CTL generated against peptide presented by APC was increased by either addition of exogenous peptide or by CD40L activation of the CLL cells. This suggests that insufficient antigen presentation as well as costimulation contribute to the low levels of cytotoxicity observed against native CLL cells. Our results showed that enhancement of costimulation by CD40 activation appear to induce autologous cytotoxic T cell responses in CLL ($P<0.05$) by $^{51}$Cr release assay. Finally, the failure of the allogeneic cell line HMY-2 to induce a significant cytolytic T cell response raises the possibility that either the patient T cells were anergic to allogeneic stimulation, or that the assay was technically inadequate.

Recently, it was shown that survivin- specific CTLs can be detected in patients with CLL or malignant melanoma and that these CTLs are able to lyse HLA- matched allogeneic breast cancer and malignant melanoma cells. This finding indicates that
survivin has the potential to be used for immunotherapy of tumours (Andersen et al., 2001a). This finding also was strengthened by the identification of a natural processed HLA-A2 binding peptide that could be used for generation of survivin specific CTLs when pulsed on dendritic cells (Schmitz et al., 2000). It is known that MHC class I molecules become unstable in the absence of binding peptides. Because T2 cells lack TAP molecules, which transport peptides into the endoplasmic reticulum, the MHC class I level on the cell surface of T2 cells is low. In the presence of binding peptides, MHC class I is stabilized leading to upregulation on the cell surface. Based on that, a binding assay was done to check the binding affinity of survivin to HLA-A2$^+$ molecules. As was shown in 4.3.2.1, an upregulation of HLA-A2 on peptide pulsed T2 cells was clearly seen. Passoni et al. (2002) used a computer assisted analysis to screen the ALK amino acid sequence for the presence of HLA-A2 binding motifs. The ability of selected ALK derived peptides to bind to HLA-A2 molecules and to form stable MHC/peptide complexes was assessed by measuring the binding affinity where 9 of the 22 predicted peptides were shown to have high affinity with strong binding to HLA-A2. Another group (Mayr et al., 2005a) have shown that all 4 FMOD peptides were to bind HLA-A2 in a binding assay using the TAP deficient cell line T2. Siegel and her group (2006) also used T2 binding assay to screen the OFA-iLR protein for HLA-A2 binding peptides in which 2 of 14 candidate peptide exhibited a high affinity to the HLA-A2 molecules.

After checking the ability of survivin peptides to bind to T2 cells, IFN-γ ELISpot assays were performed to investigate T cell responses of CLL patients to survivin peptides. The ELISpot methodology represents a strong tool to monitor peptide specific T-cell responses. However, no significant differences were observed against survivin peptides in HLA-A2$^+$ and HLA-A2$^-$ B-CLL patients. Obviously, this might simply reflect that these patients do not host a tumour specific response. However, responses in these patients may have been present but below the detection of our assays. Furthermore, other class I MHC- restricted survivin- derived peptide epitopes may dominate the response in these patients, and the presence of additional peptides processed and presented in the context of HLA-A2 cannot be excluded. Results were quite different when repeated
twice to the same B-CLL sample (CLL-23). One possible explanation could be a lower amount of IFN-γ produced per cell which might be a lower functional avidity of T cells.

Because most human cancers express high levels of survivin, immunotherapeutic strategies aiming at this antigen may have broad clinical applications. The major concern of such an approach would be the induction of autoreactive immune responses. Thus, the future of survivin based vaccination will depend on both the therapeutic efficacy and on the type of side effects that may follow immunization. The attractiveness of survivin for vaccination purposes relies not only on the universal expression among tumours of different origin, but also on the fact that down regulation or loss of its expression as a means of immune escape would slow down the progression of the tumour.

In conclusion, CD40L autologous CLL cells stimulation increased IFN-γ T cells compared with unactivated CLL cells, but gave no evidence for antigen specificity of this response. There was also no evidence if survivin specific CTL from T2/peptide data.
5. *In vitro* induction of HLA-A2 restricted survivin specific CTL in normal donor
5.1 Introduction

B-CLL is an attractive candidate to examine immunotherapeutic approaches because it is a slow growing tumour, allowing time for the generation of an immune response against the tumour cells. Immunotherapy using dendritic cells has shown encouraging results in both haematological and non haematological malignancies (Goddard et al., 2001).

The marked growth in our understanding of the immune system and its interaction with malignant disease includes the mechanism by which antigen is presented to T lymphocytes (Ben- Efraim, 1999; Pawelec et al., 1999). DCs are known to be essential for the initiation of primary immune responses and are particularly efficient at capturing and presenting antigens to T cells (Palucka et al., 2005). Tumour specific CD8+ cytotoxic T lymphocytes (CTL) constitute the most important effector cells for anti-tumour responses. CTLs recognize “processed” peptides that are derived from endogenous proteins and are presented on the cell surface in association with MHC class I molecules (Melief et al., 1995). Therefore, tumour specific CTLs can recognize and select the antigenic peptides and then kill the tumour cells in an antigenic peptide specific approach.

