Breaking T-cell Tolerance in Chronic Lymphocytic Leukaemia

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“A true lesson in perseverance lies in completing a PhD”

(Ryan Wong, Random thought)

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ABSTRACT

CLL is an incurable B-cell malignancy associated with profound tumour cell-mediated immune dysfunction. It therefore represents a challenging disease for the successful application of immunotherapeutic strategies aimed at promoting anti-tumour T-cell responses. In this study, extensive immunophenotypic analysis of T-cells from the blood of CLL patients was performed, in order to better characterise their dysfunctional status within the disease. Analysis of CLL patient blood samples revealed a skewing of T-cells towards a highly differentiated effector memory phenotype as well as the expression of markers associated with exhaustion/senescence ($CD28^-$ and $CD57^+$) and immunosuppressive molecules (PD-1 and CD200). In addition this study revealed the expansion of $CD8^+$ T-cells in a subset of CLL patients leading to an inversion in the normal CD4:CD8 ratio. The presence of an inverted CD4:CD8 ratio was subsequently shown to be associated with a shorter time to first treatment and reduced progression-free survival.

Characterisation of T-cells identified several molecules that could be targeted therapeutically in order to break T-cell tolerance in CLL patients and potentially restore normal immune responses. Investigation of the immunosuppressive molecules PD-1 and CD200 showed that they are over expressed in CLL patients, suggesting that they may be involved in maintaining T-cell tolerance in the disease. However, blockade of PD-1-PDL-1 and CD200-CD200R signalling pathways failed to enhance T-cell responses from CLL patients *in vitro*. Investigation of an alternative approach to enhance T-cell responses in CLL involved the use of a bi-specific antibody targeting CD19 and CD3 called blinatumomab. *In vitro* testing showed that blinatumomab can induce T-cell activation, promoting the release of pro-inflammatory cytokines and granzyme B secretion from both $CD4^+$ and $CD8^+$ T-cells. In

addition, blinatumomab was shown to mediate T-cell dependent killing of CLL cells requiring the formation of T-cell:CLL cell conjugates.

Finally this study provided clear evidence that blinatumomab can break T-cell tolerance in CLL and strongly advocates the progression of blinatumomab into clinical trials as a novel therapeutic agent in CLL.

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LIST OF SUPPLIERS

BD	Becton Dickinson UK Ltd., Oxford, UK
Beckman Coulter, UK	Beckman Coulter (UK) Ltd, High Wycombe, UK
Biolegend	Biolegend, London, UK
Caltag	Now Invitrogen, Paisley, UK
Dako	Dako Ltd, Ely, Cambridge, UK
Ebiosciences	by Insight Biotechnology Ltd, Middlesex, UK
Fisher	Fisher Scientific, Loughborough, UK
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ABBREVIATIONS

ACT	Adoptive cell transfer
ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired immune deficiency syndrome
ALLO-SCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
A-T	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated gene
AUTO-SCT	Autologous stem cell transplantation
BCR	B-cell receptor
BMECs	Bone marrow-derived endothelial cells
BiTE	Bi-specific T-cell engager
BTLA	B and T lymphocyte attenuator
CAR-Ts	Chimeric Antigen Receptor T-cells
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CLL	Chronic lymphocytic leukaemia
CLL^{IR}	CLL patients with an inverted ratio of T-cells (CD4:CD8 <1)
CLL^{NR}	CLL patients with a normal ratio of T-cells (CD4:CD8 ≥1)
CM	Central Memory
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T- Lymphocyte Antigen 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EM	Effector memory
EMRA	Effector (EMRA)
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorter
FCR	Fludarabine, cyclophosphamide and rituximab
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
GITR	Glucocorticoid-induced TNF receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVL	Graft versus leukaemia
HD	Healthy donor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
HTLA	Human T-lymphotrophic virus
ICAM	Intercellular Adhesion Molecule

ICS	Intracellular cytokine staining
IGHV	Immunoglobulin heavy chain variable region
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITregs	Induced Treg cells
KIRs	Killer cell immunoglobulin-like receptors
LAG-3	Lymphocyte-activation gene 3
LFA	Lymphocyte function-associated antigen
LMP	Low molecular weight polypeptide
LPS	Lipopolysaccharide
Mabs	Monoclonal antibodies
MAGE-1	Melanoma-associated antigen 1
MART-1	Melanoma-associated antigen recognized by T-cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MM	Multiple myeloma
MSC	Myeloid suppressor cells
NCR	Natural cytotoxicity receptors
NK	Natural killer cell
NKG2D	Natural killer group 2, member D
NKT	Natural killer T-cell
NLC	Nurse-like cells
N-Tregs	Natural T-regs cells
OS	Overall survival
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PD-1	Programmed death-1
PDL-1	Programmed death-1 ligand
PE	Phycoerythrin
PFS	Progression-free survival
PGE	Prostaglandin
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RIC	Reduced intensity conditioning
SCT	Stem cell transplantation
SHP	Src (Sarcoma) Homology 2 Domain Phosphatase
scFv	Single chain variable fragments
SMAC	Supermolecular adhesion complex
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TGF	Tumour growth factor
Th cell	T-helper cell
TILs	Tumour infiltrating lymphocytes
TIM-3	T-cell immunoglobulin mucin-3
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand
Treg	T regulatory cell
TSA	Tumour specific antigen

TTFT	Time to first treatment
VEGF	Vascular endothelial growth factor
ZAP-70	ζ-associated protein

CHAPTER 1

Introduction

1.1 Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is the most diagnosed leukaemia in the western world representing 30% of all leukaemias in Caucasians (Shvidel et al. 1999). It is characterised by the clonal expansion of $CD5^{+}CD19^{+}CD23^{+}$ B-cells that progressively accumulate in the peripheral blood, bone marrow and secondary lymphoid tissues. CLL is a remarkably heterogeneous disease where patients who display an indolent disease may live for over 30 years post diagnosis, whereas those patients that present with aggressive disease may die within 2-3 yrs despite aggressive therapy (Allendorf and Davis 2011, Zenz et al. 2010). Although standard chemoimmunotherapy has proven success in reducing the tumour burden in CLL patients, it does not result in long-term disease-free survival. At present there are no curative therapies for CLL, with the possible exception of allogeneic haematopoietic stem cell transplantation (allo-HSCT), but this treatment is unsuitable for the vast majority of CLL patients (Dreger 2009). Given this backdrop, there is now a surge to better understand the pathogenic mechanisms behind this disease with the aim of developing new, widely applicable, therapeutic approaches to improve the survival of patients.

1.1.1 Aetiology and Epidemiology

CLL is considered to be a disease of the elderly with a median age at diagnosis of 72 years. Less than 2% of patients are younger than 45, 9.1% between 45 and 54, 19.3% between 55 and 64, 26.5% between 65 and 74 and 42.3% greater than 75 (Gribben 2010).

Although the frequency of CLL detection has increased since the 1950s, CLL rates are thought to be relatively stable with worldwide projection rates ranging from <1 to 5 per 100,000 people (Redaelli et al. 2004).

CLL occurs at higher rates in males than in females with nearly twice as many males diagnosed with the disease (Redaelli et al. 2004). Geographic incidence varies widely with the highest rates reported in Caucasians and reduced incidences in African and Asian populations; incidence rates are 25% and 77% lower in black and Asian/pacific islander populations respectively when compared to Caucasians (Dores et al. 2007). Interestingly these lower incidences of CLL were maintained in US Asian populations suggesting racial/ hereditary factors in the occurrence of the disease (Groves, Linet and Devesa 1995).

Although the first description of CLL was made over 150 years ago, the aetiology of CLL remains unknown. Several environmental determinants including agricultural chemicals as well as viruses e.g. Human T-lymphotrophic virus (HTLV), Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) have been proposed as causative agents but no direct links have been made (Lanasa 2010). By far the most important risk factor for the development of CLL is a familial history with between 8 and 10% of newly diagnosed CLL cases having an inherited link (Sellick et al. 2007).

1.1.2 CLL pathogenesis

CLL is a lymphoproliferative disease with as much as 1% of the entire CLL clone turning over each day (Messmer et al. 2005). The expansion and survival of these cells *in vivo* is thought to be influenced by several factors including the presence of cytogenetic

abnormalities, microenvironmental signals and tumour immune evasion mechanisms (Dighiero 2005).

1.1.3 Genetic aberrations

Analysis of the whole CLL population using sensitive cytogenetic techniques such as fluorescence *in situ* hybridisation (FISH), has shown that up to 80% of individuals with CLL have cells with 1 or more genetic aberration (Döhner et al. 2000). Five categories of genetic aberrations have been defined in CLL patients: a deletion in 13q (55%), a deletion in 11q (18%), trisomy of 12q (16%), a deletion in 17p (7%) and a deletion in 6q (6%). 17p deletions or deletion 11q22-q23 were shown to be independent prognostic markers, with patients carrying these genetic aberrations having rapid disease progression and shorter survival times (Döhner et al. 2000). The vast majority of patients with 17p deletions were subsequently shown to have a mutation in the p53 gene, with predicted resistance to treatment with alkylating agents and a short median treatment-free interval of 9 months (Stilgenbauer et al. 2002). Patients with deletions of 11q22-q23 commonly have a mutation in the ataxia telangiectasia mutated (ATM) gene, which is also associated with the autosomal recessive disorder ataxia telangiectasia (A-T). Individuals with this disease have a predisposition to cancer including hypersensitivity to ionizing radiation, genomic instability and disruption of cell cycle checkpoints (Lavin and Shiloh 1997). Candidate genes responsible for the pathogenesis of other genetic aberrations in CLL are yet to be determined.

1.1.4 *IGHV* mutational status

During B-cell responses to antigen, B-cells readily undergo proliferation in germinal centres followed by a phase of somatic hypermutation whereby mutations are introduced into the immunoglobulin variable region genes (Liu et al. 2008). B-cells then enter a phase of affinity maturation where B-cells with high affinity for the specified antigen are selected for survival. In CLL two subsets of patients exist, those with mutated and those with unmutated immunoglobulin variable region genes. Variable region gene sequences with less than 98% homology to germline are considered to have undergone somatic hypermutation and therefore considered mutated (Gribben 2010). The incidence of unmutated *IGHV* genes can vary between 20-50% depending on the patients' chromosomal abnormalities and has been subsequently shown to have prognostic significance (Oscier et al. 1997). The median survival time of stage A patients with mutated *IGHV* genes was 3 times longer than that of the corresponding patient group with unmutated *IGHV* genes (Hamblin et al. 1999). Interestingly a higher incidence of mutated *IGHV* genes was found in familial CLL cases than sporadic cases (Houlston, Catovsky and Yuille 2002).

Generally the *IGHV* genes expressed by B-cell leukaemias do not tend to develop additional somatic mutations over time. The presence of two groups of CLL patients based on mutated or unmutated *IGHV* genes was therefore thought to represent two subtypes of CLL with different cytogenetics and different maturation states (Schettino et al. 1998). CLL cells with unmutated *IGHV* regions were considered to be derived from a pre-germinal centre naïve B-cell and those with mutated *IGHV* regions from an antigen experienced post-germinal central memory cell (Kröber et al. 2002).

However, gene expression profiling using micro-array technology has cast doubt on these findings showing that all CLL cells display a common and characteristic gene

expression profile that is largely independent of *IGHV* mutational status. Comparison of CLL gene expression profiles with those from healthy B-cells points to CLL cells being more related to memory B-cells than naïve B-cells (Klein et al. 2001). Moreover recent transcriptome analyses of CLL cells has indicated that they are derived from a normal $CD19^{+}CD5^{+}$ B-cell counterpart (Seifert et al. 2012).

CLL cells use a biased repertoire of V genes with the most frequently used rearranged genes being VH1-69, VH4-34, VH3-21 and VH3-7 (Mauerer et al. 2005). VH1-69 was found exclusively in unmutated CLL representing the most frequently detected *IGHV* gene in this group (25%). The VH3-21 gene was mainly used in the mutated (87%) compared to the unmutated (13%) group. The use of the VH3-21 gene was subsequently shown to be associated with poor prognosis regardless of mutational status (Tobin et al. 2002).

1.1.5 Antigenic stimulation

The role of antigenic stimulation in the pathogenesis of CLL has been widely debated with no candidate antigen as yet identified. The presence of mutated *IGHV* genes in a subset of CLL patients suggests that the CLL cell B-cell receptor (BCR) is functional and able to recognize antigen. Antibody gene sequencing has shown that CLL patients have a restricted use of *IGHV* genes (VH, D and JH) with a bias in antibody repertoires found in these leukaemic expansions. In patients with mutated *IGHV* genes, approximately 20% of the mutated *IGHV* genes display replacement mutations in a pattern indicating antigen recognition (Fais et al. 1998). 30% of CLL patients carry quasi-identical (stereotyped) receptors with 1% of CLL patients identified expressing virtually identical Immunoglobulin (Ig) (Darzentas et al. 2010). The likelihood of two CLL cells carrying identical BCRs is

extremely remote (10^{-9}-10^{-12}) suggesting that CLL B-cell development and progression may by driven by a common antigen.

Proteins derived from infectious pathogens including EBV and CMV as well as self-proteins, have all been put forward as potential antigens capable of stimulating CLL cells. An auto-antigen non muscle myosin chain IIA (MYHIIA) found on apoptotic cells was shown to be capable of binding CLL monoclonal antibodies (Stamatopoulos 2010). More recently CMV and EBV infection has been linked with CLL cases expressing the VH4-34 and CLL recombinant antibodies encoded by *IGHV* 1-69 or IGVH 3-21 have been shown to react with a phosphoprotein pUL32 derived from CMV (Kostareli et al. 2009, Steininger et al. 2012).

1.1.6 The lymph node microenvironment

CLL cells recovered from the blood of CLL patients and cultured *in vitro*, rapidly undergo spontaneous apoptosis, yet in the body they have the ability to proliferate and resist apoptosis (Collins et al. 1989). *In vivo*, CLL cells preferentially localise to the lymph nodes and bone marrow, where microenvironmental niches aid their survival and proliferation (Figure 1.1). CLL cells possess a variety of surface adhesion molecules including selectins, integrins and immunoglobulin which facilitate interactions between CLL cells, accessory cells and the extracellular matrix (ECM). These interactions ultimately promote the migration, localisation and survival of CLL cells at these sites (Vincent, Cawley and Burthem 1996).

Accessory cells present in lymphoid compartments are thought to play an important role in maintaining the CLL clone (Buggins et al. 2010). Bone marrow-derived endothelial cells (BMECs) and follicular dendritic cells (FDC) provide pro-survival signals to CLL cells

(Jewell and Yong 1997). The peripheral blood of CLL patients also contains cells capable of differentiating into nurse-like cells (NLC) *in* vitro that protect CLL cells from apoptosis (Burger et al. 2000). T-lymphocyte numbers are also elevated in CLL patients with evidence of extensive T-cell expansions in patients with poor prognostic markers (Röth et al. 2008). Furthermore, $CD4^+$ T-cells were shown to be essential for the proliferation and engraftment of CLL cells in mouse models (Bagnara et al. 2011) with activated $CD4^+$ T-cells also shown to co- localise with proliferating CLL cells in lymph node (LN) pseudofollicles (Patten et al. 2008). Activation of $CD4^+$ T-cells induces the upregulation of CD40L, which delivers activation signals to CLL cells by binding CD40 present on the CLL cell surface. T-cell derived cytokines including IL-4, IFN-γ and TNF-α have also been shown to inhibit CLL apoptosis *in vitro* (Tangye and Raison 1997).

1.1.7 Symptoms and diagnosis of CLL

The presence of small monomorphic B-lymphocytes expressing CD19, CD5 and CD23 in the peripheral blood, bone marrow or lymph nodes is used to diagnose CLL with positive samples requiring the presence of at least 5000 B-cells per µl (Gribben 2010). Most patients present with few symptoms at diagnosis but these increase during disease progression. The progression of disease is marked by an acceleration in lymphocyte doubling times, leading to the accumulation of leukaemic cells and the development of lymphadenopathy, splenomegaly and heptamegaly (Hallek and Group 2005). In a small minority of patients autoreactive antibodies to blood components have been shown to develop, which can result in anaemia and thrombocytopenia. Other symptoms of CLL include unintentional weight loss, fever, night sweats, extreme fatigue and infections (Binet et al. 1981). Infections remain a major issue in CLL patients, primarily due to immune dysfunction induced by the tumour and

therapy related immunosuppression. Patients are at risk of developing infections caused by bacterial pathogens such as streptococcus or staphylococcus, fungal pathogens such as candida and aspergillus, and viruses such as herpes viruses (Morrison 2007).