Based on that, in this chapter I investigated whether human CD8+ T lymphocytes from a healthy donor could be specifically activated against survivin peptides in vitro. Monocyte derived DC’s from a healthy, HLA-A2+ individual were derived in vitro, pulsed with survivin peptides known to encode a HLA-A2’ restricted CTL epitope, and were used to stimulate autologous T cells in vitro, in an attempt to generate a survivin-specific CTL line, that could then be used to assess antigen processing and presentation of survivin by unactivated and activated B-CLL cells.
5.2 Materials and Methods

5.2.1 Generation of Human Dendritic Cells (DCs) Culture

Blood was obtained from a HLA-A2+ healthy donor, and PBMC were purified from 60 ml of heparinized whole blood by density gradient centrifugation over Lymphoprep as was described in section 2.2.2. PBMC from the buffy coat were washed five times in washing media (RPMI 1640+ 2mM L-glutamine, 1000U/ml penicillin) (Sigma, UK) by centrifugation at 1450 rpm for 10 minutes to remove the platelets. To generate DCs, monocytes were isolated from the PBMC by positive selection using immunomagnetic cell separation with anti-CD14 antibody-conjugated magnetic microbeads, according to the manufacturer’s instructions (MACS, Miltenyi Biotech, UK) and essentially as was described in 2.2.3 for CD19+ cell isolation. Isolated cells were greater than 95% pure as determined by immunofluorescent flow cytometry analysis (FACS) following staining with CD14+ monoclonal antibody (mAb). The CD14-negative cell fraction was frozen in aliquots in liquid nitrogen for use as a source of responder T cells. DCs were generated in two different cultures (4 days culture and 8 days culture) to check the optimal time for DC maturation. The only difference between both cultures was the duration in which the cytokines were added. Purified CD14+ monocytes (2-3x10^7) were cultured in Growth medium (RPMI 1640 + 10% FCS, 2mM L-glutamine, 1000U/ml penicillin) in the presence of 2000 units/ml granulocyte macrophage colony-stimulating factor (GM-CSF) and 1000 units/ml IL-4 (both from R&D systems, UK). Monocytes were incubated in 80cm tissue culture flasks at 10^6/ml cells in a 7ml final volume at 37ºC, 5% CO2 in a fully humidified incubator for 2 days in the 4 days culture or 5 days in the 8 days culture. After the required period of incubation, DCs were cultured for additional 2 days (in the 4 days culture) or 3 days (in the 8 days culture) in the presence of 2000 units/ml GM-CSF, 1000 units/ml IL-4, 10ng/ml tumour necrosis factor-alpha (TNF-α) (R&D systems, UK) and 1µg/ml prostaglandin E2 (PGE2) (Sigma, UK) in growth medium for further maturation. After day 4 and day 8 of the protocol of each culture, cells were collected and checked for maturity by staining with (FITC)-conjugated anti-CD80 and anti-MHC Class II, and (PE)-conjugated anti-CD83, anti-CD86 and anti-
CD14 monoclonal antibodies (all mAbs from Beckman Coulter Instrumentation Laboratory, UK).

5.2.2 In vitro Generation of Survivin- Specific T Lymphocytes

CD8+ T lymphocytes from the same normal donor as the dendritic cells, were enriched by positive selection from the CD14 negative cell fraction of the PBMC’s using an anti-CD8 antibody coupled to magnetic microbeads (MACS, Miltenyi Biotech, UK), according to manufacturer’s instructions. Following isolation, those CD8+ T cells were stimulated in vitro with peptide-pulsed autologous dendritic cells (DCs) as follows. Mature DC’s were split into two tubes and spun down, to add HLA-A2 binding survivin peptides to them (ELTGEFLKL and LMLLGEFLKL, the same survivin peptides described in section 4.2.2.1). Both peptides were dissolved in sterile distilled water and were added at 50μg/ml in 0.5 ml serum free medium to the mature DCs, and incubated for four hours at 37°C. After the four hours incubation, DCs were washed and resuspended in growth medium at 5x10^5 DCs/ml (2ml medium per peptide pulsed DCs). One ml peptide pulsed DC was added to each of two wells of a 24 well plate for each peptide. To each well of DCs, CD8+ autologous T cells were added (3x10^6 in 1 ml growth medium) and both were cultured together at 37°C in 5% CO2 incubator. Seven days later, cultures were washed and viable T cells were counted and adjusted to 2x10^6/ml in growth medium. 1 ml of T cells was added to wells of a clean 24 well plate and restimulated with freshly prepared survivin peptide- loaded DCs as described above at a responder to stimulator ratio 2:1. The cultures were supplemented with 25 units/ml recombinant human IL-2 and 10ng/ml recombinant IL-7 (both from R&D systems, MN) and medium was refreshed on day 4. An aliquot of cells was removed each week, prior to restimulation, on which to perform a viable cell count. After 5 cycles of stimulations, the cultures were tested for survivin specific T cells by pentamer staining as described next in section 5.2.4, and by ELISpot assay using peptide pulsed T2 cells (as was described in section 4.2.2.1).
5.2.3 Stimulation for autologous survivin CTL in B-CLL patients by ELISpot

ELISpot was performed after 5 restimulations of in vitro culture to find out if T cells were able to specifically recognize a survivin-derived peptide contained in DC-survivin peptide stimulated specific T cells culture. ELISpot assays were carried out essentially as was described before in 4.2.1.2. Unstimulated B-CLL cells and CD40L activated B-CLL cells from HLA-A2+ B-CLL patient and T2 cells pulsed with one or other of survivin peptides, were used as stimulators and were added in triplicates. T2 cells were washed 3 times and resuspended in washing media to a final concentration of $3 \times 10^6$ cells/ml and transferred into Eppendorf tubes to be pulsed with one or other of survivin peptides at 50μg/ml plus 3μg/ml human β2-microglobulin (Sigma, UK), and were incubated at 37°C, 5% CO₂ in humidified air for 4 hours. Cells were then washed twice by centrifugation at 1450 rpm and were diluted to desired concentrations. Unstimulated B-CLL cells and CD40L activated B-CLL (following the same steps of activation mentioned earlier in section 2.2.8.1) after MitC treating them for an hour and washing them twice by centrifugation at 1450 rpm, were counted and also diluted to the same required concentrations. Effector cells (the attempted survivin CTL cells) were added to stimulators in 3:1 ratio. Plates were then incubated at 37°C for 48hours. After five washes with PBS, the biotynilated secondary antibody 7-B6-1 Biotin was added and incubated for two hours. Streptavidin-alkaline phosphatase was then added for an hour and finally the substrate solution (BCIP/ NBT- plus) (all reagents from Mabtech, Sweden) was used and formed spots were counted using a dissection microscope.