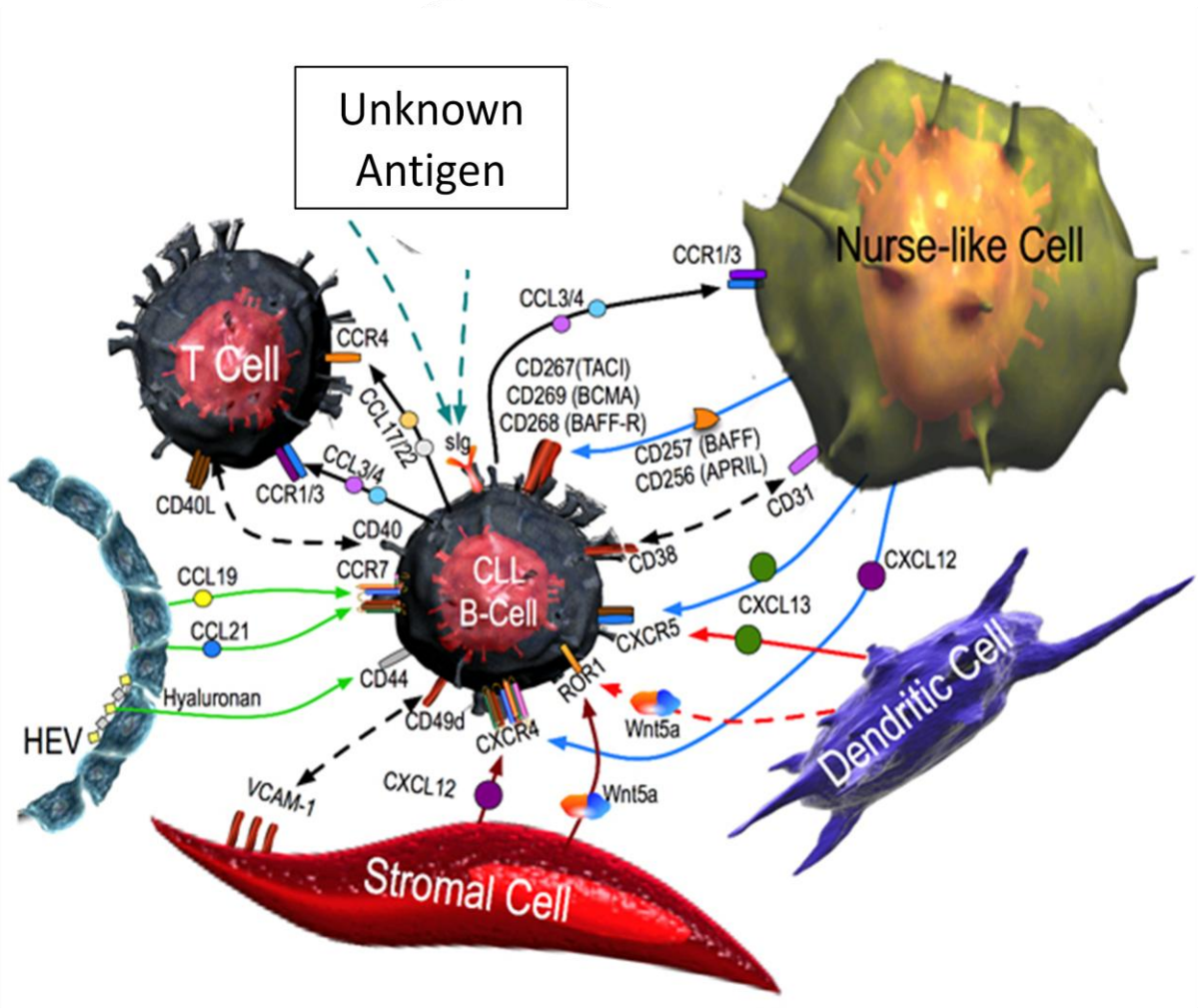


Figure 1.1 The CLL tumour microenvironment *(Fecteau and Kipps 2012)*. Accessory cells within the tumour microenvironment provide signals in the form of chemokines, cytokines and receptor-ligand interactions to CLL cells. These interactions are crucial in supporting CLL cell survival and proliferation.

1.1.8 Staging and prognostic markers in CLL patients

The standard system for estimating prognosis in CLL is the clinical staging systems developed by Binet (Binet et al. 1981) and Rai (Rai et al. 1975). These systems are used to define early (Binet stage A, Rai 0), intermediate (Binet stage B, Rai 1 and 2) and advanced (Binet stage C, Rai 3 and 4) disease. At the time of diagnosis patients are 25% stage 0, 50% stage 1 to 2 and 25% stage 3 or 4 (Gribben 2010). Although these staging systems assess risk, they are unable to predict an individual's risk of disease progression when diagnosed with early stage disease (Herishanu and Polliack 2005).

In order to get a clearer prediction of a patient's clinical course, additional prognostic markers can be used. Several prognostic markers have been defined in CLL including lymphocyte doubling time (Viñolas et al. 1987), *IGHV* mutational status, CD38 expression (Damle et al. 1999), ZAP-70 expression (Dürig et al. 2003), bone marrow histological pattern (Rozman et al. 1984), serum soluble CD23 levels (Sarfati et al. 1996), serum beta-2 microglobulin (Di Giovanni et al. 1989) and cytogenetic aberrations including 17p deletions (Furman 2010).

1.1.9 Treatments

Current treatment strategies for CLL include the use of chemotherapeutic agents and immunotherapy in the form of monoclonal antibodies. In rare cases radiotherapy or surgery are used to debulk or remove tissues with extensive CLL cell infiltration e.g. lymph nodes or spleen. The clinical management of CLL patients is usually based on the 'watch and wait' approach, whereby treatment is only administered upon the development of symptoms (Gribben 2010).

1.1.9.1Chemotherapy

Alkylating agents

Chlorambucil and cyclophosphamide are the most widely used alkylating agents in CLL. The precise mode of action through which these drugs work remains unclear. Chlorambucil is however capable of binding to several cellular structures including membranes, proteins, RNA and DNA with DNA cross-linking thought to be the most important factor for its anti-leukaemic activity (Begleiter et al. 1996). Clinical trials of chlorambucil, administered with or without prednisone, showed initial response rates of between 60% and 70% but no significant complete responses (Byrd, Stilgenbauer and Flinn 2004). Furthermore, chlorambucil did not prolong the survival of stage A CLL patients (Dighiero et al. 1998). When the clinical effect of chlorambucil was assessed against cyclophosphamide there was no significant difference in overall survival, complete response rates or duration of responses (Raphael et al. 1991).

Purine analogues

Several purine anologues are currently used as treatments in CLL including fludarabine, cladribine and pentostatin. The incorporation of nucleotide phosphates from these compounds into the DNA of proliferating tumour cells induces cell death by apoptosis (Johnson and Thomas 2000). Fludarabine has been shown to inhibit enzymes involved in DNA synthesis including, DNA polymerase, DNA primase and DNA ligase. The enzyme ribonuclease, responsible for maintaining the cellular deoxynucleotide pool is also inhibited which increases the likelihood of fludarabine phosphate being incorporated into cellular DNA (Parker et al. 1988). Separate studies have shown that fludarabine and cladribine used as

single agents had similar clinical activity in CLL, whilst pentostatin showed less activity in this disease (Ricci et al. 2009). Several studies have also evaluated the effect of fludarabine in comparison to alkylator-based regimes in CLL. Patients treated with fludarabine had significantly higher complete response rates than alkylator-based chemotherapy (Zhu et al. 2004).

1.1.9.2 Monoclonal antibody therapy

Despite encouraging response rates with chemotherapy there are still several challenges to treating CLL patients. Chemotherapy alone has not led to an improvement in overall survival in CLL patients. The introduction of the monoclonal antibodies (mAbs) has revolutionised the treatment of CLL as well as other human malignancies (Held, Schubert and Pfreundschuh 2008). Rituximab and alemtuzumab are mAbs that target antigens on the surface of the CLL cells, inducing apoptosis through mechanisms including antibody directed cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). mAbs are considered to have lower toxicity than chemotherapeutics, probably due to the targeted nature of the antibodies which avoids damage to healthy tissue (Adams and Weiner 2005). Rituximab is a chimeric murine-derived mAb, capable of highly specific associations with its target antigen CD20. Although expressed weakly, CD20 can be found on virtually all CLL cells. Clinical trials using rituximab as a single agent showed higher response rates in untreated patients compared to relapsed or refractory patients. Furthermore, complete response rates remained low at 9% (Hainsworth et al. 2003).

Alemtuzumab is an antibody directed against CD52 found on a variety of haematopoietic cells including monocytes, macrophages, a small subset of granulocytes as

well as normal and malignant T- and B-lymphocytes (Fraser et al. 2007). The ubiquitous expression of CD52 means alemtuzumab usage is associated with increased toxicity when compared with other monoclonal antibodies such as rituximab. Common side effects include neutropenia, lymphopenia and infectious complications e.g CMV reactivation (Christian and Lin 2008). Clinical trials with alemtuzumab showed an overall response rate of 33% in fludarabine-refractory CLL patients and a 40% clinical response rate in patients with p53 mutations and deletions (Keating et al. 2002). These studies suggest that alemtuzumab may be effective in subgroups of CLL patients with high-risk cytogenetic markers and may induce CLL cell death through a p53-independent mechanism. Alemtuzumab is now approved for the treatment of fludarabine refractory CLL patients (Montillo et al. 2008).

Novel antibodies that are currently in development for CLL include two anti-CD20 antibodies, GA101 and ofatumumab which have shown improved efficacy when compared to rituximab (Robak 2008). In addition antibodies designed to block CLL cell migration to secondary lymphoid organs; which is necessary for CLL cell survival and proliferation are now being tested. These include natalizumab which targets the alpha 4 integrin CD49d and plerixafor which blocks the chemokine receptor CXCR4 (Burger et al. 2009).

1.1.9.3 Combination therapies in CLL

Currently most patients receive intermittent treatment with periods of stable and progressive disease, however very little is known about when to start treatment or what combinations of drugs result in the best clinical outcome. Combining monoclonal antibodies with chemotherapy has been actively investigated, with the aim of improving treatment of CLL patients. Several studies have investigated the synergistic effect of fludarabine with

monoclonal antibodies including rituximab and alemtuzumab, with both found to have a synergistic effect with fludarabine in killing CLL cells (Di Gaetano et al. 2001). A recent study has also shown that the combination of fludarabine, cyclophosphamide and rituximab (FCR), induced an overall response rate of 74% in relapsed CLL patients including those with chromosome 17 abnormalities (Badoux et al. 2011). Furthermore a recent clinical trial showed that FCR treatment increased progression-free survival (PFS) and overall survival (OS) in symptomatic patients (Molica 2011). Currently FCR is regarded as the 'gold standard' for CLL treatment, but with new studies combining current therapeutic drugs as well as the advent of new novel therapies, future changes to the way CLL patients are treated seems inevitable.

Novel therapeutic agents in development include monoclonal antibodies as mentioned previously as well as BCR signalling antagonists including Bruton's tyrosine kinase (BTK) inhibitors and PI3 kinase inhibitors. Both have shown promising and ongoing clinical responses in early phase I/II clinical trials in CLL and have shown efficacy even in 'high risk' CLL patients (Woyach, Johnson and Byrd 2012). Blockade of BCR signalling may prevent upregulation of anti-apoptotic proteins such as BCL-2 necessary for CLL cell survival *in vivo* as well as interfering with signalling through chemokine receptors such as CXCR4 (Burger 2011). Future clinical trials will help to establish whether remissions with such therapies can be maintained long-term of whether resistance mechanisms will develop.

1.1.9.4 Stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is not a suitable treatment option for most CLL patients, in particular the frail and elderly due to the high treatment related

morbidity and mortality (Gribben 2009). It does however remain a potential treatment for high-risk CLL patients who are younger and likely to die from their disease (Mauro et al. 1999). High-risk patients are defined as those with p53 mutations, purine analogue refractoriness or have early relapse after purine analogue combination therapy. There are two types of stem cell transplant available autologous (auto-SCT) and allogeneic (allo-SCT). Auto-SCT requires the infusion of haematopoietic stem cells derived from the patient's blood or bone marrow, with the cytotoxicity seen in patients thought to be due to the accompanying high-dose chemotherapeutic regimen (Böttcher, Ritgen and Dreger 2011). Allo-SCT involves the transfer of stem cells from an HLA-matched donor and relies on an immune-mediated anti-host response or graft versus leukaemia (GVL) effect to kill CLL cells (Dreger and Montserrat 2002). Although allo-SCT is characterised by high treatment-related mortality compared to auto-SCT, it does have some significant advantages. Unlike auto-SCT, allo-SCT can overcome poor prognostic markers (e.g. p53 mutations) and has a lower incidence of relapse, with survival curves plateauing in the long-term suggesting a potential curative effect (Esteve et al. 2002). A recent study also showed that there was no difference in overall survival between patients receiving auto-SCT as first-line therapy compared to chemotherapy (Sutton et al. 2011). Further work is needed to try and identify prognostic markers that identify patients at sufficiently high risk as to warrant the use of allo-SCT, and to investigate the use of reduced intensity conditioning (RIC) allo-SCT in patients with poor performance status (Gribben 2009).

1.1.9.5 The clinical efficacy of therapeutic treatments in CLL

In the past decade significant improvements in the way clinicians treat CLL has led to increased response rates and durations of responses in patients. This has included clinical

trials looking at the effects of combination drug treatments, at when to start treatment and at which patient groups are likely to benefit for such treatments (Byrd et al. 2004). Despite this CLL remains a largely incurable disease for the majority of patients, where almost 70% of CLL patients will die from causes related to CLL, with older patients (>80 years) nearly twice as likely to die than those between 40 and 59 (Diehl, Karnell and Menck 1999). The future development of novel agents to treat CLL is therefore warranted.

1.2 The immune system

The immune systems' central role is to protect the body from invasion by infectious pathogens. It is made-up of two separate arms, innate immunity and adaptive immunity. Innate immunity provides a rapid, non-specific, response to infection by pathogens that does not result in immunological memory. Alternatively, adaptive immunity is initiated in instances where the innate immune system is unable to resolve infection, resulting in the expansion of pathogen-specific effector cells. After pathogen clearance, a subset of these effector cells will persist in the body capable of responding to pathogen re-challenge (Janeway et al. 2008).

1.2.1 Innate Immunity

The innate immune system is an evolutionary ancient part of the host defence system, which is made up of several components including anatomical barriers, secreted molecules and effector cells. Initial infection is prevented by the skin epithelium that provides a physical barrier to infectious agents. This barrier is aided by mucus and cilia found in such areas as the respiratory and gastrointestinal tract, which helps to trap and remove pathogens before they

cause damage. Furthermore secreted factors found in sweat, saliva and tears including lysozyme, phospholipase and defensins also have antimicrobial activity (Janeway et al. 2008).

Once a pathogen penetrates into the tissues, humoral factors play an essential role in inducing acute inflammation characterised by oedema and the recruitment of innate immune effector cells. Components of the coagulation and complement systems aid the removal of the pathogen by increasing vascular permeability and attracting phagocytic cells to the site of infection. Activated complement can also lysis bacteria directly or facilitate opsonisation of antibody-bound pathogens by phagocytic cells.

Whereas the adaptive immune response relies on receptors encoded by rearranged gene segments, innate immune cells use germ-line encoded receptors capable of recognising molecular structures essential for microbe survival (Janeway 1989). These molecular structures or pattern-associated molecular patterns (PAMPs) are conserved by a number of pathogens e.g. Lipid A of Lipopolysaccharide (LPS) and are recognised by receptors termed pattern recognition receptors (PRRs) (Janeway and Medzhitov 2002). Several PRRs have been identified including mannose receptors (bacterial and viral carbohydrate), scavenger receptors (anionic polymers and acetylated low-density lipoproteins) and Toll-like receptors (various bacterial and viral components). Binding of infectious agents via PRRs can result in phagocytosis and the release of pro-inflammatory cytokines including IL-1, TNF-α and IL-6 by phagocytes (Janeway et al. 2008).

Innate immune cells include natural killer (NK) cells, eosinophils, mast cells, neutrophils, macrophages and dendritic cells. These cells can differentiate into short-lived effector cells in response to inflammation and usually clear the infection without the need for an adaptive immune response (Medzhitov and Janeway 1997). Macrophages are mononuclear

phagocytes that mature from monocytes in the blood before moving to the tissues. They are the first cell to encounter antigen, after which short-lived neutrophils are recruited from the blood to the site of infection. Both cell types are important phagocytic cells able to recognise, ingest and destroy pathogens.