5.2.4 Human MHC class I- peptide pentamer staining

Pro5™ pentamers comprise five MHC- peptide complexes assembled through a coiled-coil domain. Due to their configuration, all five MHC-peptide complexes in the pentamer are available for binding to complementary T cell receptors (TCRs). Each Pro5™ pentamer also comprises up to five fluorescent or biotin tags for bright and efficient labeling (Figure 5.1). R-PE labeled Pro5 MHC class I pentamer (Proimmune Limited, UK) is used for detecting and enumerating CD8+ single antigen-specific T cells using flow cytometry. HLA-A2 pentamers incorporating both (ELTGEFLKL) and
survivin peptides were used. In this chapter, pentamers were used to count survivin specific CD8\(^+\) T lymphocytes and determine their frequency.

![R-PE labeled Pro5\textsuperscript{TM} MHC Pentamers Structure](image)

**Figure 5.1** R-PE labeled Pro5\textsuperscript{TM} MHC Pentamers Structure. They consist of five MHC-peptide complexes and five fluorescent tags with a coiled coil domain in between.

Staining was performed according to the manufacturer's instruction. 2-5 \(\times 10^5\) T cells were incubated with 10\(\mu\)l of labeled pentamers (Promimmune Limited, UK) for 10 minutes at room temperature (RT) in the dark, after spinning the pentamer at 1450 rpm for 3 minutes to remove any protein aggregates that might contribute to non specific binding. Cells were then washed with 2ml washing buffer (0.5% BSA in PBS) and centrifuged at 1450 rpm for 5 minutes. Fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibody (mAb) (1\(\mu\)l, PromImmune Limited, UK) was added for 20 minutes of additional incubation on ice and shielded from light. Cells were then washed as described above and fixed by 200\(\mu\)l fix solution (0.5% Paraformaldehyde). Samples were immediately acquired after fixation on a FACSCalibur flow cytometer and analysed using CellQuest software (both, Becton- Dickinson Ltd, UK). 50,000 total events were acquired per sample, which was gated on living lymphocytes. In the histogram plot, X and Y axes showed pentamer\(^+\) cells and CD8\(^+\) living cells respectively. As a negative control, unstimulated PBMC from the same HLA-A2\(^+\) healthy control
were similarly stained with the same pentamers for comparison. Pentamers were titrated to minimize background staining and were used at a final dilution of 1/50 (10µg/ml).

5.3 Results
5.3.1 Induction of survivin specific CTL line by peptide pulsed, HLAA2⁺ DC in normal donor

Survivin is overexpressed in almost all human malignancies and, therefore, represents a possible universal target for the development of cancer vaccination therapies. As demonstrated by RT-PCR, flow cytometry and western blotting in chapter 2, survivin expression was detected in B-CLL samples after activation of the cells by CD40 ligation, but was not expressed or expressed only weakly in non activated CLL cells. To analyze the recognition of survivin derived peptides by CTLs, the generation of survivin specific CTL line was attempted. As a first step to achieve so, autologous DCs derived from monocytes of HLA-A2⁺ healthy donor were used, and two different DC cultures were generated as described earlier in 5.2.1.

5.3.1.1 Generation of DC by cytokines: 4 days and 8 days culture

Circulating DCs in human blood are rare (< 1% of human PBMC) and difficult to maintain in culture. Therefore, monocytes are commonly used as progenitors for the in vitro generation of DCs (Sallusto et al., 1994; Romani et al., 1994). DCs were generated by isolating PBMC from an HLA-A2⁺ healthy donor, and monocytes were then separated and cultured in the presence of GM-CSF, IL-4 for 2 days or 5 days to generate immature DCs. Immature DCs were then matured for another 48 hours or 72 hours respectively by adding PG-E2 and TNF-α. The expression of the surface molecules on mature DCs was confirmed by staining using the following mAbs: anti-CD80, anti-CD83, anti-CD86, anti-MHC class II. The phenotype of mature DC was then analyzed by flow cytometry on the last day of each culture. An immunophenotypic profile consistent with a mature DC phenotype (CD80, CD83, CD86, MHC class II) was homogeneously expressed by DC’s following both the 4 day and the 8 day maturation protocols, as shown in Figure 5.2. Immature DCs were capable of differentiation into
mature DCs as reflected by the upregulation of DC maturation markers (CD80, CD83, CD86 and MHC class II) and markedly reduced expression of CD14. The Δ mean fluorescence intensity (ΔMFI) was obtained by dividing the MFI of the mature DCs by the MFI of the immature DCs. Results did not show much difference between 4 days and 8 days cultures in the expression of DCs maturation markers (CD80, CD83 and CD86). However, the ΔMFI of MHC class II was twice that in 8 days culture compared with the 4 days culture, and for that we chose to generate DCs using the eight day culture protocol for the experiments described.

5.3.1.2 Generation of a survivin- specific T- cell line

To prime survivin- specific lymphocytes, peptide pulsed autologous DCs derived from healthy donor PBMCs were used after their in vitro maturation, confirmed by flow cytometric analysis for DC surface markers: CD80, CD83, CD86 and MHC class II. 5x10^5 DCs were pulsed with 50μg/ml synthetic survivin peptides for 4 hours, washed and incubated with 3x10^6 autologous CD8^+ T cells from the same healthy donor. After 7 days of culture, T cells were restimulated with autologous survivin peptide- pulsed DCs and 10ng/ml human recombinant IL-7 and 25U/ml IL-2 (R&D Systems, UK) were added on day 5. In attempt to generate autologous survivin specific T lymphocytes from HLA-A2^+ healthy donor, T cells were checked and counted weekly, starting with 3x10^6 CD8^+ T cells for the primary stimulation. Following the 2nd and 3rd restimulations, there were between 4- 4.5x10^6 cells, whereas 7.5x10^6 T cells were generated after five weeks of in vitro culture. However, a decrease in the number of the surviving T cells (5x10^6) was observed after the fifth restimulation (Table 5.1). It was therefore described to analyse the antigen specificity of the T cell culture by ELISpot array and pentamer staining on day five following the fifth in vitro stimulation with survivin peptide pulsed DCs. Figure 5.3 shows pictures for clusters of T cells noted around survivin peptide pulsed DCs, taken after the third cycle of restimulation.
Figure 5.2 Phenotypic characterisation of DC maturation markers (CD80, CD83, CD86 and MHC class II) on DC’s derived from an HLA-A2+ healthy donor at 4 days and 8 days culture. The open histogram shows the profiles obtained with an isotype control mAb. The mAb staining at day 0 prior to cytokine stimulation is shown by the pink histograms. Staining with the maturation markers at day 4 and 8 is shown by the blue histograms.
Table 5.1 Proliferation of autologous T cells from HLA-A2+ healthy donor on stimulation with survivin peptide loaded DCs in the presence of IL-2 and IL-7. Shown are T cell numbers during the 5 restimulations: 7, 14, 21, 28 and 35 days of in vitro culture.

<table>
<thead>
<tr>
<th>Number of restimulation</th>
<th>T cells count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3x10^6</td>
</tr>
<tr>
<td>2</td>
<td>4x10^6</td>
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<tr>
<td>3</td>
<td>4.5x10^6</td>
</tr>
<tr>
<td>4</td>
<td>7.5x10^6</td>
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<tr>
<td>5</td>
<td>5x10^6</td>
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Figure 5.3 (A) An image showing clusters of lymphocytes surrounding mature dendritic cells which were loaded with survivin peptides, taken after the 3rd restimulation of the culture. Original magnification x 100. (B) A higher power image that shows the dendritic cells interacting with T cells. Original magnification x400.
5.3.1.3 Estimation of survivin-specific CTL by ELISpot

To determine the frequency of survivin-specific T cells in the DC-stimulated T cell line from an HLA-A2\(^+\) healthy individual, ELISpot assay was performed using the IFN-\(\gamma\) ELISpot kit (Mabtech AB, Sweden) according to the manufacturer’s instructions (as described in section 4.2.1.2). This was also done to analyse the recognition of survivin-specific derived peptides by CTLs.

After 5 weeks of in vitro culture, peptide pulsed T2 cells were used to disclose survivin reactive T cells. T2 cells were pulsed with the two different HLA-A2 binding peptides derived from survivin (ELTGEFLKL and LMLLGEFLKL). T2 cells without adding any peptides were used as a negative control. In addition, reactivity was tested against a HLA-A2\(^+\) CLL sample, unactivated or activated by CD40 ligation. The data are shown in Figure 5.4. Unstimulated T cells showed very low background IFN-\(\gamma\) release. T2 cells without peptide stimulated a significant increase in IFN-\(\gamma\) release compared with unstimulated T cells. However, the T cells obtained did not show any peptide specificity against either of the survivin peptides, as T2 cells loaded with survivin peptides did not stimulate an increase in IFN-\(\gamma\) secretion by the T cells, compared with T2 cells without peptide. In fact, T2 cells pulsed with LMLLGEFLKL peptide showed a significantly lower stimulation than T2 cells alone (P<0.001). Unactivated HLA-A2\(^+\) CLL cells did not stimulate T cell IFN-\(\gamma\) release, whereas CD40L activated CLL cells stimulated a significant increase (P<0.0001) in IFN-\(\gamma\) secreting T cells, compared with both unstimulated T cells or T cells stimulated with unactivated, HLA-A2\(^+\) CLL cells. The antigen specificity of this response, however, was not indicated.

Overall, the ELISpot assay did not indicate the presence of survivin-specific, HLA-A2 restricted CTL in the DC-survivin peptide stimulated T cell culture.
Figure 5.4 Analysis of peptide specific T cells contained in DC- survivin peptide stimulated specific T cells culture. HLA-A2+ unactivated CLL cells (yellow bar), HLA-A2+ CD40L activated B-CLL (blue bar) and T2 cells separately pulsed with one or other survivin peptides (the two purple bars) were used as stimulators. T2 cells without peptides were used as a control (the pink bar) (**P< 0.001, ***P<0.0001).

5.3.1.4 Estimation of survivin specific CTL by pentamer staining

To validate the data obtained by ELISpot analysis, the expanded T cells were stained with HLA-A2 pentamers specific for survivin after the 5th restimulation, which is another method to identify antigen specific T cells. Pentamer staining was performed according to the manufacturer’s instructions as was described in section 5.2.3. The two colour flow cytometry with anti-CD8 mAb and HLA-A2/ survivin pentamer complexes did not reveal any CD8+ T cells specific for either of the survivin peptides used (0.02% using ELT peptide and 0.021% using LML peptide) although 10.25%- 10.5% were the percentages of total CD8+ T cells gated population using each peptide respectively (Figure 5.5), giving a frequency of only 0.02% of survivin peptide specific cells within the CD8+ T cell population. These data are in line with ELISpot results, and they confirm the lack of survivin specific CTLs in the DC/survivin peptide induced T cell line.
Figure 5.5 HLA-A2 pentamer/peptide staining of CD8\(^+\) T cells in survivin CTL culture. CD8\(^+\) lymphocytes from an HLA-A2\(^+\) healthy donor were subjected to five rounds of stimulation with survivin peptide loaded DCs prior to pentamer staining. Left hand plots show staining with HLA-A2 pentamer+ ELT peptide, whilst right hand plots show staining with HLA-A2 pentamer+ LML peptide. Numbers given in the upper right quadrant are the percentage of cells reacting with survivin HLA-A2 pentamer/peptides, and numbers given in the upper left quadrant are the percentage of CD8\(^+\) T cells of total gated population (R1).