NK cells play an important role in the detection of stressed, infected or malignant cells. NK cells receive signals through surface activating receptors such as (NKG2D) or inhibitory receptors (Killer cell immunoglobulin-like receptors (KIRs), which can bind to Major histocompatibility complex (MHC) class I molecules (Pegram et al. 2011). During infection the balance of these signals can become disrupted in favour of activation, inducing proliferation, IFN-γ production and release of cytotoxic granules from NK cells (Lakshmikanth et al. 2009).

1.2.2 Adaptive immunity

Unlike the innate immune system, the adaptive immune response requires some time to react to antigen, is antigen specific and demonstrates immunological memory, capable of rapid responses to subsequent re-challenge with the same antigen. The adaptive immune system is composed of two components, antibody-producing B-cells forming the humoral component and T-lymphocytes the cell-mediated component. Both T- and B-lymphocytes differentiate from a common lymphoid progenitor in the bone marrow with T-cells then migrating to the thymus to complete their maturation process. Following maturation, both T- and B-cells enter the circulation with functional maturation of B-cells occurring in the secondary lymphoid organs (e.g. lymph nodes and spleen) (LeBien and Tedder 2008).

1.2.2.1 Humoral responses

B-cells are lymphoid cells that are produced in the bone marrow and develop in lymphoid tissues such as the LNs and spleen. They are characterised by their expression of surface markers including CD19 and CD20. CD19 is expressed on virtually all B-cell lineage cells and is a regulator of intercellular signal transduction, whilst CD20 is expressed on mature B-cells and functions as a membrane calcium channel. CD5 is found on a subset of IgM-secreting B-cells in healthy individuals and has been shown to be highly expressed on tumour B-cells in CLL patients (LeBien and Tedder 2008).

The protection of extracellular spaces is mediated by B-cell derived antibodies, which contribute to the destruction of extracellular pathogens, and prevent the spread of intracellular pathogens. Antigens activate B-cells through their engagement with surface BCR causing B-cell differentiation into antibody-secreting plasma cells and memory B-cells. The process of B-cell activation usually requires additional signals provided by T helper cells in the form of CD40-CD40L interactions and cytokines.

Antibodies act through multiple mechanisms in order to facilitate the destruction of pathogens. They can bind toxins and antigens on the surface of pathogens preventing their spread and entry to cells or facilitate opsonisation of the antigen by phagocytic cells possessing FC receptors, which are capable of recognizing the antibody C-region. Alternatively pathogen-bound antibodies can activate proteins of the complement system which can lyse microorganisms directly or facilitate opsonisation of the pathogen by binding to complement receptors on phagocytic cells (Janeway et al. 2008).

1.2.2.2 Cell mediated immune responses

The main cellular subset that mediates adaptive cellular immunity are T-lymphocytes which express αβ T-cell receptors (TCRs) on their surface, capable of recognising antigens in the form of short peptides presented on the surface of cells. These foreign peptides are delivered and presented onto the surface of cells by glycoproteins called major histocompatibility complex (MHC) molecules. There are two classes of MHC molecules, MHC class I molecules are presented on all nucleated cells whereas MHC class II molecules are normally only expressed on antigen presenting cells (APCs).

Generally T-cells can be categorised into two groups based upon their surface expression of CD4 or CD8. Peptides derived from intracellular pathogens present in the cytosol are carried to the cell surface and presented on MHC class I molecules to $CD8^+$ T-cells. Peptide antigens from pathogens that multiply in intracellular vesicles or are ingested from an extracellular source are processed and presented to $CD4^+$ T-cells on MHC class II molecules (Janeway et al. 2008).

Priming T-cell responses

Upon activation, professional APCs including macrophages, dendritic cells (DC) and B-cells are all capable of activating naïve T-cells to produce armed effector cells. The most important APCs for T-cell priming are the DCs that reside in peripheral tissues, which are common sites of infection (e.g. skin, mucosal surfaces). In peripheral tissues, DCs are constantly sampling the environment, taking up both self and non-self-antigens, degrading and processing and presenting them on MHC class I and II molecules (Banchereau et al. 2000). Upon infection inflammatory cytokines help activate DCs, whilst PAMP recognition

by DC surface PRRs induce expression of the co-stimulatory molecules CD80/CD86. Activated DCs expressing co-stimulatory (CD80/CD86) and adhesion molecules (Intercellular Adhesion Molecule 1(ICAM-1, ICAM-3) and Lymphocyte function-associated antigen (LFA-3)) migrate to lymph nodes, where they fully mature into highly effective APCs capable of interacting with and activating naïve T-cells (Guermonprez et al. 2002). Both macrophages and B-cells can be similarly induced by PRRs on their surface to upregulate co-stimulatory molecules and act as APCs. Thus the initiation of an innate immune response hastens the movement of APCs to the LNs where they can interact with T-cells.

Upon arrival in the LNs, DCs lose their ability to capture new antigen and distribute throughout the cortex in the T-cell areas. Naïve T-cells circulate continuously from the blood to the lymphoid organs directed by the chemokine CCL21 (Flanagan et al. 2004), where they come into contact with thousands of APCs allowing sampling of MHC:peptide ligands (signal 1). The recognition of a specific MHC:peptide by the naïve T-cell TCR is by itself not sufficient for T-cell activation. Activation is dependent on a secondary signals (signal 2) provided through CD28 ligation on the T-cells to CD80/CD86 on the activated DCs. Naïve T-cells that receive both signal 1 and signal 2 then cease to migrate and undergo several rounds of expansions before becoming armed effector cells capable of leaving the lymph node and migrating to the site of infection (Janeway et al. 2008).

$CD4^+$ T-cells

$CD4^+$ T-cells are a heterogeneous population of cells including effector T-cells important in protection against pathogens, and regulatory T-cells (Tregs, TH3, TR1) that

protect against autoimmunity (Parish and Liew 1972, Jonuleit and Schmitt 2003). In 1986 Mosmann *et al*, identified the existence of two main subsets of effector $CD4^+$ T-cells, Th type 1 (Th1) and Th type 2 (Th2), from which several subgroups including Th0, Th17, Th9 and Th22 have been subsequently identified (Mosmann et al. 1986, Jutel et al. 2001). The polarisation towards a Th1 or Th2 response was found to be dependent on environmental cytokines, with Th1 responses critical for immunity to intracellular microorganisms favoured by the presence of IFN-γ and IL-12 and Th2 responses important for immunity to extracellular pathogens favoured by IL-4 and IL-25 (Paul and Seder 1994).

Th1 cells produce several pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2. Initial production of IL-12 from APCs in response to specific activation signals acts on NK cells inducing the production of IFN-γ. In turn IFN-γ acts on $CD4^+$ T-cells inducing the expression of IL-12Rβ2 and later IL-18Rα on Th1 cells. This allows Th1 cells to respond directly to IL-12, which can synergise with IL-18 to induce IFN-γ production from Th1 cells even in the absence of TCR stimulation (Yang et al. 2001). Th1 cells activate macrophages enabling destruction of intracellular organisms and on B-cells to promote the production of strongly opsonising antibodies (IgG1 and IgG3) (Herbst, Schaible and Schneider 2011). Th2 cells can produce cytokines such as IL-4, IL-5, IL-9 and IL-13. Th2 cells also aid the activation of B-cells promoting IgE class switching in the presence of IL-4 whilst Th2 cell derived IL-5 is a major eosinophil-differentiation factor promoting proliferation and survival (Zhu and Paul 2008).

$CD4^+$ T-cells also play a crucial role in the initiation of $CD8^+$ T-cell responses. Activation of $CD4^+$ T-cells induces the upregulation of CD40L, which can bind to CD40 on APCs promoting their activation and upregulation of co-stimulatory molecules including CD80/CD86. This process facilitates the activation/licensing of DCs allowing them to present

already captured antigens to $CD8^+$ T-cells. Activated $CD8^+$ T-cells can then synthesise IL-2, which acts in an autocrine fashion by binding to surface IL-2R to drive proliferation and differentiation. Moreover, the presence of IL-2 during $CD8^+$ T-cell priming has been shown to be crucial for $CD8^+$ memory T-cell development (Williams, Tyznik and Bevan 2006). $CD4^+$ T-cell are also able to produce their own cytokines such as IL-2 and IFN-γ as well as stimulating DCs to produce IL-12 and IL-15. Together these cytokines can promote $CD8^+$ T-cell proliferation, survival and enhance CTL cytotoxicity (Zhang, Zhang and Zhao 2009b).

Regulatory T-cells

Regulatory T-cells (Tregs) play an important role in maintaining peripheral tolerance including; prevention of autoimmunity, modulating immune responses to pathogens or environmental stimuli, and maintaining immune homeostasis (Sakaguchi et al. 2009). They may however have a negative role in preventing immune responses to tumours, with increased Tregs cells found in several solid cancers (lung, breast, pancreatic, liver) and haematological malignancies (Hodgkin's lymphoma, CLL) (Beyer and Schultze 2006). Subsequent studies have identified correlations between increased Tregs and poor prognosis in several cancers (Zhou et al. 2009). Tregs are defined by their expression of surface CD4, CD25, CTLA-4, glucocorticoid-induced TNF receptor (GITR) and intracellular expression of FOXP3. This transcription factor is essential for the function, maintenance and development of regulatory T-cells, but its presence does not necessary indicate a regulatory phenotype (Hori, Nomura and Sakaguchi 2003). Two types of regulatory cells have been identified; those that originate in the thymus called natural Tregs cells (nTreg) and those that are generated in the periphery named adaptive or induced Treg cells (iTreg). No markers have yet been identified to differentiate between both types, but iTreg development has been

shown to require TCR stimulation as well as the cytokines TGF-β and IL-2 (Curotto de Lafaille and Lafaille 2009).

The exact mechanism of Treg mediated suppression of immune responses is still unknown but is thought to occur in a contact-dependent manner with soluble mediators also contributing. Tregs produce inhibitory cytokines IL-10, IL-35 and TGF-β, with IL-10 and TGF-β production shown to be crucial in Treg regulation in asthma and allergy models (Joetham et al. 2007). Tregs may also disrupt normal effector T-cell development by sequestering IL-2 and inhibiting IL-6 expression responsible for promoting pro-inflammatory Th17 cell development (Oukka 2007). Human Tregs also have cytotoxic activity and are able to induce apoptosis of effector T-cell and NK cells through both granzyme-dependent and TRAIL-DR5-dependent pathways (Cao et al. 2007). Other surface markers including the immunosuppressive molecule CTLA-4, are constitutively expressed on Tregs and may play a role in suppressing not only T-cells but also important activators of effector T-cells including DCs (Read, Malmström and Powrie 2000).

NK T-cells and Gamma delta (γδ) T-cells

NK T-cells and γδ T-cells play a crucial role in bridging the gap between the innate and adaptive immune responses. NK T-cells are a population of T-cells that share properties of NK cells and are thought to have a regulatory role in controlling immune responses to infections and tumours (Guven et al. 2003). They express an invariant TCR composed of an α and β chain which recognises CD1d in conjunction with hydrophobic ligands. Upon TCR ligation NK T-cells have been shown to secrete several cytokines including IL-4, IL-10 and IFN-γ (Godfrey et al. 2000).

Gamma delta T-cells express γδ T-cell receptors and typically represent between 1-10% of human T-lymphocytes (Kunzmann and Wilhelm 2005). In contrast to αβ T-cells, γδ T-cells recognize small non-peptidic phospohorylated metabolites which do not require processing and presentation by MHC class molecules. These metabolites are produced by a broad range of microorganisms including bacteria and parasites. Several functions have been described for γδ T-cells including elimination of intracellular microorganisms (bacteria, parasites and viruses) and tumour cells, as well as roles in regulating immune homeostasis. Upon activation γδ T-cells can also act in a similar fashion to APCs, presenting antigens and providing co-stimulatory signals to $CD8^+$ T-cells (Brandes et al. 2009).

$CD8^+$ T-cells

The detection of cells infected with intracellular pathogens e.g. viruses, bacteria and parasites is mediated by cytotoxic $CD8^+$ T-cells through the recognition of foreign peptide bound to surface MHC class I molecules on APCs. Peptides derived from pathogens are generated in the cytosol through degradation by the proteasome. These peptides translocate into the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP), where they associate with newly formed MHC class I molecules. These peptides: MHC class I complexes can then be transported to the cell surface, where the short 8-10 amino acid peptides are presented to $CD8^+$ T-cells (Pamer and Cresswell 1998).

The initial binding of $CD8^+$ T-cells to an APC is a non-specific interaction mediated by adhesion molecules LFA-2 and CD2. Levels of the molecules are up to four-fold higher on armed effector T-cells compared to naïve T-cells, allowing efficient binding to cells expressing low levels of ICAMs and CD58. Upon recognition of antigen-specific peptide:

MHC class I complexes, $CD8^+$ T-cells form an immunological synapse. The T-cell receptor and associated co-receptor cluster at the point of contact called the central supramolecular adhesion complex (SMAC) and are flanked by adhesion molecules including LFA-1 making up the peripheral SMAC (Ramsay et al. 2008). Clustering of the TCR at the contact point initiates reorganisation of the cell's actin cytoskeleton, aligning the effector cell's secretory apparatus towards the target cell (Valitutti et al. 1995).

Activation of $CD8^+$ T-cells induces 2 key effector functions; the release of cytokines and the induction of target cell death. Cell killing can be mediated through the release of cytolytic granules containing perforin, serine proteases (granzymes) and granulysin. Perforin disrupts the target cell's membrane integrity whilst granzymes activate caspases, which ultimately lead to DNA degradation and cell apoptosis. Granulysin causes direct target cell membrane damage as well as mitochondrial depolarisation, resulting in caspase-3 activation (Kaspar et al. 2001). $CD8^+$ T-cell expression of these granule components requires specific cytokine signals delivered through the upregulation of IL-2 and IL-6R expression upon TCR-stimulation (Russell and Ley 2002).

The existence of an alternative $CD8^+$ T-cell killing mechanism was made upon discovery that effector T-cells from perforin knockout mice still retained the ability to lyse target cells (Kägi et al. 1994). This pathway is widely thought to be mediated by the calcium independent Fas/FasL pathway with other apoptotic pathways such as TNF-related apoptosis inducing ligand (TRAIL) playing a lesser role in target cell elimination (Johnstone, Frew and Smyth 2008). The Fas/FasL pathway is responsible for clearance of T-cell clones after antigen elimination, inactivation of autoreactive peripheral T-cells, allograft rejection and clearance of damaged or abnormal cells (Henkart 1994). Upon T-cell activation, FasL is up regulated onto the surface which can engage Fas expressed on target cells leading to

activation of caspase-8 and the subsequent downstream activation of caspase-3 (Waring and Müllbacher 1999).

Activated $CD8^+$ T-cells are also capable of secreting several cytokines including IFN-γ, TNF-α and TNF-β, that act to enhance pathogen recognition and destruction. IFN-γ can inhibit viral replication directly as well as enhancing antigen presentation by increasing MHC class I expression on the surface of target cells. TNF-α and TNF-β have also been shown to synergise with IFN-γ to promote macrophage activation allowing them to move to sites of infection and increase their antigen presenting capability (Janeway et al. 2008).

Memory T-cell responses

Following the resolution of infection, T-cells that have been activated and expanded undergo apoptosis, with a small population of antigen-specific T-cells known as memory cells persisting and forming the basis of immunological memory. These memory cells show a low activation threshold and are capable of proliferating and acquiring effector function in response to a second encounter with a pathogen, resulting in long-term protective host immunity. Unlike naïve T-cells, memory T-cells are capable of proliferating in an antigen-independent manner in the presence of IL-7 and IL-15, which is crucial in the maintenance of both memory $CD4^+$ and $CD8^+$ T-cells (Seddon, Tomlinson and Zamoyska 2003).

Two types of memory T-cell have been defined based on their ability to mount effector function and their expression of homing receptors to secondary lymphoid organs (Sallusto et al. 1999). Central memory T-cells (TCM) express CD45RO, as well as CCR7 and CD62L, which are required for migration into secondary lymphoid organs. TCMs can be stimulated by mature DCs present in the LNs, where upon activation they produce mainly IL-2 before

differentiating into $CCR7^-$ effector cells capable of secreting IFN-γ or IL-4. Effector memory T-cells (TEM), express CD45RO but unlike TCM cells have lost the expression of CCR7. These cells display chemokine receptors and adhesion molecules facilitating migration to inflamed tissue (gut, lungs and liver) where upon activation, they have rapid effector function (Campbell et al. 2001). Highly differentiated effector memory cells or TEMRA are closely related to TEM cells but have lost their expression of CD45RO and instead re-express CD45RA. Both TEM and TEMRA have a lower proliferative potential than TCM and are characterised by increased levels of senescent T-cells. Analysis of TEM cells showed they carry large amounts of perforin with TEMRA shown to carry the highest levels. $CD4^+$ and $CD8^+$ TEM also secrete a broader spectrum of cytokines than naïve T-cells including IFN-γ, IL-4 and IL-5.