5.4 Discussion

Advances in our understanding of the processes by which the immune system recognizes and eliminates cancer cells have led to a renewed optimism for the development of effective immunotherapies for cancer. A range of human tumour associated antigens has been identified, which contain HLA class I-restricted epitopes recognized by tumour specific CTL. These antigens represent attractive targets for use in cancer vaccination, and clinical trials have been undertaken using a number of these antigens (or peptides derived from them) as immunogens.
Tumour specific activation of T cells depends on adequate presentation of tumour associated peptides. The most effective way to present antigenic peptides is by the use of DCs, which are known to be crucial for the initiation of primary T cell responses. DCs stand out by their capacity to present peptides derived from exogenous antigens both on MHC class II molecules and on MHC class I molecules. In consequence, a number of experimental strategies to induce tumour specific T cell responses are based on the appropriate use of DCs. DC’s can be pulsed with synthetic peptide epitopes derived from tumour associated antigens such as MUC-1, survivin, MAGE, Melan-a/MART and p53 (Fong et al., 2000; Rosenberg, 2001; Schuler et al., 2003). In vitro studies have demonstrated that DC loaded with tumour antigens can induce CTL responses against melanoma (Imro et al., 1999), chronic myeloid leukaemia (Neida et al., 1998), acute myeloid leukaemia (Choudhury et al., 1999), and pancreatic cancer (Peiper et al., 1997).

Schmitz et al. (2000) described survivin as capable of inducing specific CTLs in vitro when the protein was processed and presented by DCs. A second group (Andersen et al., 2001b) scanned the survivin protein for the presence of HLA-A2 binding motifs, to use to search for specific T cell responses in cancer patients. However, neither of these studies demonstrated the capacity of survivin- reactive T cells to lyse tumour cells of different tissue origins. Based on that, after checking the autologous CLL responses/survivin T2 in the previous chapter, I aimed to test the potential of survivin peptides to elicit specific T cell responses by using DCs as APCs. If that was successful, the resulting survivin specific CTL line was to be used to see if HLA- A2+ B-CLL cells processed and presented survivin to antigen- specific CTL, and to test their capability to lyse B-CLL cells. As the generation of DCs for the experiments required repeated bleeding of the same individual, the in vitro generation of a survivin- specific T cell line was attempted using peripheral blood T cells and DC’s derived from a volunteer, normal HLA-A2+ individual, rather than from a patient with CLL.
Mature DCs were generated from CD14\(^+\) selected monocyte from PBMC of the healthy donor. Following selection and culture in a combination of maturation factors, I was able to generate DCs with a fully mature immunophenotype. An immunophenotypic profile consistent with a mature DC phenotype (CD80, CD83 and CD86) was expressed by day 4 in 4 days culture and by day 8 in 8 days culture, with related down-regulation in the expression of CD14. However, 8 days culture displayed higher level of MHC class II molecules, and that led to me using 8 days as the optimal time for generating DCs. Ohminami et al. (2000), Mayr et al. (2006) and Bund et al. (2006) used the same method to generate DCs from normal HLA-A2\(^+\) peripheral blood monocytes which were then pulsed with the peptide of interest and used as APC for CTL, to explore if antigen-specific CTLs can be generated \textit{in vitro}. Ohminami and his group were able to establish a WT-1 specific CD8\(^+\) CTLs by stimulating CD8\(^+\) T lymphocytes from a healthy individual repeatedly with WT1 peptide-pulsed autologous dendritic cells, and examined its immunologic actions on leukemia cells. Those WT-1 specific CD8\(^+\) CTL line were able to lyse autologous cells loaded with a WT-1 peptide consisting of the HLA-A2 binding motifs, and were cytotoxic to HLA-A2\(^+\) leukemia cells expressing WT1, but not to HLA-A2\(^+\) lymphoma cells that did not express WT1, HLA-A2\(^-\) leukemia cells, or HLA-A2\(^+\) normal cells. The other group (Mayr et al., 2006) generated MDM2 specific CTL from two different HLA-A2\(^+\) healthy donors which were able to lyse CLL cells. In alignment with the previous studies, Bund et al. (2006) were able to identify CD229 derived peptides that bind to HLA-A2, and reported the generation of peptide specific CTLs \textit{in vitro} with the use of autologous DCs derived from monocytes of HLA-A2\(^+\) healthy donors. These T cells not only recognised HLA-A2 binding CD229 derived peptides presented by T2 cells, but also CD229 overexpressing B-CLL cells in an MHC-I restricted manner.

In the experiments that I carried out, the DC’s used exhibited a mature phenotype, and formed clusters with T cells in culture. After checking the full maturation of DCs, these DCs were loaded with one or either of survivin- derived peptides, to check if they were able to present survivin peptides and to efficiently induce survivin specific CTLs from lymphocytes of a healthy donor. After five cycles of weekly restimulation, the
specificity of T lymphocytes cultures for survivin peptides was evaluated by ELISpot and pentamer staining. Although the two methods used to detect survivin specific T cells are of different principles, the HLA-A2 pentamer staining (phenotypic measurement of T cells carrying the same antigen specific TCR) and the IFN-γ ELISpot assay (identification of functional IFN-γ secreting antigen specific T cells), gave the same results. No CTL response against either of the survivin derived peptides, (ELTGEFLKL) and (LMLGEFLKL), HLA-A2 restricted epitopes was detected.