Both TEM and TCM cells are thought to represent different states of memory T-cell activation. TCM cells closely resemble naïve T-cells which are maintained in a state of low-level activation with low relative turnover and expression of activation markers (Sallusto et al. 1999). In comparison TEM resemble effector cells, overtly activated with the expression of integrins and chemokine receptors necessary for entry to non-lymphoid organs. There is also evidence to suggest that the cytokine milieu and site of T-cell activation can influence the expression of homing receptors on memory T-cells (Baron et al. 1993).

The lineage of memory T-cell development has not yet been fully characterised. Three pathways have been proposed, the first being the linear pathway of memory T-cell development whereby memory T-cells develop from effector cells generated in response to infection (Figure 1.2). Several studies have subsequently supported this theory with one identifying a population of effector cells with high expression of CD127 and IL-7Rα as precursors for $CD8^+$ memory T-cells (Kaech et al. 2003). The second, named the divergent

pathway suggests naïve T-cells can give rise to daughter cells capable of differentiating into both effector and memory T-cells (Kaech, Wherry and Ahmed 2002). This direct process of memory T-cell development may occur during the end of an infection when T-cells still receive antigen and co-stimulatory signals but there is an absence of inflammatory milieu e.g. IL-12, IFN-γ and IL-21 (Kalia et al. 2006). The last model suggests that memory T-cell development can be dictated by the length of antigen stimulation on effector cells with short periods of stimulation favouring central memory T-cells and longer periods favouring effector memory T-cell development.

During periods of extended antigenic stimulation, T-cells can become dysfunctional and enter a state of exhaustion. T-cell exhaustion is defined by loss of proliferative capacity, expression of inhibitory receptors (e.g. PD-1, TIM-3 and LAG-3) and poor effector function (Freeman et al. 2006, Sakuishi et al. 2010). Exhaustion has been demonstrated in various mouse models as well as during chronic infections and in cancer. It effects both $CD8^+$ and $CD4^+$ T-cells and represents a distinct state of T-cell differentiation different from effector or memory T-cells (Yi, Cox and Zajac 2010). The cause of T-cell exhaustion remains unclear but it is believed, the persistence of antigen, presence of suppressive cytokines IL-10 and TGF-β and inhibitory receptor signals have a role in promoting T-cell exhaustion (Wherry 2011).

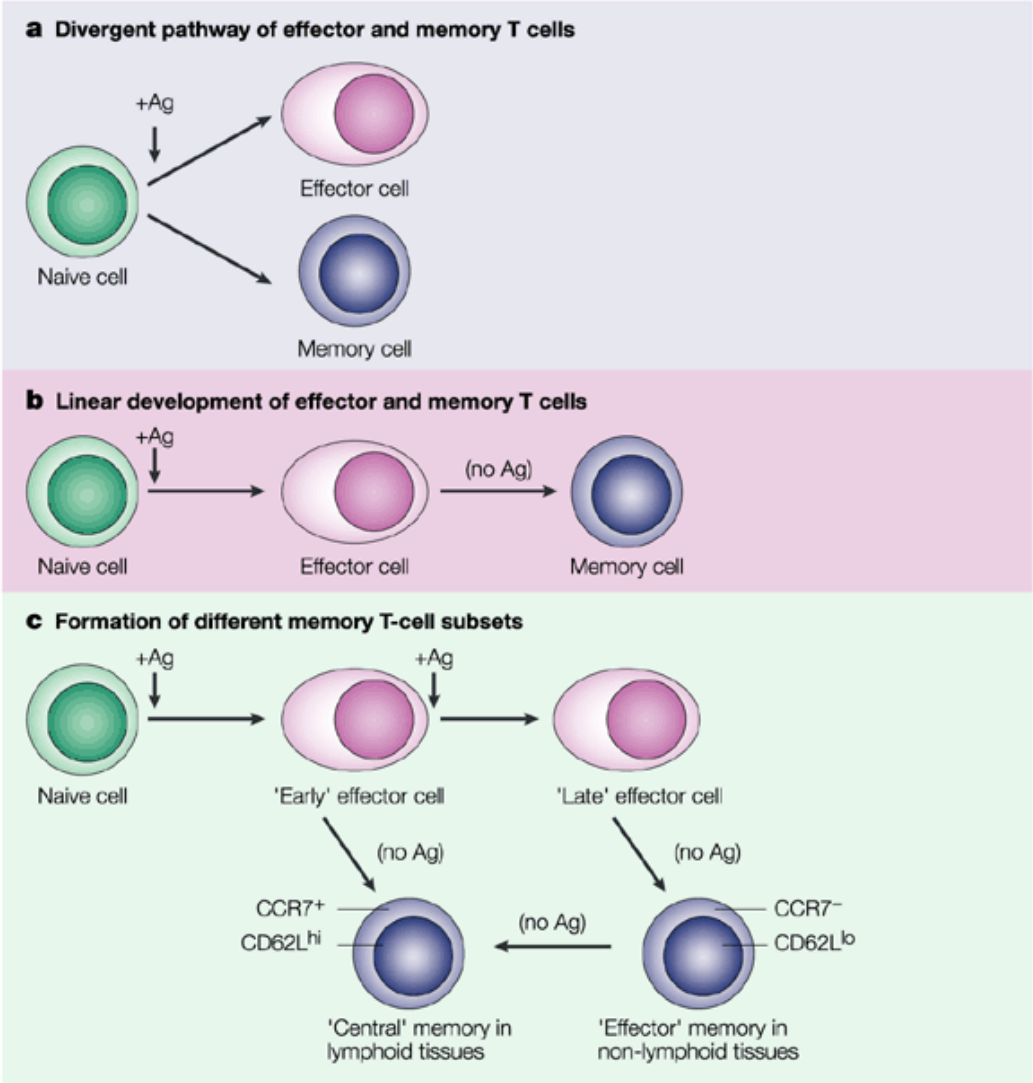


Figure 1.2 Models of memory T-cell development ***(Adapted from Kaech et al. 2002).*** The first model represents a divergent pathway whereby antigen stimulation gives rise to both effector and memory cells (a). The second model represents the linear pathway with antigen stimulation of naïve cells initially giving rise to effector cells which may develop into memory cells once the antigenic stimulation has gone (b). In the last model the generation of a memory phenotype is dictated by the length of antigen stimulation: short stimulation favours central memory T-cells and longer stimulation favours effector memory T-cell development.

1.3 Cancer immunology

The concept of the immune system playing a role in the recognition and destruction of tumours was first conceived by Ehrlich *et al* in the early 20th century and later developed by Thomas and Burnet into the hypothesis of cancer immunosurveillance (BURNET 1957). This hypothesis suggested that the development and accumulation of tumour cells with new antigenic markers may induce an effective immunological reaction which clears the tumour before symptoms become apparent. In mouse models protection from tumour formation was found to be dependent on IFN-γ and perforin, with both contributing independently towards the anti-tumour effector function (Kaplan et al. 1998). IFN-γ and perforin are secreted by several lymphocytes including $CD8^+$ T-cells and NK cells which pointed to specific cell types being important in tumour surveillance. Using genetic, immunochemical and functional ablation methods to deplete cell subtypes in mouse models, both innate (γδ T-cells) and adaptive (NK and αβ T-cells) cellular responses were subsequently found to be important in tumour surveillance (Shankaran et al. 2001).

Whether this theory of immunosurveillance can be directly applied to humans remains to be determined. Early follow up studies of immunosuppressed individuals including transplant and AIDS patients as well as those with immunodeficencies show a significantly higher risk of tumour development, however these are often virus associated malignancies (Dunn et al. 2002). There is however little evidence of immunosurveillance for non-viral cancers such as melanoma and pancreatic cancer.

The fact that tumours can develop in individuals with intact immune systems demonstrates that immunosurveillance is not 100% effective. In 2002 Dunn *et al*, put forward the idea of immunoediting, which suggested that tumour development is shaped by three phases; elimination, equilibrium and escape. Tumour cells that are not initially eliminated by

the immune system enter a second phase of equilibrium where they are immunologically shaped to produce new tumour variants able to escape immune recognition. The malignant cells can then grow out and become detectable in the escape phase which is associated with immunological anergy and tolerance towards the tumour (Dunn, Old and Schreiber 2004). If these series of events are correct they pose several key questions for immunologists a) what is the cause of this switch between host protection and no protection and b) can we reverse this immunological anergy and tolerance in order to restore host protection?

1.3.1 Immune responses to cancer cells

In mammals several cell types including B, T, NK cells and macrophages have proven to have tumouricidal effects. B-cells are capable of binding auto-antigens expressed on apoptotic tumour cells and have been shown to infiltrate tumour sites. Naturally occurring anti-tumour antibody responses are associated with improved survival in breast cancer and may be enhanced after chemotherapy (Karanikas et al. 2009). Macrophages are capable of direct cytotoxic responses either through the release of lytic factors such as serine proteases and reactive nitrogen intermediates into the neoplastic cell or through antibody dependent cellular cytotoxicity (ADCC). They can also influence immune responses indirectly through the release of pro-inflammatory cytokines (IFN-γ, TNF-β and IL-1β) which can help stimulate the anti-tumour activity of NK and T-cells (Gough et al. 2001).

NK cells as well as T-cells play a crucial role in the defence against tumour cells, virally infected and some bacterially infected cells. NK cells possess both activating and inhibitory receptors, which synergise to help NK cells respond to changes in MHC class 1 molecules found on most somatic cells. MHC class 1 receptor: ligand interactions can induce

inhibitory signals to be sent to NK cells, blocking activation through the recruitment of tyrosine phosphatase SHP-1 to the ITIM in the receptor's cytoplasmic tail (Rajagopalan and Long 2010). Down regulation of MHC class 1 molecules during viral infection and carcinogenesis leads to the removal of this inhibitory signal and allows the activation of the NK cell (Adam, Odhav and Bhoola 2003). NK cells also possess natural killer receptors (natural cytotoxicity receptors (NCR) or DNAX assessory molecule-1 (DNAM-1)), which bind ligands expressed on the surface of tumour cells (Lakshmikanth et al. 2009). They can also release pro-inflammatory cytokines such as IFN-γ and TNF-α that promote the development of Th1 type responses.

1.3.1.1 T-cells in tumour surveillance

T-cells play a crucial role in preventing the development and progression of neoplastic cells. T-cell surveillance of tumour development and growth depends on T-cell recognition of tumour-associated antigens (TAA) in the form of peptides, processed and presented by neoplastic cells on MHC molecules. These TAAs can be self-antigens or modified self-antigens, which are over expressed on tumour cells but are barely detected on healthy tissue. There are 3 different classes of TAA: the first are neoantigens which are peptides derived from mutations in tumour cells, the second are self-antigens which are mainly proliferation and differentiation markers over expressed by tumour cells and the third group are modified self-antigens due to tumour-specific post translational modifications (Palena and Schlom 2010). In cases where tumour formation is associated with infection with DNA or RNA oncogenic viruses, (10-15% of cancers worldwide) viral protein can act as TAAs if broken down and presented on MHC molecules (Martin and Gutkind 2008).

T-cells with high affinity receptors for MHC:self-peptides are normally deleted during T-cell development in the thymus with T-cells in the periphery that are reactive to TAAs restricted to those with low affinity T-cell receptors that may be tolerant to the tumour (Töpfer et al. 2011). T-cell responses to TAAs have however been identified in solid tumours (Melanoma: MAGE-1 and MART-1) as well as blood borne tumours (CLL: MDM2, Fibromodulin) (Traversari et al. 1992). Interestingly the frequency of these TAA specific T-cells are higher in cancer patients than in healthy donors; where they are generally of a naïve phenotype. However both T-cells from patients and healthy donors have the potential to kill tumour cells (Marincola et al. 1996). *In vitro* priming of T-cells from melanoma patients using peptide loaded DCs revealed a subset of T-cells capable of killing autologous tumour cells (Yee et al. 2002). T-cell tolerance to tumours may therefore be relative but not absolute, with even low affinity T-cells capable of discharging effector function if properly activated (Pardoll 2002).

Both $CD4^+$ and $CD8^+$ T-cells play an important role in anti-tumour immune responses. $CD8^+$ T-cells however are thought to be more important in the detection of tumour cells due to the fact that most non-haematopoietic tumours express MHC class I and the activation of $CD8^+$ T-cells results in a direct cytotoxic response towards the target cell. $CD4^+$ T-cells are thought to support $CD8^+$ T-cell anti-tumour responses. CD40L present on $CD4^+$ T-cells can engage CD40 on APCs helping activate tumour-specific $CD8^+$ T-cells through cross priming. $CD4^+$ T-cells were also shown in mouse tumour models, to produce both Th1 type cytokines such as IFN-γ; which can activate macrophages and Th2 cytokines e.g. IL-4; which recruits anti-tumour effector cells such as tumourcidal eosinophils. $CD4^+$ T-cells can also recognize tumour antigens presented by MHC class II molecules on macrophages (Hung et al. 1998). It

is however important to point out certain subsets of $CD4^+$ T-cells can have a negative effect on anti-tumour immune responses as highlighted by $FOXP3^+$ Tregs.

1.3.2 Tumour evasion of T-cell responses

As discussed previously, selective pressures put on tumour cells by the immune system may select for tumour variants able to escape immune recognition. Tumour cells display multiple immunosuppressive mechanisms that can directly or indirectly suppress T-cell responses. This includes; creation of a tumour microenvironment that promotes Treg production, increased surface expression of immunosuppressive molecules and reduced expression of molecules involved in antigen presentation. All of these mechanisms could in isolation or combination facilitate immune escape (Zou 2005) (Figure 1.3). The tumour microenvironment has been shown to induce the tolerance of both tumour specific $CD4^+$ and $CD8^+$ T-cells including skewing cytotoxic T-lymphocyte (CTL) maturation towards a preterminally differentiated state preventing effective CTL responses (Staveley-O'Carroll et al. 1998). Tumour suppressive mechanisms are also thought to co-operate in both early and advanced stages of cancer leading to a loss of immunological control and tumour progression.

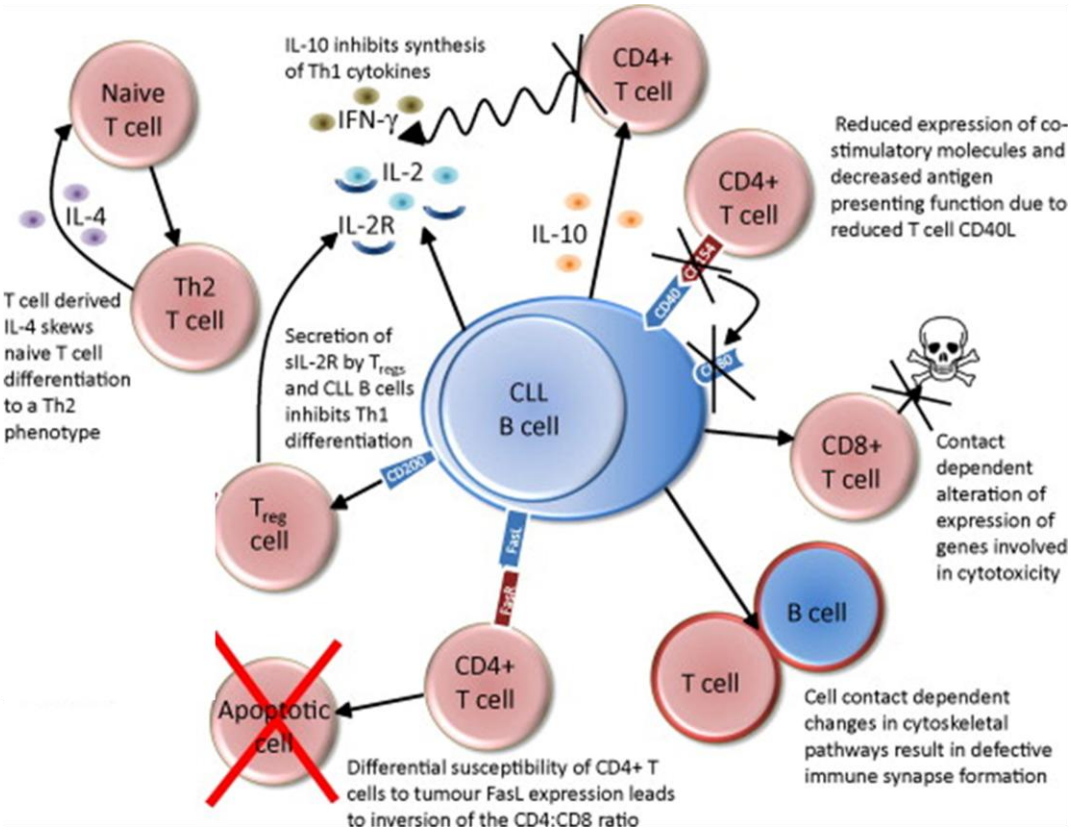


Figure 1.3 Suppression of T-cells mediated by CLL cells *(Riches, Ramsay and Gribben 2010).* Tumour cells display a reduced level of surface co-stimulatory molecules and increase surface expression of immunosuppressive molecules to block T-cell activation. Contact-dependent changes in genes involved in cytoskeleton rearrangements and cytotoxicity in T-cells can inhibit anti-tumour immune responses. Tumour cells also secrete suppressive cytokines such as IL-10 that promote the development of a Th2 phenotype.

1.3.2.1 Reduction in tumour antigen presentation capacity

One of the best-studied immune evasion strategies deployed by tumours is the impairment of antigen presentation (Rivoltini et al. 2002). Complete loss and/or down regulation of surface MHC class I expression has been observed in several cancers including colorectal, prostate, cervical carcinoma and melanoma. This down regulation can inhibit $CD8^+$ T-cell recognition of the tumour. The genetic instability of cancer cells can lead to a

loss of MHC class I expression through the accumulation of genetic abnormalities including mutations in the HLA class I genes and beta 2-microglobulin gene (Hicklin, Marincola and Ferrone 1999). Defects in cytokine signalling pathways in cancer lines have also been shown to reduce MHC class I upregulation upon IFN-γ stimulation (Respa et al. 2011).

Antigen presentation in tumour cells can also be effected by abnormal peptide processing which has been observed in lung and renal cancer and lymphoma (Hicklin et al. 1999). Defects in proteosome subunits LMP2/LMP7 and TAP can affect the spectrum of peptides presented on MHC class I allowing the preferential expression of peptides which disfavour CTL recognition and activation.

For optimal T-cell activation T-cells not only require antigen-specific triggering of their TCR but also additional co-stimulatory signals through CD28:CD80/CD86 ligation. In the absence of co-stimulation T-cells undergo anergy and become tolerised to the tumour. Down regulation of CD80 and CD86 molecules have been described in several solid and haematological malignancies including CLL that may inhibit T-cell recognition and activation (Fujiwara et al. 2004, Dai et al. 2009, Grzywnowicz et al. 2012). Furthermore CD80/CD86 expression has been associated with better overall survival in nasopharyngeal carcinoma (Chang et al. 2007).

1.3.2.2 Expression of immunosuppressive molecules

As well as co-stimulatory signals, the B7 family can provide negative signals that control and suppress immune responses. B7-H1 (PDL-1) is expressed on several human cancers where upon binding to its counter-receptor PD-1 can induce apoptosis, anergy or exhaustion of T-cells (Rabinovich, Gabrilovich and Sotomayor 2007). PD-1 belongs to a

family of proteins that negatively regulate T-cell responses including CTLA-4 and BTLA. PD-1 ligation on T-cells leads to the recruitment the protein tyrosine phosphatase SHP-2 which interfere with TCR signalling by dephosphorylating downstream effector molecules syk, ZAP70 and PI3K (Keir et al. 2008). Tumour cells can also release soluble forms of PDL-1 with high serum levels associated with worse prognosis in renal cell carcinoma (Frigola et al. 2011).

CD200 is the ligand for the inhibitory immune receptor CD200R which is highly expressed on a variety of cancer cells including solid tumours e.g. melanoma and ovarian cancer and haematological malignancies e.g. acute myeloid leukaemia (AML), multiple myeloma (MM) and CLL (Rygiel et al. 2011, El Desoukey et al. 2012, Palumbo et al. 2009). Although the role of CD200-CD200R interactions in tumour immunology remains unclear, CD200 expressed on tumour cells has been shown to inhibit anti-tumour T-cell responses. This inhibition may occur through direct interaction of CD200 with CD200R expressed on T-cells or through the inhibition of CD200R expressing cells of the macrophage lineage, which is associated with Th2 cytokine production and Treg induction (Hoek et al. 2000).

1.3.2.3 Secretion of immunosuppressive cytokines and soluble inhibitory factors

Tumour cells are known to secrete an array of immunosuppressive cytokines capable of inhibiting T-cell responses and favouring tumour progression. Analysis of cytokines present in the tumour microenvironment identified low levels of GM-CSF, IL-4, IL-12 and IFN-γ that are essential for DC maturation and T-cell priming, and an abundance of IL-6, IL-10, TGF-β that have suppressive properties (Freedman et al. 2004, Buggins et al. 2008). IL-10 and TGF-β secreted by tumour cells can act in an autocrine fashion promoting tumour cell

growth, whilst inducing the differentiation of tumour reactive $CD4^+$ T-cells to become regulatory $FOXP3^+$ T-cells (Chen, Benoist and Mathis 2005). TGF-β is also known to inhibit T-cell activation, proliferation and transcription of genes encoding perforin and granzyme A and B necessary for cytotoxic T-cell responses (Thomas and Massagué 2005). Elevated levels of TGF-β have been associated with poor prognosis in breast and lung cancer (Walker and Dearing 1992).

1.3.2.4 Resistance to T-cell killing mechanisms

Tumour cells possess a series of mechanisms aimed at preventing and protecting them from cytotoxic T-cells. The loss of FAS and TRAIL receptors due to tumour-associated mutations and the secretion of soluble decoy receptors (sCD95) and TRAIL-R4/R5, help tumour cells avoid CTL death receptor-mediated killing (Park et al. 2001, Shin et al. 2001). Resistance mechanisms to granzyme/perforin mediated CTL attack have also been described including the expression of serine protease inhibitor PI-9/SPI-6 in murine and human cancers resulting in resistance of tumour cells to granzymes (Bird et al. 1998).

The over expression of anti-apoptotic proteins including members of the BCL-2 family BCL-X and MCL-1 in tumours as well as reduced expression of pro-apoptotic proteins have been described in several cancers including CLL (Findley et al. 1997, Pepper et al. 2008). A high level of the anti-apoptotic regulator FLICE inhibitory protein (C-Flip) in melanoma cells is associated with resistance to TRAIL-mediated but not perforin/granzyme-mediated killing (Griffith et al. 1998). In addition, increased levels of serine protease inhibitor (serpins) have also been shown in haematological malignancies which may protect them from granzyme B mediated killing (Riewald et al. 1998).

1.3.2.5 Contact dependent inhibition of T-cells

Direct suppression of T-cells upon contact with tumour cells has been described in CLL. T-cells/tumour cell contact in CLL led to defective actin polymerisation and impaired immunological synapse formation between T-cells and tumour cells. Furthermore differential gene expression was seen in both $CD4^+$ and $CD8^+$ T-cells including genes involved in cytotoxicity (Ramsay et al. 2008). Increased surface expression and secretion of FAS-L and TRAIL by tumour cells may also induce T-cell death by interacting with the corresponding receptor on T-cells (Shiraki et al. 2005).

1.3.2.6 Stimulation of regulatory and suppressive cell populations

Elevated numbers of Tregs have been found in both solid tumour as well as haematological malignancies (Ustun et al. 2011). Stage-dependent increases in Tregs have been shown in CLL (Giannopoulos et al. 2008), whilst in solid tumours the appearance of Tregs at the tumour site has been linked to worse prognosis (Beyer and Schultze 2006). Although the precise mechanism of inhibition is unclear, Tregs inhibit T-cell, DC and NK cell function in a contact and dose-dependent manner (Trzonkowski et al. 2004). At least part of Treg suppressive activity is thought to be due to the surface expression of inhibitory molecules PD-1 and CTLA-4 and the secretion of IL-10 and TGF-β. Both activated natural Tregs as well as induced Tregs; derived from tumour-specific $CD4^+$ T-cells at the tumour site are believed to mediate suppression (Linehan and Goedegebuure 2005). Tregs express CCR4 allowing them to migrate towards CCL22 secreting tumour cells and local macrophages whilst in lymphoid tissues they can be found in close proximity to $CD11c^+$ DCs, CD4 and $CD8^+$ T-cells (Ishida et al. 2006).

A group of myeloid suppressor cells (MSC) have been identified in pancreatic, colon and breast cancer, which are found in the lymph node and also at the tumour site where they inhibit naïve T-cell responses. MSCs are a heterogeneous cell population including immature DCs, granulocytes and macrophages. MSCs deplete the milieu of L-arginine, which is essential for T-cell function including responses to IL-2 and development of T-cell memory phenotypes (Rodriguez et al. 2004).

1.3.2.7 Accessory cell dysfunction

BM-derived APCs, particularly DCs have been shown to play an important role in the development of tumour antigen-specific $CD4^+$ T-cell tolerance (Sotomayor et al. 2001). The tumour microenvironment contains increased levels of IL-10, vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2) which are known to inhibit DC maturation (Gabrilovich et al. 1996). Without DC maturation and the upregulation of co-stimulatory molecules, presentation of tumour antigens to T-cells may result in T-cell tolerance. Although DC levels are reduced in cancer patients, there is an increase in DCs with a regulatory phenotype inducing T-cell defects through the production of IL-10 and indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan depletion (Liu et al. 2009).

1.4 Cancer Treatment

1.4.1 Cancer immunotherapy

Cancer immunotherapy covers a broad range of strategies aimed at generating anti-tumour responses in patients. These include the transfer of tumour-targeting antibodies or

tumour reactive lymphocytes to cancer patients, cancer vaccines, adjuvants and pro-inflammatory molecules (Brody et al. 2011).

1.4.1.1 Therapeutic Cancer Vaccines

Vaccines aimed at preventing or treating malignancies provide an attractive model for cancer prevention. These vaccines aim to treat patients with existing cancers by stimulating the body's immune system to recognise and target the malignant cells. Therapeutic cancer vaccines tested in clinical trials include peptide vaccines, whole cell vaccines, DC vaccines and heat shock protein vaccines. So far results of these trials have being relatively poor with an overall objective response rate of 3.3% seen in 1306 vaccine treatments tested for metastatic cancer (Itoh et al. 2009).

Peptide-based and whole cell vaccines

Cancer vaccines incorporating tumour specific antigens could lead to the induction of both effector and memory immune responses capable of targeting malignant cells. Several groups of candidate tumour antigens have been identified on the surface of cancer cells including cancer testis antigens (MAGE-A1, NY-ESO-1), differentiation antigens (Melan-A, Mart-1), and over expressed antigens (survivin, hTERT). So far only a limited number of peptide vaccines have been tested in clinical trials, most of which contain antigens recognised by $CD8^+$ T-cells in melanoma patients. Melanoma peptides MART-1 and gp100 were the first to be tested in phase I and II studies as a vaccine in metastatic melanoma resulting in clinical responses in between 10-30% of patients (Rosenberg et al. 1998). Although peptide

vaccines have been shown to induce tumour specific T-cell responses *in vivo* this has not translated into significant clinical responses.

The relative failure of peptide vaccines to induce clinical responses in cancer patients may be due to several factors. The use of vaccines targeting a single antigen may result in the expansion of tumour variants that have lost that antigen and are resistant to vaccine-induced immune responses. Consequently improved vaccines incorporating multiple immunogenic epitopes have been investigated (Perez et al. 2010). Whole tumour cells have the potential to present multiple tumour antigens on their surface to T-cells. Vaccination using whole tumour cells derived from autologous or allogenic sources has been tested in several cancer types including colorectal and prostate cancer and melanoma (Simons et al. 2006). Alternatively polyepitope peptide vaccines including vaccines using long peptides; generated by the chemical linkage of multiple immunogenic epitopes have been tested in clinical trials in colon and non-small cell lung cancer (NSCLC) (Perez et al. 2010). A report by Neller *et al* published the combined results of 177 clinical trials for patients with a wide range of solid cancers receiving either molecular defined synthetic antigens or autologous or allogeneic tumour cell vaccines. They found that 8.1% (138 of 1711 patients) receiving immunotherapy with whole tumour or tumour extracts had an objective clinical response compared to 3.6% (63 of 1733 patients) that received molecularly defined antigen vaccines (Neller, López and Schmidt 2008).

Collectively these studies suggest that whole tumour vaccines can generate superior clinical responses compared to molecularly defined antigen vaccines but overall clinical responses remain low.

Dendritic cell-based vaccines

New technologies have allowed for the generation of high numbers of DCs from $CD34^+$ progenitor cells derived from patient bone marrow or peripheral blood (Bai et al. 2002). Mature DCs can be loaded with tumour peptides or pulsed with tumour cell lysates or apoptotic debris, which theoretically may contain multiple tumour antigens. These DCs can then be transferred back into the patient where they can present tumour antigens on both MHC class I and class II molecules.

Patients with metastatic cancer vaccinated with autologous DCs loaded with 4 class 1 HLA-restricted melanoma peptides resulted in enhanced immune responses to one or more melanoma antigens in 16 out of 18 patients, with tumour regression evident in 7 patients (Banchereau et al. 2001). The ability of DC vaccines to induce CTL production and induce anti-tumour responses is dependent on multiple factors including the site of vaccination and the origin of the DCs used. In addition, using a mixture of $CD8^+$ and $CD4^+$ T-cell epitopes and including cytokines such as IL-12 can improve DC vaccine efficacy (Parmiani et al. 2002).

Although DC-vaccines appear to generate stronger CTL responses than standard tumour-peptide vaccines only a small proportion of patients show clinical responses. That said there has been some success for a DC based vaccine called ‘Dendreon’ which became the first licensed cancer vaccine in the US, after it showed a 4 month median survival advantage in advanced prostate cancer. It is composed of autologous DC cells cultured *in vitro* and pulsed with a prostatic acid phosphatase (PAP) antigen present on 95% of prostate cancer cells (Kantoff et al. 2010). Efforts to improve the efficacy of DC-based vaccines in prostate cancer as well as other cancers is ongoing including combining DC vaccines with TLR-ligands and cytokines to enhance DC presentation of antigens *in vivo*.

1.4.1.2 Improving the immunogenicity of cancer vaccines

Cancer vaccines have been shown to induce increased frequencies of tumour specific T-cells *in vivo* but this has not always correlated with clinical responses in patients. Novel strategies of improving the immunogenicity of cancer vaccines including the use of cytokines and adjuvants are now being considered. Indeed it is likely that combinatorial approaches using vaccines that allow for improved T-cell priming and antigen presentation as well as therapies that block the suppressive effects of the tumour microenvironment may be necessary for optimally and effective cancer immunotherapy.

Enhancing Antigen presentation

As discussed previously, tumour cells are poor antigen presenting cells, expressing reduced levels of MHC and co-stimulatory molecules necessary for T-cell activation. Several strategies aimed at improving the antigen presentation capacity of tumour cells are now being considered. Tumour cells genetically modified to express co-stimulatory molecules such as CD80, CD70 and LIGHT have been shown to induce cytotoxic anti-tumour responses *in vitro* (Douin-Echinard et al. 2000). Alternatively, presentation of tumour antigens can be enhanced using CD40L and TLR-agonists such as cytosine-phosphate-guanine (CPG) DNA. CD40L and CPG DNA are potent stimulators of B-cells and DCs, inducing the upregulation of MHC and B7 co-stimulatory molecules as well as the release of Th1-type cytokines (Teleshova et al. 2006). Both soluble forms of CD40L and tumour cells genetically modified to express CD40L have been tested in clinical trials. CLL patients infused with genetically modified CLL cells expressing CD40L resulted in significant drops in tumour cells *in vivo*, however no

complete responses were observed (Wierda et al. 2000). CPG DNA is being investigated in animal models and clinical trials as a vaccine adjuvant (Klinman 2004, Bode et al. 2011).

Blocking immunoinhibitory molecules

Increased expression of immunoinhibitory molecules known to inhibit T-cell activation including PD-1, CTLA-4 and TIM-3 have been identified on the surface of T-cells from cancer patients (Fourcade et al. 2010, Curran et al. 2010). Immunotherapeutic approaches using blocking antibodies to disrupt the interaction of the molecules with their respective ligands are being investigated in clinical trials to try and enhance anti-tumour T-cell responses. The FDA recently approved a CTLA-4-targeted antibody ipilimumab, which improved the median overall survival of metastatic melanoma patients by 3.7 months (Hodi et al. 2010). Synergistic effects of blocking multiple immunoinhibitory molecules have been shown in mouse models where combining CTLA-4 and PD-1 blocking antibodies increased circulating $CD107a^+$ $CD8^+$ T-cells compared to either antibody alone (Mangsbo et al. 2010). Clinical trials are ongoing to assess the clinical benefit of blocking antibodies including the kinetics of clinical responses, potential side effects e.g. autoimmunity and potential for combination with radiotherapy and hormone therapy (Sharma et al. 2011).