It is not clear why I was unsuccessful in generating a survivin specific CTL line. The proportion of CD8⁺ T cells in the cultures overall (10% of live gated cells) was unexpectedly low, given the starting population of CD8⁺ T cells and the peptide stimulation protocol. As was shown in Figure 5.1, DCs were generated successfully. However, failure of generating survivin specific CTL line might be due to multiple factors. Schmidt et al. (2003) generated DCs from normal HLA-A2⁺ peripheral blood monocytes in the presence of GM-CSF, IL-4 and TNF-α, pulsed with survivin peptides and used as APCs for CTL priming. The survivin peptide specific CTL lines obtained from several weekly restimulations were able to lyse not only target cells pulsed with the antigenic peptide but also recognised tumour cells expressing survivin in an HLA-A2 restricted manner. By comparing this group culture conditions with mine, some alterations in the culture conditions might have helped, including low-dose GM-CSF and IL-4. Other groups have used IL-2 and IL-7 instead (Bund et al. 2006), and did not use PG-E2 in the DC culture. A second potential explanation for the diverse antitumor immune response generated by DCs primed with tumors prepared by different methods is potential variations in the mechanism of antigen presentation. Specifically, studies by Berard et al. (2000) clearly demonstrate that monocyte-derived human DCs primed with allogeneic melanomas are capable of stimulating naive T cells to recognize shared melanoma antigens. These data suggest that cross-priming, defined as the presentation of exogenous antigens on class I MHC molecules, is an important mechanism. In addition, DCs primed with irradiated tumor may present antigen by spontaneous tumor DC fusion and bystander effects, in which DCs provide appropriate costimulation for tumor-stimulated T cells. Krackhardt et al. (2002) data also suggested that T cells from patients with CLL are capable of killing allogeneic CLL cells, and further that DCs from patients
with CLL are capable of providing sufficient antigen presentation to initiate this response. These results support the use of a patient whose tumour expresses the antigen, rather than a normal donor blood, and may reflect differences in antigen presentation and/or costimulate for the activation of antigen-primed as opposed to antigen-naïve T cells. The starting T cell population could therefore also be of a reason. However, Bund et al. (2006), who were able to generate CD229 specific CTL line from a normal donor, used the same conditions and starting number of cells (at least $2 \times 10^6$ cells/ml) to generate their cell line.

The ELISpot methodology represents a strong tool to monitor peptide specific T cell responses. However, although it has been shown that ELISpot reactivity in most cases correlates with the capacity to lyse the target cell, it does not assess cell mediated cytotoxicity directly, as IFN-γ secretion is not limited to only cytolytic CD8 T cells. Anderson et al. (2006), therefore, have used perforin ELISpot to provide an estimation of cytotoxic effector cell frequency. They measured T cell responses against survivin in 8 patients (two breast cancer patients, two melanoma patients and four renal cancer patients) and were able not only to detect an IFN-γ response but also a perforin response. On the other hand, Schmidt et al. (2003) assessed the cytotoxicity of the in vitro induced CTL in a standard $^{51}$Cr release assay. Survivin specific CTL line obtained after several weekly restimulations demonstrated peptide specific killing. T cells only recognised T2 cells coated with survivin peptide, whereas they did not lyse target cells pulsed with the control peptide (adipophilin), confirming the specificity of the cytolytic activity, but a failure to lyse antigen expressing but MHC marked cells.

A peptide vaccine approach involves several steps which are aimed firstly at identifying the appropriate peptide, secondly at checking for its immunogenecity and relevance as a tumour associated antigen in vitro, and thirdly at formulating a safe product to be used clinically. Several tumour specific, and some leukaemia specific, peptides have so far been identified, and studies aimed at evaluating the potential clinical benefit of peptide vaccines in cancer patients have begun. Among the reasons that make a peptide vaccine strategy interesting are several unique advantages that peptide immunization offers over other vaccine approaches:
1. Peptide vaccines permit specific targeting of the immune response against one or more unique antigens.

2. Emerging technology has made it simple, rapid and inexpensive to sequence and prepare large quantities of tumour antigen peptides for both laboratory and clinical use.

3. Use of synthetic peptides greatly reduces the possible risk of bacterial or viral contamination that might derive from autologous or allogeneic tissue for immunization.

On the other hand, the main disadvantages of peptide immunization are:

1. Lack of universal applications, as each peptide is restricted to a single HLA molecule.

2. Poor immunogenicity of most native peptides.

3. Risk of inducing antigenic tolerance.

Mature DCs are recognized as the most potent antigen-presenting cells. Multiple techniques of antigen priming have been advocated to create a tumor-specific DC vaccine as mentioned in section 1.5. Several approaches for delivery of TAA into DC have been developed. In the clinical protocol of vaccination by DC, both synthetic peptides corresponding to tumour antigens and tumour eluted peptides have been used for DC mediated antigen presentation (Nestle et al., 1998). While synthetic peptides represent only the limited antigenic repertoire of the presently known tumour antigens, tumour eluted peptides may reflect a wider antigenic spectrum. Another potential disadvantage of using defined synthetic peptides to activate tumour reactive T cells is that the generated peptide specific T cells may not recognise tumour cells expressing the antigen of interest and processing and presenting it naturally, through the MHC class I antigen processing pathway. Loading DC with cocktails of different synthetic peptides, corresponding to different tumour antigens expressed by the same tumour, has been demonstrated to be a clinically effective procedure (Suresh et al., 2005). Nevertheless, it is possible that the synthetic peptide approach will limit patient selection on the basis of the HLA phenotype, and will prevent the possibility of
activating both CD4 and CD8 T cells directed to different epitopes of the same antigen. To avoid these disadvantages, several alternative methodologies using a mix of TAA have been developed, for example using DCs pulsed with whole tumour lysate or apoptotic cells to present derived antigen in an HLA-A1 restricted manner (Albert et al., 1998). However, a possible limitation of these approaches is the need for large numbers of primary samples or tumour cell lines, and the lack of control of the nature of the antigens that are being presented by the DCs. The use of RNA instead of protein could constitute a good alternative since it could be amplified in vitro (Boczkowski et al., 1996). A further possibility is the engineering of DC with expression vectors carrying TAA genes (e.g. viral vectors, retroviral, adenoviral and vaccinia vectors).

In conclusion, after investigating survivin peptides for their potential to induce cytotoxic T cell responses, I was unable to generate peptide specific CTLs from a normal individual in vitro or to demonstrate their ability to lyse B-CLL cells. The data suggest that there were no T cells in the culture able to recognize the two peptide antigens derived from survivin in the context of HLA-A2, following 5 rounds of stimulation of T cells with peptide- pulsed autologous DCs in vitro.
6. General Discussion
B-CLL is a disease of the elderly, for whom combination chemotherapeutic approaches and stem cell transplants may not be appropriate. There is marked heterogeneity in terms of disease progression. Many individuals will require no therapy for many years whilst others will die with chemotherapy resistant disease. B-CLL is a chronic disease and it is therefore important not to poison normal haemopoietic cells and thus compromise any further therapeutic attempts. Since B-CLL is a slowly progressive disease, there are often extended periods of time where a patient is off therapy with stable disease. These characteristics make B-CLL an attractive candidate for testing novel immunotherapeutic strategies. However, several limitations require specific attention. Immunotherapeutic challenges to B-CLL include the fact that B-CLL cells are: 1) ineffective APC in the case of direct priming (Krackhardt et al., 2002), 2) B-CLL cells can down-regulate the level of stimulatory T cell surface molecules typically induced in response to antigen (Romano et al., 2003) and 3) the poor immunogenicity of B-CLL cells may be attributed to a low level of co-stimulatory and adhesion molecules required for the initiation of an effective immune response (Scrivener et al., 2003). In addition, defects of host T cell function have been defined, such as an increase in absolute numbers of CD4+ and CD8+ T cells with abnormal CD4+/CD8+ ratio which correlates with disease progression, and diminished response to mitogens (Scrivener et al., 2003). These findings suggest that the T-cells in B-CLL patients may be unable to start, maintain and complete an immune response against malignant B cells and other antigens, and may be involved directly in sustaining the tumour. Therefore, an effective therapeutic anti-tumour vaccine will likely have to overcome immune tolerance by enhancing both antigen presentation and effector pathways.

The majority, if not all, tumour cells express proteins that the immune system is capable of recognizing as antigenic but fail to present these antigens to the immune system to generate effective antitumour immune responses. One explanation for this is that the cells from which the tumours arise are poorly immunogenic because of their inability to deliver the signals that are required for the activation of antigen specific T cells. These signals include the costimulatory receptor/ligand interactions CD28/B7 and CD40/CD40 ligand, and are further enhanced by additional accessory receptor/ligand
interactions, e.g. lymphocyte function associated antigen-1 (LFA-1)/ intracellular adhesion molecules 1 (ICAM-1), and by the presence of certain cytokines, e.g. IL-2, IL-7 and IL-12.

Several factors determine the suitability of a protein as an antigen for tumor vaccines (Lim et al., 2002). In addition to expression in tumour cells, restricted normal tissue expression of the protein will provide tumour specificity and hence reduced toxicity from a tumour vaccine based on the protein. Furthermore, the protein has to be immunogenic in vivo in cancer bearing patients who may be immunosuppressed. Other considerations include the appropriate processing and presentation of the antigenic peptide in association with the MHC molecules on the cell surface for T-cell recognition, and optimal tumour-effector cell interaction. Survivin, a member of the IAP family, meets these criteria. The fact that it is over-expressed in tumour cells may provide a survival benefit for tumour cells, and that its expression is almost completely restricted to malignant tissues makes this protein a useful tool for the development of applicable vaccination therapies.

The expression of survivin was investigated in resting B-CLL samples by RT-PCR, flow cytometry and western blotting. Studying the mRNA expression of survivin, the RT-PCR method showed that most of the samples tested (8 out of 11) did not express survivin (with weak expression in 3 out of 11) (Figure 2.2). Determining the protein expression of survivin in resting B-CLL cells showed no evidence of survivin expression by western blotting in all six samples tested (Figure 2.5). Flow cytometry results, however, showed positive expression in two out of the six samples tested (Figure 2.4).

To determine and compare the effects of different ways of activating B-CLL cells (CD40L, CpG-ODN, or combination of CD40L and CpG-ODN), extracellular staining for the relevant activation markers (CD54, CD80 and CD86) was performed prior to checking activation role on survivin expression in B-CLL cells. A significant upregulation of the surface markers in response to CD40 ligation, CpG-ODN and them both, was detected after the optimum three days of activation period (Figure 2.7 and
Table 2.2). However, CD40L activated B-CLL cells showed the highest upregulation in the adhesion molecule CD54, and costimulatory molecules CD80 and CD86 compared to cells treated with CpG-ODN or a combination of CD40L plus CpG-ODN. To also study the effect of the different ways of activation on the ability of B-CLL to induce T cell proliferation, B-CLL cells were activated and left for the same period of activation. CD40L activated B-CLL showed the highest ability to stimulate allogeneic T cells to increase their cellular proliferation. B-CLL cells activated with CD40L and CpG-ODN also showed highly significant increase compared to CpG-ODN activated cells in which T cell proliferation was low. Survivin expression was then assessed using the same methods as before (RT-PCR, flow cytometry and western blotting) to check whether its expression was induced by the different means of stimulation. CD40 ligation, which showed the strongest upregulation of the surface markers and T cell proliferation in the AMLR, also induced survivin bands of the predicted size on RT-PCR, indicating expression of survivin in all the 11 samples tested, with a faint band in one of them. There was a good correlation between RT-PCR and western blotting data as survivin protein expression was induced by CD40 stimulation. However, CD40L+CpG-ODN activated cells showed the highest expression of survivin by western blotting. Using flow cytometry, CD40 ligation as well as CD40 ligation + CpG-ODN of B-CLL cells resulted in a quite similar degree of upregulation of survivin expression. Taking the flow cytometry and western blotting data together, the combination of CD40 ligation plus CpG-ODN had a higher enhancement of survivin expression than CD40 ligation on its own.