Cytokines as vaccine adjuvants

Since cytokines play an important role in shaping immune responses in the tumour microenvironment, it is possible that cytokine therapy may break immune tolerance to cancer cells. Several cytokines including IFN-α, IL-2, IL-12 and GM-CSF have been evaluated in clinical trials (Dranoff 2004). Administration of high dose IL-2 was shown to have an

antitumour effect in 20% of metastatic melanoma and renal cancer patients (Fyfe et al. 1995). The clinical benefit of cytokine therapy is however limited by significant systemic toxicity associated with high dose of cytokines administered. Thus investigators have focused on directly modifying the cytokine microenvironment at the tumour site through local infusions of cytokines. Localised injection of cytokines, particular IL-2, can have therapeutic benefit coordinating innate and adaptive immune responses at the tumour site in mouse models (Dranoff 2004). In humans, cytokine linked antibodies (Immunocytokines) are now in clinical trials designed to direct cytokines to the tumour site. In a study of neuroblastoma patients, 22% had a complete response after receiving an antibody targeting GD2 disialoganglioside on neuroblastoma cells combined with IL-2 (Shusterman et al. 2010).

The application of gene-transfer techniques has also allowed tumour cells to be directly modified to express particular cytokines. Several cytokine secreting tumour vaccines are being investigated to determine whether they can augment immune responses towards wild-type tumours. GM-CSF vaccines have been tested in multiple cancers including metastatic melanoma, where localised infusion induced both T-cell and B-cell accumulation in resected lesions and extensive tumour necrosis in 3 out of 6 patients (Salgia et al. 2003).

1.4.1.3 Adoptive T-cell transfer

The anti-tumour effect seen in patients successfully treated with allo-HSCT has encouraged the use of adoptive immunotherapy as a tool for treating cancer. Adoptive T-cell transfer is a type of transfusion therapy whereby T-cells pre-selected for reactivity to the tumour are infused into a patient. Ideally these T-cells should be able to proliferate, home and

persist at the tumour site and maintain effector function capable of eliminating the tumour and preventing its reoccurrence (June 2007).

Two approaches have been identified for isolating tumour reactive T-cells from cancer patients. The first relies on identification and isolation of tumour specific T-cells before expansion *in vitro*. The second relies on the assumption that tumour specific T-cells exist in the patient's body which are already primed, thus polyclonal T-cell activation *in vitro* will also result in expansion of these T-cells. Tumour specific T-cells can be expanded using irradiated allogeneic PBMC (feeder cells) acting as APCs or by creating artificial APCs using anti-CD3 and anti-CD28 antibodies (Powell and Levine 2008). MHC class I molecules loaded with viral or tumour-specific peptides have also been used to generate antigen-specific T-cell expansions. MHC tetramers presenting peptides for tumour antigens MART1, gp100 and virus (EBV) have been used to isolate high avidity T-cells which can be expanded *in vitro* (Dunbar et al. 1999).

Adoptive transfer of virus specific T-cells has already proved successful in reconstituting viral responses following allo-HSCT and can induce clinical responses against virally transformed tumours (Riddell et al. 1992). Treatment of EBV-positive type II latency tumours with autologous EBV-specific T-cells resulted in 2 complete and 1 partial remission out of 6 patients (Straathof et al. 2005).

The investigation of TAA-reactive T-cells in adoptive therapy has been limited by the low number of identified tumour-associated antigens and the difficulty in expanding known TAA-reactive T-cells from the blood of cancer patients. However adoptive transfer of T-cells targeting MART-1/Melan A and gp100 were shown to migrate to tumour sites and eliminate antigen positive tumour cells in metastatic melanoma (Yee et al. 2002). In myeloma, adoptive

T-cell transfer combined with vaccination with tumour antigens hTERT and survivin, resulted in immune responses to the tumour antigen vaccine (Rapoport et al. 2011).

Despite the promising results in a few studies, the majority of early clinical trials with adoptively transferred T-cells showed only modest and often transient responses (Rosenberg et al. 1994). Successful application of ACT is hampered by several factors including the limited knowledge of how to culture T-cells which are likely to traffic to the tumour site, persist and exert effector function *in vivo* and the tolerising effects of the tumour microenvironment exerted on T-cells.

Currently there are no FDA-approved T-cell therapies for cancer despite over 60 years of research. However lessons learned from adoptive transfer in lymph depleted hosts, in Treg depleted patients and the use of improved culturing techniques may help to improve ACT efficacy and bring it into routine practice in clinical medicine (June 2007). Indeed Rosenberg's group reported response rates of 52% and 72% in patients with metastatic melanoma receiving chemotherapy or total body irradiation prior to ACT (Dudley et al. 2008).

Engineering T-cells to recognise tumour cells

One current limitation of ACT is that many tumours are poorly immunogenic, so naturally occurring tumour specific T-cells occur at very low frequencies. This makes it difficult to expand high avidity tumour-specific T-cells in sufficient numbers for transfusion. Using gene transfer techniques, T-cells of any specificity can be reprogrammed to target tumour antigens. In particular molecular determinants on the surface of malignant cells which are over expressed and crucial for tumour cell survival, would be attractive targets. This

would overcome tumour escape mechanisms designed to avoid surveillance by the patient's own T-cells. Furthermore tumour-specific T-cells obtained from the blood of patients for ACT are often tolerised and after several rounds of expansion may be driven to a terminally differentiated phenotype with a replicative senescence phenotype (June 2007). By genetically engineering T-cells, tumour specificity can be conferred to selected T-cells, preferably with high proliferative capacity (long telomere length), a TCM phenotype and lacking markers associated with a senescent/exhausted phenotype.

Chimeric Antigen Receptor T-cells (CAR-Ts)

One approach is to manipulate T-cells to express chimeric antigen receptors on their surface conferring new antigen specificity. These CAR-Ts consist of the antigen recognition domain of the antibody combined with the intracellular domain of the CD3 zeta chain or FCγR1 protein allowing the T-cell, upon recognition of the appropriate antigen, to become activated (Porter et al. 2011). These constructs allow T-cells to target tumour cells in an antigen specific but MHC-independent manner via the intracellular signalling pathway of the endogenous TCR. Previous *in vivo* studies using adoptive transfer of CAR-Ts in neuroblastoma and ovarian cancer have reported poor clinical activity with rapid clearance of CAR-Ts from the body (Jena, Dotti and Cooper 2010). Several factors are thought to prevent the persistence of CAR-Ts *in vivo* including the presence of host humoral and cellular immune responses, the immunotoxic effects of the tumour microenvironment and the lack of co-stimulatory signals necessary for long-term T-cell survival upon tumour recognition (Lamers et al. 2011).

TCR gene transfer

T-cells can be genetically engineered to express natural αβ TCRs heterodimers with known specificity for tumour antigens (Schumacher 2002). Killing of leukaemia cells has been observed *in vitro* and in mouse models with T-cells genetically modified to express TCRs (Tsuji et al. 2005). $CD8^+$ T-cells genetically engineered to express TCRs reactive to tumour peptides including MART, gp100, NY-ESO-1 and p53 were shown to be capable of recognizing HLA-A2-matched tumours in melanoma, lung and breast cancer. Furthermore adoptive transfer of genetically engineered lymphocytes transduced with a TCR recognising gp100 in metastatic melanoma patients resulted in durable engraftment and objective regression of metastatic lesions in two patients (Morgan et al. 2006).

Limitations of TCR engineering technology include the potential pairing of the transgenes with the endogenous TCR chains leading to novel TCR specificities and increased risk of autoreactivity, low cell surface expression of tumour specific TCRs, and the transduction efficacy of the retroviral delivery system.

1.4.1.4 Antibody therapy

Monoclonal antibodies

Monoclonal antibodies are used at several stages in cancer therapy including diagnosis, treatment and monitoring of patients. In terms of treatment, monoclonal antibodies can be used to target specific antigens on the tumour cell surface, which are absent or have reduced expression on normal tissue. Upon binding monoclonal antibody can induce indirect killing of tumour cells through ADCC by recruiting phagocytic cells including monocytes and macrophages or can directly bind complement-inducing tumour cell death through CDC.

MAbs can also function to inhibit cell signalling pathways. In the case of trastuzumab it can block HER2-mediated signalling in breast cancer cells leading to an upregulation of p27kip1 protein resulting in cell cycle arrest (Le, Pruefer and Bast 2005). Multiple mAbs have been approved or in clinical trials for use in solid cancers (trastuzumab: HER-2/neu antigen, Edrecolomab: 17-1A antigen) as well as haematological malignancies (Rituximab: CD20, Alemtuzumab CD52, Gemtuzumab CD33 and anti-idiotype antibodies). Solid cancers have however proved harder to treat than haematological malignancies due to the lack of identified tumour specific antigen targets and the difficulty of mAbs penetrating into solid tumours (Oldham and Dillman 2008).

The potential for mAbs to block molecules important in tumour cell survival are also being investigated in clinical trials. mAbs capable of blocking growth factor receptors on the tumour surface including Epidermal growth factor (EGF) receptors has been shown to induce proliferative arrest, apoptosis and inhibition of angiogenesis in tumour cells (Sharkey and Goldenberg 2006). Several blocking mAbs to immune-modulatory molecules are also being tested in clinical trials with the aim of promoting immune responses to tumours cells. These includes blocking antibodies to CTLA-4, which has been found to antagonize early T-cell activation and IL-2 production and PD-1 which has been shown to promote T-cell anergy upon binding to the ligand PDL-1 (Fife et al. 2009).

Alternative approaches include conjugating monoclonal antibodies to anti-cancer drugs, radioisotopes or toxins that has allowed treatments to be delivered to the surface of the tumour cells. This ensures that treatments are concentrated towards tumour cells whilst limiting toxic effects on surrounding tissue. A randomised trial comparing rituximab (anti-CD20) mAb to an anti-CD20 mAb conjugated to yttium-90, showed enhanced overall response rates in those patients treated with the antibody conjugate (Witzig et al. 2002).

There are several obstacles to the implementation of monoclonal antibodies in the clinic. Firstly generating high yields of antibodies necessary for infusion can be laborious and expensive. Secondly murine monoclonal antibodies induce the production of anti-mouse antibodies *in vivo*, which limits the half-life of any therapeutic mAb. This problem has been somewhat reduced through the development of chimeric and fully humanised antibodies (Oldham and Dillman 2008).

Bi-specific antibodies

The successful application of monoclonal antibodies as cancer treatments has resulted in a drive to enhance the efficacy of these molecules through new antibody engineering approaches. Engineering of mAb FC fragments has been used to try and improve the efficacy of existing mAbs by enhancing ADCC or CDC killing mechanisms. Other research has focussed on the development of a new class of antibodies termed bi-specific antibodies capable of dual specificity. Such molecules can induce the retargeting of immune effector cells towards target cancer cells by binding surface antigens expressed on both cell types resulting in the killing of the tumour cells. Bi-specific antibodies capable of retargeting innate immune cells (e.g. macrophages, neutrophils) as well as acquired immune cells (e.g. T-cells and NK cells) have been described (Chames and Baty 2009). Importantly bi-specific antibody mediated cytotoxicity does not require MHC:peptide presentation by tumour cells. Such antibodies have also shown anti-tumour activity both *in vivo* and in animal models.

Early bi-specific antibodies were formed using chemical cross-linking or quadromas but clinical application was limited by the difficulty of producing large homogeneous batches of mAbs. Furthermore patients often developed anti-mouse Ab responses, preventing

repeated mAb administration. Subsequent advances in antibody engineering led to a reduction in the size of bi-specific antibodies by producing small antibody fragment representing the entire recognition region of the parent molecule. These single chain variable fragments (scFv) include the variable domains of the antibody heavy and light chains which can be fused to another scFv through a peptide linker to form a single polypeptide chain (Wolf et al. 2005) (Figure 1.4).

The efficacy of bi-specific antibodies has been demonstrated in clinical trials in patients with solid cancers and haematological malignancies. Bi-specific antibodies targeting CD3 and HER2/neu (Ertumaxomab) or EpCAM (Catumaxomab) have shown clinical activity in breast and ovarian cancer (Kiewe et al. 2006). In 2009 Catumaxomab became the first bi-specific antibody approved by the European commission for treatment of malignant ascites in patients with EpCAM-positive cancers. In haematological malignancies bi-specific antibodies targeting the B-cell antigen CD19 have been tested in clinical trials of acute lymphoblastic leukaemia and non-Hodgkin's lymphoma (NHL) patients. Seven patients with NHL treated with a bi-specific antibody targeting CD19 showed tumour regression and elimination of tumour cells from the bone marrow and liver (Bargou et al. 2008). Bi-specific antibodies are also being evaluated in myeloid leukaemias targeting surface antigens such as CD33 (Aigner et al. 2012).

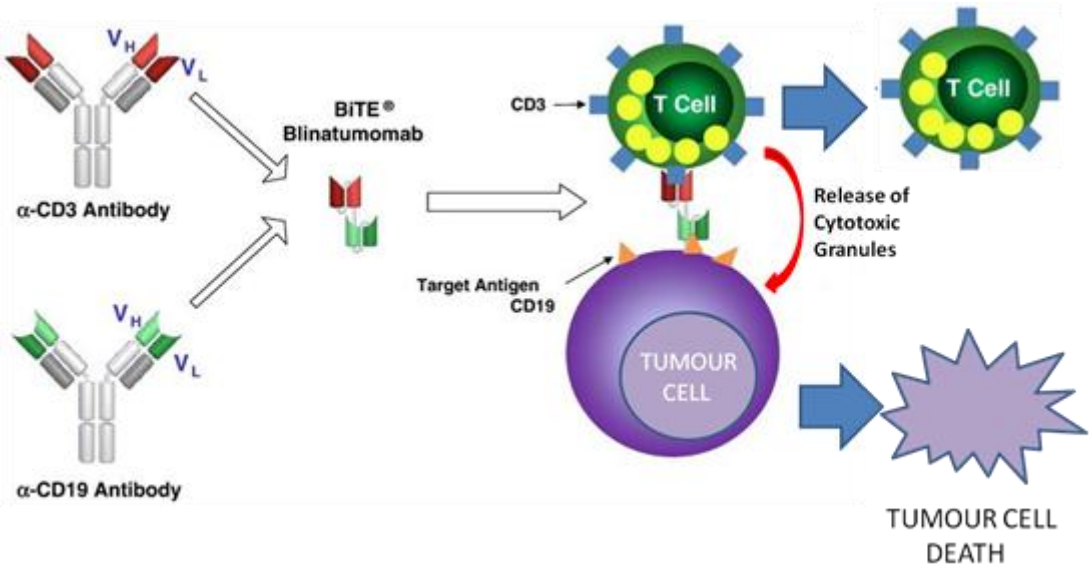


Figure 1.4 Schematic diagram detailing the formation of a bi-specific antibody and its mode of action on effector cells and target cells *(Adapted from Baeuerle and Reinhardt 2009)*. Bi-specific T-cell engager (BiTE) antibodies are formed by the fusion of two single chain variable fragments from two different antibodies using by a short peptide linker. The BiTE antibody binds both the effector cell (T-cell) and tumour cell triggering a cytotoxic killing mechanism to be directed towards the tumour cell.

1.5 Aims of this project

The use of alkylating agents and purine analogs has proven highly successful in inducing remissions but therapy does not result in long-term disease free survival in CLL patients. Novel therapeutics aimed at improving the treatment and overall survival of CLL patients is therefore warranted.

Immunotherapy has emerged as an exciting tool in the treatment of CLL, with a recent study showing for the first time that immunotherapy alongside chemotherapy can increase progression free survival (PFS) and overall survival (OS) of CLL patients (Molica 2011). The objectives of this project were to characterise T-cell abnormalities in CLL patients with the aim of using this information to develop immunotherapeutic strategies capable of breaking T-

cell tolerance within the disease. Such therapeutic strategies will subsequently be investigated to determine whether they can improve T-cell immune response *in vitro* and potentially restore anti-tumour immunity.

CHAPTER 2

Materials and Methods

2.1 Tissue culture basics

2.1.1 Media and Buffers

AB MEDIA

Roswell Park Memorial Institute (RPMI) 1640 (Sigma) was supplemented with 2mM L-Glutamine (Invitrogen), 25mM Hepes buffer (Sigma), 100units/ml Penicillin and 100µg/ml streptomycin (Invitrogen) and 5% AB serum (Welsh blood service).