As CD40 activation was an effective method to induce the highest proliferation response of allogeneic T cells, its ability to stimulate autologous T cells was investigated on B-CLL samples, using ELISpot and $^{51}$Cr release assay. Autologous T cell responses were detected with the coincubation of CD40L autologous B-CLL cells compared to unactivated B-CLL cells by ELISpot (Figure 4.1) and by $^{51}$Cr release assay (Figure 4.2) in which a significant increase in lysis was detected in CD40 ligated cells compared to unactivated cells.
To further study autologous T cell responses in HLA-A2 B-CLL patients, T cells were stimulated with T2 cells pulsed with survivin peptides or T2 cells on their own. This stimulation did not show any significant difference in the presence of T cell responses specific for either of the peptides in the B-CLL patients tested (5 HLA-A2⁺ patients and 4 HLA-A2⁻) compared to T cell responses to T2 cells on their own.

It was shown that survivin reactive T cells can be found in the peripheral blood of patients with malignant melanoma or CLL and that these CTLs when isolated by magnetic beads can lyse allogeneic HLA-matched breast cancer and melanoma cells (Tamm et al., 1998; Schmitz et al., 2000; Anderson et al., 2001; Altieri, 2001). However, the survivin specific lysis of autologous targets or tumours remains to be demonstrated (Schmidt et al., 2003). Based on these studies, an attempt to generate a survivin specific CTL line to check the ability of T cell responses to lyse unactivated and CD40L activated B-CLL cells was examined, using peptide pulsed autologous monocyte derived dendritic cells from HLA-A2⁺ healthy donor. Unfortunately, the attempt was not successful and there was no survivin specific T cells in the culture detected by ELISpot or pentamer staining.

As the clinically successful antigen specific cancer immunotherapy depends on the identification of TAA, a second aim of the project was to investigate the expression of several TAA (MAGE-A1, MAGE-A3, Proteinase-3, NY-ESO-1, WT-1, MUC-1 and PRAME) in B-CLL patients by the method of conventional RT-PCR, and to check the effect of CD40 ligation on their expression. No evidence of expression of MAGE-A1, MAGE-A3, Proteinase-3 and WT-1 was detected in B-CLL samples pre- and post CD40 activation. 8 samples out of the 20 samples tested showed positive PRAME expression on CD40 ligated B-CLL cells compared with what in unactivated CLL cells, whilst an upregulation in NY-ESO-1 expression was seen in one sample after activation. MUC-1 was expressed in 4 out of the 20 samples tested before and after activation, with no marked upregulation detected on CD40L activated B-CLL cells.
The efficacy of cancer vaccines is affected by the immunogenicity of antigens, by the route of immunisation, and by tumour features making them susceptible to immunologic recognition. Therefore, more efficient vaccine approaches are dependent on the analysis of the specific interactions of vaccine induced immune responses at the site of antigen expressing cancers (Jager and Knuth, 2002). Based on that, future perspectives of tumour vaccination are focused on the definition of more potent strategies of immunization. Whole tumour proteins containing multiple, antigenic epitopes may increase the chance of polyvalent B and T cell activation. Adjuvants might enhance the immunogenicity of peptides and proteins by activating costimulatory factors and mediating the production of cytokines. DCs loaded with peptides or proteins in vitro, or transduced with the relevant genes, might effectively stimulate both MHC class I and II restricted T cells in vivo. The identification of new tumour antigens will provide a broader basis for polyvalent immunization to prevent the escape of antigen loss variants. As the clinical effectiveness of cancer vaccination becomes more established, antigen specific immunotherapy might be considered as an alternative for adjuvant treatment of patients with cancer.

The establishment of sensitive and complementary methods for the evaluation of antigen- specific immune responses will help to improve current vaccination strategies of cancer vaccine trials. The prognostic significance of individual tumour features (e.g. antigen and MHC class I/II expression) reflecting the susceptibility of cancer cells to be targeted by antigen specific T cells will be explored, and a better selection of patients for antigen specific immunotherapy will hopefully result. The applicability of cancer vaccines remains limited to a small population of cancer patients with a matched repertoire of antigens expressed by the individual tumour. However, approaches designed according to individual patient and disease characteristics may teach us successful ways of antigen specific vaccination against human cancer (Jäger et al., 2003).
Future directions:

- Given the rapid progress in CLL research, a rapid change in the management of CLL can be predictable. Genetic parameters will allow the physician to predict both prognosis and response to treatment more precisely. As a consequence, treatment of CLL will become much more diverse and individualized. While some patients might benefit from the traditional monotherapy treatments, others will require different approaches including chemoimmunotherapy followed by a maintenance treatment with monoclonal antibodies or immune gene therapy using the transfer of the CD40 ligand gene into CLL cells for example. This might lead us to further study the role of CD40 activation in the immunotherapy of B-CLL.

- As B-CLL is not a homogenous disease, and although common antigens may be present in a proportion of B-CLL patients, other B-CLL patients may have unique antigens. Therefore, identification of antigens in B-CLL and studying whether CD8+ T lymphocytes can be specifically activated against them are needed.

- As generating survivin specific CTL line using peptide pulsed monocyte derived DCs was unsuccessful, testing other culture conditions and studying the role of DCs to initiate T cell immune responses are to be demonstrated.

- Looking at survivin specific lysis of autologous targets or tumours also needs further investigation.

B-CLL represents a promising disease for the development and use of immunotherapies, including cancer vaccination. However, the data presented in this thesis indicates that there is still much work to be done before this potential be realised.
7. Bibliography


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