FIBROBLAST MEDIA

Dulbecco's Modified Eagle Medium (DMEM, Sigma) was supplemented with 10% foetal calf serum (FCS), 100units/ml Penicillin and 100µg/ml streptomycin (Invitrogen), 2mM L-Glutamine (Invitrogen) and 1mM Sodium pyruvate (Invitrogen).

FREEZING MEDIA AND FREEZING MIX

MEDIA- 2mM L-Glutamine (Invitrogen), 100units/ml Penicillin and 100µg/ml streptomycin (Invitrogen) was added to RPMI.

MIX- 40% Freezing media, 50% FCS and 10% Dimethyl Sulfoxide (DMSO, Sigma).

FACS BUFFER

1% FCS was added to Phosphate buffered saline (PBS, Invitrogen)

MACS BUFFER FOR IMMUNOMAGNETIC CELL ENRICHMENT

0.5% Bovine serum albumin (BSA-Sigma) and 2mM Ethylenediaminetetraacetic acid (EDTA, Sigma) was added to 100ml of PBS. The buffer was then sterile filtered using a 0.2µm bottle top filter (Millipore).

FIX/PERM BUFFER

3 parts Fix/Perm diluent + 1 part Fix/Perm Concentrate (Ebiosciences)

PERMEABILISATION BUFFER

1 part perm wash concentrate (Ebiosciences) was diluted in 9 parts distilled water.

RED BLOOD CELL LYSIS BUFFER

Red blood lysis solution (Miltenyi Biotec) was diluted 1/10 in distilled water.

10% NP40

10g Tergitol-type NP40 (nonyl phenoxypolyethoxylethanol) (Sigma) was dissolved in 100ml of water.

2.1.2 Tissue culture plastics

Culture Flasks (T25, T75 and T225) (Nunc), Pipettes and Falcon tubes (15ml, 50ml) (Corning Incorporated), Tips (1000, 200, 20, and 2µl) (Star lab), plates (98, 48, 24, 12 well) (Nunc and Greiner bio-one). FACS tubes (BD biosciences).

2.1.3 Cell viability staining

Trypan blue staining

Cell viability was assessed using the live cell exclusion dye trypan blue. Dead cells take up the dye giving them a distinct blue colour under the microscope. Live cells, which appear white, can then be counted allowing the total number of viable cells in suspension to be measured. Cell counting was performed using the Beckman Coulter Vi-cell or a Haemocytometer (Immune systems).

Beckman Coulter Vi-cell counting- Briefly 50µl of sample was added to 450 µl of PBS (1/10 dilution) in a cell counting container and placed into the Vi-cell carousel. The cell type, dilution and name of sample were entered into the machine. The machine was then set to run, allowing the sample to be mixed with trypan blue and the number of viable cells per µl calculated.

Haemocytometer- Twelve microlitres of cell suspension was mixed with an equal volume of trypan blue in a 96 well plate. Twelve microlitres was then pipetted under the grid of a disposable haemocytometer. The total numbers of viable cells were counted in 3 squares divided by 3 and then multiplied by 20,000 to give the number of cells per ml.

Aqua staining

Exclusion of dead cells during flow cytometric analysis was performed using the amine-reactive fluorescent dye aqua. Cells were washed twice in 1ml of PBS by centrifugation at 756 x g for 2 minutes. The aqua dye (Invitrogen) was diluted 1/40 in PBS

and 4µl added to each sample. The cells were incubated at 4°C for 15 minutes in the dark before being washed twice in 1ml of FACS buffer to neutralise the reaction.

2.2 CLL patient blood samples

Professor Chris Fegan obtained blood samples from CLL patients attending clinics at the University Hospital Wales and Llandough hospital. Additional samples were obtained from Birmingham University in collaboration with Dr Guy Pratt. All samples were taken with informed consent from patients and the appropriate ethical approval obtained. Healthy, age-matched control samples were obtained by Professor Chris Fegan with informed consent, or from volunteers from within the department.

2.3 Cell lines

Non-transfected mouse fibroblasts (NTLs) and CD40 ligand transfected mouse fibroblasts TL(CD40L) were kindly donated by Dr Aneela Majid (University of Leicester).

2.3.1 Growing cell lines in culture

NTL and TL(CD40L) cell lines were cultured in 20ml of fibroblast media in a T175 flask. 2-4x10^6 fibroblast cells were used for the initial seeding of the flasks and allowed to grow for 3 days until a confluent monolayer was formed. The cells were then washed in PBS and incubated for 5 minutes at 37°C in the presence of 5ml EDTA-trypsin (Invitrogen). Once the cells had detached from the flask surface, the cells were washed in 15ml of fibroblast media and spun down at 272 x g for 5 minutes. The supernatant was removed and the cells

re-suspended in 10ml of fibroblast media. 1ml was used to reseed the flask and the rest of the cells were used for co–culture experiments or frozen down.

2.3.2 Cell line and PBMC cryopreservation

Between 1-5x10^6 fibroblast cells or PBMCs were suspended in 1ml of freezing mix and stored in cryovials overnight at -80ºC. The cells were then transferred and stored in liquid nitrogen until required.

2.3.3 Co-culture of cell lines with CLL PBMC

TL (CD40L) and NTL cell lines were suspended in 5ml of fibroblast media and irradiated (8000 RADs). 5x10^4 irradiated NTL or TL (CD40L) cells were then seeded into a 48-well plate in 1ml of AB media. The plate was then incubated for 24 hours at 37°C to allow the fibroblast cells to adhere to the well surface. 1x10^6 CLL patient PBMCs were suspended in 500µl of AB media and added to the surface of the irradiated NTL and TL (CD40L) cells and incubated for 3 or 7 days.

2.4 Cell separation

2.4.1 Peripheral blood mononuclear cell (PBMC) separation

Blood samples were collected and immediately processed for PBMC extraction. Whole blood was layered onto a lymphoprep (Axis-shield) gradient at a 1:1 ratio and centrifuged at 756 x g for 20 minutes without the use of the brake. The buffy coat interface containing

PBMCs was carefully collected and the cells washed in 15ml of PBS by centrifugation at 272 x g for 10 minutes. The supernatant was discarded and any red blood cell contamination removed by re-suspension of the pellet in 1ml of red blood cell lysis buffer and incubation at room temperature for 10 minutes. Cells were washed again in 15ml of PBS and pelleted by centrifugation at 272 x g for 5 minutes. The supernatant was removed and the cells re-suspended in 10ml of fresh PBS and counted by trypan blue staining.

2.4.2 $CD3^+$ T-cell isolation

$CD3^+$ T-cells were enriched using an indirect magnetic bead labelling system designed to deplete non T-cells (Miltenyi Biotec). Cells were washed in PBS and spun down at 272 x g for 10 minutes. The supernatant was removed and the cell pellet re-suspended in 40µl of MACS buffer per 10^7 cells. 10µl of a Biotin-Antibody Cocktail against CD14, CD16, CD19, CD36, CD56, CD123 and CD235a (Glycophorin A) was then added per 10^7 cells. The cell suspension was vortexed and incubated at 4-8°C for 15 minutes. 30µl of additional MACS buffer and 20µl of anti-biotin microbeads per 10^7 cells was then added. The cell suspension was again incubated for 15 minutes at 4-8°C before being washed in 6ml of MACS buffer. The cells were centrifuged at 272 x g for 10 minutes, the supernatant removed and the pellet re-suspended in 500µl of cold (4°C) MACS buffer ready for magnetic column separation (See section 2.4.4).

2.4.3 $CD19^+$ B-cells isolation

CLL cells were isolated by depleting non B-cells (Miltenyi Biotec). Cells were washed in PBS and spun down at 272 x g for 10 minutes. The supernatant was removed and the cell

pellet re-suspended in 40µl of buffer per 10^7 cells. 10µl of B-CLL biotin-antibody cocktail against CD2, CD4, CD16, CD36, Anti-IgE, and CD235a (Glycophorin A) was added and the cell suspension, vortexed and incubated for 10 minutes at 2-8°C. The cells were then washed by adding 1-2ml of buffer and centrifuged at 272 x g for 10 minutes. The supernatant was removed and the pellet re-suspended in 80µl of MACS buffer. 20µl of anti-biotin microbeads was added and the cells again incubated for 15 minutes at 4-8°C. Cells were then washed in PBS, centrifuged at 272 x g for 10 minutes and the supernatant removed. The pellet was re-suspended in 500µl of MACS buffer ready for loading onto a magnetic column.

2.4.4 Magnetic column separation of cell fractions

The cells were suspended in 500µl of MACS buffer and loaded onto a magnetic MS column or LS columns (Miltenyi) in the presence of a strong magnetic field (MiniMacs Separators). The cell suspension was allowed to run through the column until the column reservoir was empty. The column was then washed by adding 500µl of MACS buffer to the top of the column, allowing it to completely run through the column. This wash step was repeated 3 times. In cases of negative selection the effluent was collected in a 15ml falcon tube. The number of viable cells was counted by trypan blue staining and the purity of the collected cells determined using single colour flow cytometry.

2.4.5 Measuring the purity of collected cell fractions

$1x10^5$ viable cells were taken from the collected effluent and stained with a FITC-conjugated anti-CD3 or PE cy-7-conjugated anti-CD19 antibody. A purity of >95% $CD3^+$ T-cells within the viable gate was used as the threshold for a pure $CD3^+$ T-cell population.

Samples with a CD3 purity less than 95% were put back through a MS column and the purity of the effluent fraction tested again using the CD3 staining protocol. For $CD19^+$ B-cells, a purity of 99% or greater was used as the threshold for CLL cell purity.

2.5 Antibody staining and flow cytometric analysis

2.5.1 Flow cytometric compensation set-up

Cells were analysed using a BD FACS Canto II or an Accuri C6 flow cytometer (BD Accuri). For analysis on the BD FACS Canto II, compensation between the fluorochrome emission spectra was done automatically using anti-mouse Ig/negative control compensation beads and FACSDiva software. Briefly 3 drops of anti-mouse Ig and Negative control comp beads were added to 700µl of FACS buffer. The beads were vortexed for 3 seconds and then 100µl was added to 1 FACS tube. Fluorochrome conjugated antibodies were then added at optimised concentrations (1 antibody per tube) and incubated for 5 minutes at room temperature (RT). A further 900µl of FACS buffer was then added to each tube. These antibodies were used as single-colour compensation controls. Each tube was analysed using the BD FACS Canto II by gating on the single bead population using forward side scatter, then gating both the positive and negatively stained populations on a histogram plot. These populations were then used to automatically calculate the compensation between antibodies using FACSDiva software.

2.5.2 Single and multi-colour immunofluorescence staining

Fluorochrome conjugated antibodies were directly incubated with 1-1.5x10^6 PBMC from CLL patients. Antibody concentrations used in this study were optimised by testing neat, ½ dilution and ¼ dilutions of the manufacturers' recommendations. The antibodies were incubated with the cells for 15 minutes at 4°C. Cells were then washed twice in FACS buffer by centrifuging at 756 x g for 2 minutes and discarding the supernatant. Cell pellets were re-suspended in 200-300µl of FACS buffer and analysed using a BD FACS Canto II or an Accuri C6 flow cytometer (BD Accuri).

2.5.3 FOXP3 staining

Intracellular FOXP3 staining was used in the identification of regulatory T-cells. 1x10^6 CLL PBMC were suspended in 100µl of FACS buffer and surface stained for 30 minutes on ice with antibodies to CD3-(AM Cyan), CD4-(Pacific Blue), CD25-(PE) and CD45RA-(APC). Cells were then washed twice in 1ml of FACS buffer and re-suspended in 500µl of Fix/Perm buffer (Ebiosciences). In order to fix the cells the samples were incubated on ice for 40-60 minutes. After incubation, the cells were washed twice in 2ml of permeabilisation buffer (Ebiosciences) and re-suspended in 30µl of permeabilisation buffer containing 2% normal rat serum. The cells were then incubated on ice for a further 15 minutes to allow permeabilisation of the cell membranes. Without washing, 15µl of a FOXP3-FITC conjugated antibody was added to the cells and the incubation continued for a further 30 minutes on ice in the dark. The cells were then washed twice in 1ml permeabilisation buffer and once in 1ml FACS buffer before being re-suspended in 300µl of FACS buffer.

2.5.4 Ki-67 proliferation assay

Intracellular Ki-67 staining was used to identify cells in active phases of cell cycle. $1x10^6$ CLL PBMC were washed once in 1ml of FAC buffer and surface stained with antibodies to CD4-(Pacific Blue), CD8-(APC-CY7), CD45RA-(APC) and CCR7-(PE). Cells were incubated at 4°C for 15 minutes before being washed twice in 1ml of FACS buffer. 50µl of Reagent A (Fixation Medium, Invitrogen) was added and the cells, incubated again for 15 minutes at 4°C to fix the cells, before being washed twice in 1ml of FACS buffer. Fifty microlitres of reagent B (Permeabilisation Medium, Invitrogen), 0.5µl of 1% NP40 and 3µl of an anti-Ki-67-(FITC) antibody were then added to the cells. The cells were incubated at 4°C for 15 minutes, then washed twice in 1ml of FACS buffer and re-suspended in 300µl FACS buffer.

2.5.5 CFSE proliferation assay

Carboxyfluorescein succinimidyl ester (CFSE) was reconstituted in DMSO to a concentration of 5mM. $1x10^6$ PBMCs were suspended in 1ml of RPMI containing 1% FCS and CFSE to a final concentration of 2µM. The cells were then incubated at 37ºC in the dark for 5 minutes before being washed in 10ml of ice cold RPMI containing 10% FCS. The cells were centrifuged at 272 x g for 5 minutes and the wash step repeated before being counted. $1.5x10^6$ PBMCs were added to separate sterile 5ml FACS tubes in 1ml of AB media. CD3/CD28 beads were then added at a ratio of one bead to one T-cell. The cells were then incubated for 3 or 7 days with the addition of blocking antibodies (PDL-1, 10µg/ml, Ebioscience, Clone MIH1), CD200 (20µg/ml, BD Pharmingen, Clone MRC OX-104) and an isotype control (20µg/ml, Ebioscience, Clone P.3.6.2.8.1) at day 0, 2 and 4 time points.

2.5.6 Intracellular cytokine, CD107 and Granzyme B staining

T-cell intracellular cytokine and granzyme B expression and surface CD107 were used to determine the activation status and cytotoxic potential of T-cells from CLL patients. $3x10^6$ CLL PBMCs were seeded per well in a 24-well plate in 2ml of AB media. Each well received PBS, 10ng/ml blinatumomab (Amgen) or 7.5µl of Human T-Activator CD3/CD28 Dynabeads (Invitrogen). The cells were then incubated for 7 days before being removed into 15ml falcon tubes and washed in 1ml of fresh AB media. The number of viable cells in each tube was determined using trypan blue staining and $\leq 1x10^6$ viable cells were added to two FACS tubes. One FACS tube would be later stained for IFN-γ and TNF-α and the other tube for granzyme B and CD107. The cells were then centrifuged at 756 x g for 2 minutes, the supernatant removed and the pellet re-suspended in 190µl of AB media. 1µl of golgi plug and 0.7µl golgi stop (BD biosciences) was added to 198.3µl of PBS and 20µl was added to each FACS tube. 10µl of CD107-(FITC) was added to those tube measuring CD107 and granzyme B, and all the tube were incubated for 5 hours at 37°C.

After the incubation, human T-activator CD3/CD28 beads were removed with a magnet and 1ml of PBS added to each tube. The cells were centrifuged at 756 x g for 2 minutes, the supernatant removed and the cells stained with the fluorescent dye aqua. The cells were then surface stained with antibodies to CD5-(PERCP-Cy5.5), CD4-(Pacific Blue) and CD8-(AM Cyan) followed by incubation at 4°C for 15 minutes. Cells were again washed twice with 1ml of FACS buffer then incubated for 20 minutes in the dark in 200µl of fixation/permeabilisation solution (BD biosciences). Perm/Wash Buffer (BD biosciences) was diluted 1/40 in PBS and 2x 200µl used to wash the cells before re-suspension in 50µl of the Perm/wash buffer. Each tube then received antibodies to IFN-γ-(PE) and TNF-α-(APC) or

CD107-(FITC) and Granzyme B-(PE). Cells were again incubated at 4°C for 15 minutes, washed twice in FACS buffer and re-suspended in 300µl of FACS buffer.

2.6 Cytokine assays

2.6.1 Supernatant transfer assay

PBMCs were incubated for three days in the presence or absence of CD3/CD28 beads (7.5µl). One ml of media was then carefully removed and stored at -20°C. Purified B-cells were isolated from CLL patients using a CLL isolation kit using the procedure outlined previously. B-cells ($1x10^6$ aliquots) were added to FACS tubes in 250µl of AB media. The supernatants were then thawed with 1 tube receiving 250µl of supernatant from the untreated culture, 1 tube receiving 250µl of supernatant from the CD3/CD28 bead treated culture and the last tube receiving 250µl of fresh AB media. The cells were then incubated at 37°C for 24 hours and the CLL cells measured for activation markers (HLA-DR and CD69) and PDL-1 expression by flow cytometry.

2.6.2 B-cell Cytokine Assay

Purified CLL cells were incubated in the presence or absence of 7 cytokines IFN-γ (10ng/ml), TNF-α (5ng/ml), IL-2 (10 IU/ml), IL-8 (10ng/ml), IL-4 (5ng/ml), IL-10 (5ng/ml) and IL-12 (5ng/ml). Briefly, $1x10^6$ CLL cells were suspended in 500µl of AB media in sterile FACS tubes and single cytokines added to separate tubes at the final concentrations indicated. The cells were then incubated at 37°C for 24 hours and the CLL cells measured for activation markers (HLA-DR and CD69) and PDL-1 expression by flow cytometry.

2.6.3 Cytokine bead array

This assay was used to determine the type of cytokines secreted by CLL PBMC after incubation for 3 days in the presence of blinatumomab. One ml of supernatant from 10 CLL patient PBMC cultures treated with or without 10ng/ml blinatumomab for 3 days was stored at -20°C.

Standards and sample preparation

Lyophilised cytokine standards were reconstituted in distilled water according to the label on the standard vial. Ten microlitres of each cytokine standard was added to a vial labelled standard 1 and filled up to a final volume of 200µl with 1x assay buffer (50ml of 10x assay buffer concentrate (Ebiosciences) diluted in 450ml distilled water). One hundred microlitres of assay buffer was added to 6 separate tubes labelled standard 2 to 7 and 50µl of standard 1 used to serial dilute the initial standard mixture. Twenty five microlitres of each standard mixture dilutions 1 to 7 was then added to 7 separate tubes. Meanwhile the supernatants from the 3 day PBMC cultures were thawed and 25µl of supernatant from the untreated and treated cultures added to separate labelled tubes.

Cytokine Bead mixture

Beads that would bind the cytokines in the supernatant were prepared by calculating the final bead mixture volume required for all samples (25µl x number of tests for standard curves, blank and samples). The individual cytokine bead vials were then vortexed and 1/20 of the total bead mixture volume required of each bead set added to a vial labelled ‘bead

mix'. Reagent dilution buffer (Ebiosciences) was then added to the vial labelled 'bead mix' to make up to the total bead mixture volume. The vial was then centrifuged at 3000 x g for 5 minutes and the supernatant removed leaving 50µl of liquid in the vial. The volume of liquid removed was replaced with the same volume of fresh reagent dilution buffer. Twenty-five microlitres of the bead mixture was then added to all the standard and sample tubes including a blank tube.

Preparation of Biotin-conjugate and strepavidin-PE solution

A biotin-conjugated second antibody mixture was made up in order to bind cytokines that had been capture by the cytokine bead mixture. Briefly each biotin conjugated antibody was added to a vial labelled biotin-conjugate mix at 1/20 of the final volume required (final volume= 50µl x number of tests for standard curve, blank and samples). The vial was then made up to the final volume by adding reagent dilution buffer. 50µl of the biotin-conjugate mixture was added to all tubes including standards, samples and the blank and the tubes left at RT for 2 hours in the dark.

After the incubation the tubes were washed twice by centrifugation at 200 x g for 5 minutes in 1ml of assay buffer. The streptavidin-PE solution was diluted in assay buffer and 50µl added to each tube. The tubes were then incubated for 1h at RT in the dark followed by two wash steps with assay buffer as outlined previously. Five hundred microlitres was added to each tube and the samples analysed by flow cytometry (BD FACS Canto II). Cytokine concentrations were calculated using FlowCytomix Pro 2.2 Software (Ebiosciences).

2.7 FACS-based cytotoxicity assay

2.7.1 Measuring absolute counts of cells

The absolute number of viable T-cells and CLL cells was assessed after incubation in the presence or absence of blinatumomab (10ng/ml). An agonistic anti-CD3 antibody (27.7ng/ml) or CD3/CD28 beads (7.5µl) were used as controls. CLL PBMC ($3x10^6$) were incubated in 2mls of AB media in the presence of BiTE, anti-CD3 antibody or CD3/CD28 beads for 7 days at 37°C. The cells were then resuspended by repetitive pipetting and 1ml of the cell suspension added to a 5ml FACS tube. The cells were then centrifuged at 756 x g for 2 minutes and 900µl of the supernatant carefully removed from each tube. Two hundred microlitres of binding buffer was then added to each tube and the cells surface stained with antibodies to Annexin V-(FITC), CD5-(Percp-cy5.5), CD8-(APC-CY-7) and CD4-(Pacific-blue) before being incubated for 15 minutes at 4°C. After incubation, 100µl of cytocount beads (Dako) was added to each tube and analysed by flow cytometry. In total $3x10^6$ events were recorded and the absolute number of cells was determined using the following equation:-

$$\text{Volume counted during acquisition} = \frac{\text{No. of beads counted}}{\text{No. of beads added per sample}} \times \text{Total volume of cell suspension}$$

$$\text{Absolute no. of cells} = \frac{\text{No. of viable cells counted}}{\text{Volume counted during acquisition}} \times \text{Total volume of cell suspension} \times \text{Dilution factor}$$

2.7.2 T-cell cytotoxicity assay in co-culture with TL (CD40L)

CLL PBMCs were incubated in the presence or absence of NTLs or TL (CD40L). Each well containing NTLs received PBS (untreated control) or blinatumomab (10ng/ml or 100ng/ml). The procedure was repeated for the wells containing CD40L cells and the wells containing media only. The plate was then incubated at 37°C for 7 days. On day 7, CLL PBMCs were carefully removed from the surface of the fibroblast monolayer, washed twice in PBS and surface stained with the dead cell stain aqua. The cells were re-suspended in 200µl of binding buffer (Ebiosciences) and labelled with antibodies against Annexin V, CD20, CD5, CD8 and CD4 for 15 minutes before analysis by flow cytometry. CLL cell death based on 2-4x10^5 events, was determined by measuring the percentage of Annexin V^+ CLL cells in each sample.

2.8 T-cell and CLL cell imaging

2.8.1 Immunofluorescence labelling of T-cells and CLL cells

Purified T-cells were labelled with 15mM red CFSE (Invitrogen) and purified CLL cells labelled with 10mM Green CFSE (Invitrogen) in 1ml of RPMI supplemented with 1% FCS. Cells were incubated at 37°C for 10 minutes before being washed twice in RPMI supplemented with 10% FCS. Labelled cells were then added to a 48 well plate in a total of 1ml of AB media at a ratio of 1 T-cell to 10 CLL cells. The cells were incubated in the presence or absence of blinatumomab (10 or 100ng/ml) for 12 hours.

2.8.2 Preparation of cells for phase contrast imaging

$1.5x10^6$ CLL PBMC were incubated in the presence or absence of blinatumomab (10, 100ng/ml), CD3 (27.7ng/ml, 277.7ng/ml) or CD3/CD28 beads (7.5µl). Cells were incubated for 12hours in a 48-well plate in 1ml of AB media

2.8.3 Phase contrast and confocal microscopy

Phase contrast and confocal microscopy were performed using a Leica RS2 microscope and analyzed using Leica confocal software (Leica Microsystems, Milton Keynes, UK) and Image J software (National institutes of Health (NIH) USA). For 3-D image reconstruction of clusters, 50 confocal sections were taken and assembled using Imaris imaging software (BITPLANE scientific software Zurich Switzerland).

2.9 Statistical analysis

All statistical analysis was performed using Graphpad Prism 5.0 software (Graphpad Inc.). For variables between independent groups a non-parametric Mann-Whitney U test was used. For statistical analysis between 1 group measuring two nominal variables, a paired t-test was used when the data were shown to conform to a Gaussian distribution. Correlations between CD4:CD8 ratios and other prognostic markers were determined using a Fisher's exact test or Chi-squared test. Time to first treatment and progression-free survival were calculated from the patients' date of diagnosis and curves constructed using Kaplan and Meier methodology.

CHAPTER 3

Immunophenotypic characterisation of T-cells from CLL patients

CLL is a disease characterised by profound immune dysfunction whereby CLL cells exert immunosuppressive mechanisms in order to avoid immune recognition (Ramsay and Gribben 2009). Furthermore, the relative failure to mount effective immune responses in CLL patients likely contributes to the pathogenesis of the disease including the progressive accumulation of tumour cells and the increased susceptibility to infection (Ravandi and O'Brien 2006). As yet the mechanisms behind CLL cell subversion of immune effector cells in particular T-cells, has not fully been characterised.

Flow cytometry is an established tool in the diagnosis, characterisation and monitoring of haematological malignancies and in viral infections including HIV (Barnett et al. 2008, Craig and Foon 2008). The objectives of this chapter were to use multi-colour flow cytometry to undertake phenotypic analysis of T-cells from the peripheral blood of 50 CLL patients, in order to identify any disease-associated T-cell abnormalities. Data generated from the analysis of these 50 CLL patients was subsequently pooled with a series generated by Dr Claudia Nunes, to obtain a cohort of over 100 CLL patients and a smaller cohort of 22 healthy age-matched normal controls.

There is growing evidence to suggest T-cells play a key role in CLL pathogenesis. Increased numbers of activated T-cells have been documented in CLL, particularly in those patients with advanced disease (Tötterman et al. 1989). Furthermore, a recent report showed that the telomere length of T-cells was shortened in advanced stage CLL patients suggesting

that T-cells may indeed be proliferating in response to the growth of the CLL clone (Röth et al. 2008). In this study, an in-depth retrospective analysis was performed on T-cells using immunophenotyping data collected from over 100 CLL patients with the aim of identifying poor prognostic patients based on T-cell abnormalities. The identification of T-cell abnormalities which affect prognosis may be crucial in directing the development of future therapies in CLL.

3.1 Lymphocyte populations in CLL patients

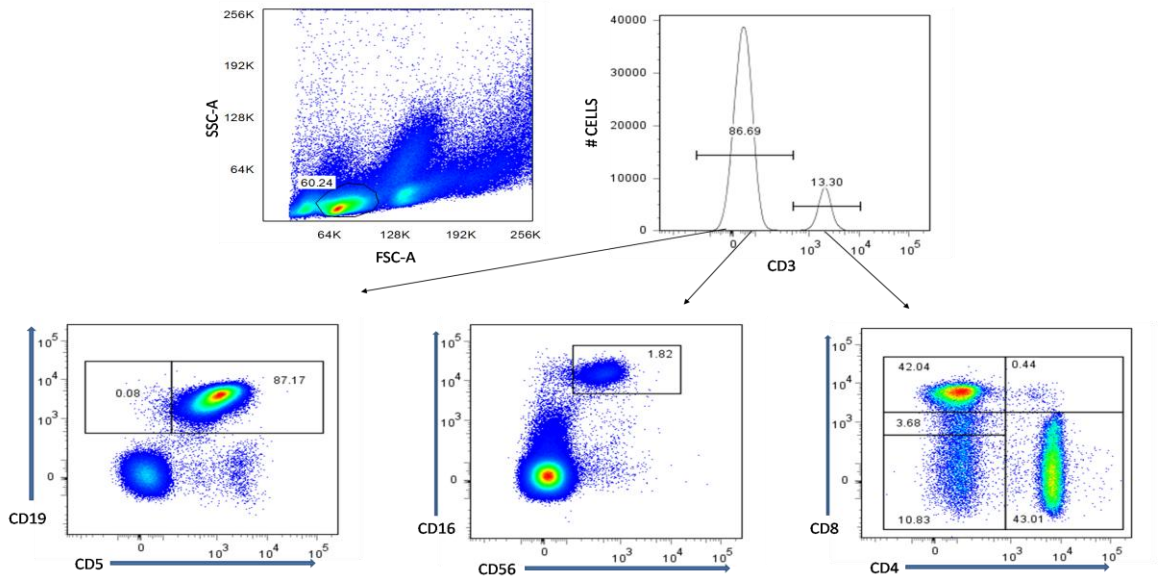
To confirm the findings of previous studies, the frequency of lymphocyte populations including $CD19^+CD5^+$ B-cells, T-cells ($CD4^+$ and $CD8^+$) and NK cells were enumerated in the peripheral blood of CLL patients and healthy age-matched donors (Figure 3.1 and 3.2). As expected the frequency of $CD19^+CD5^+$B-cells were significantly increased in the blood of CLL patients, when compared to healthy controls (Mean: 77.2% vs. 4.4%, $P<0.0001$). In contrast the percentage of $CD3^+$ T-cells was decreased in CLL patient lymphocytes compared to control samples (Mean: 12.1% vs. 61.1%, $P<0.0001$). Within the $CD3^+$ T-cell compartment, CLL patients had a significantly lower percentage of $CD4^+$ T-cells when compared to healthy donors (Mean 40.3% vs. 57.2%, $P=0.002$). Conversely the percentage of $CD8^+$ T-cells in the CLL cohort was significantly higher than the healthy donors (Mean 41.6% vs. 30.9%, $P=0.03$). Measurement of absolute T-cell counts for 50 CLL patients and 10 healthy donors revealed increased numbers of $CD4^+$ and $CD8^+$ in CLL patients (Figure 3.11).

NK cells are another population of lymphocytes capable of recognising and targeting tumour cells. Analysis of the NK population in CLL patients showed that there was a

significantly lower percentage of NK cells within the CLL samples compared to the healthy control group (Mean 1.5% vs. 6.7%, $P<0.0001$).

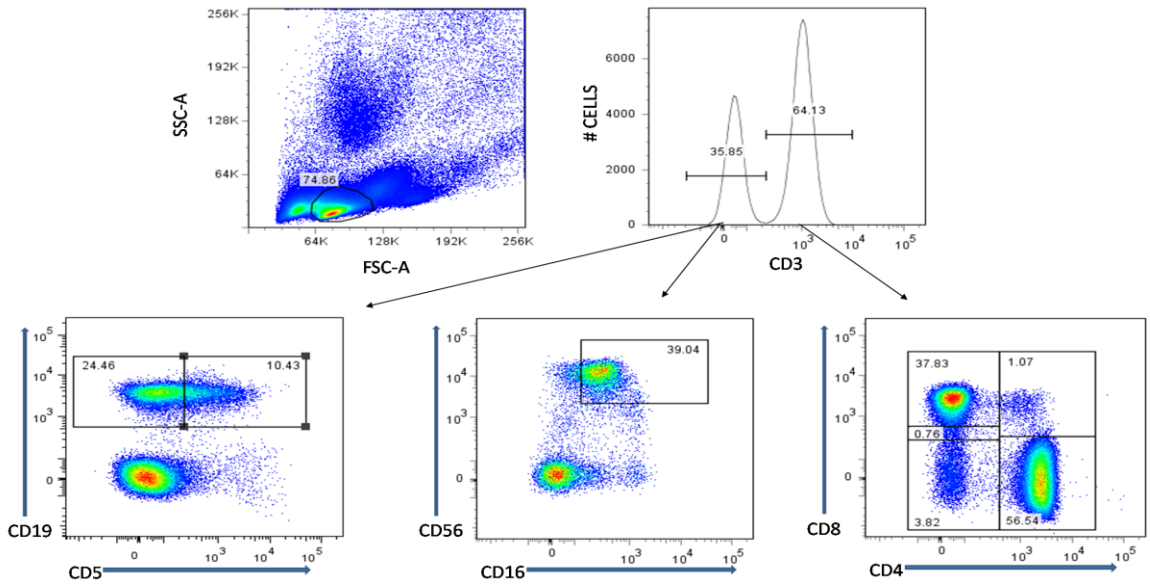
Overall these results show that $CD19^{+}CD5^{+}$ B-cells dominate the lymphocyte population in CLL patients. In addition, they show that the frequency of both NK cells and $CD3^{+}$ T-cells are significantly reduced in CLL patients with a trend towards increased $CD8^{+}$ T-cells and reduced $CD4^{+}$ T-cells.

(A) CLL PATIENT



B-cells NK cells $CD4^+$ and $CD8^+$ T-cells

(B) HEALTHY DONOR



B-cells NK cells $CD4^+$ and $CD8^+$ T-cells

Figure 3.1. Gating strategy for the identification of B, T and NK cells from CLL patients (A) and healthy age-matched controls (B) The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter and separated on the cell surface expression of CD3. Within the CD3 negative population both $CD19^+CD5^+$ B-cells and $CD56^+CD16^+$ were defined. In the CD3 positive population both $CD4^+$ and $CD8^+$ T-cells were gated.

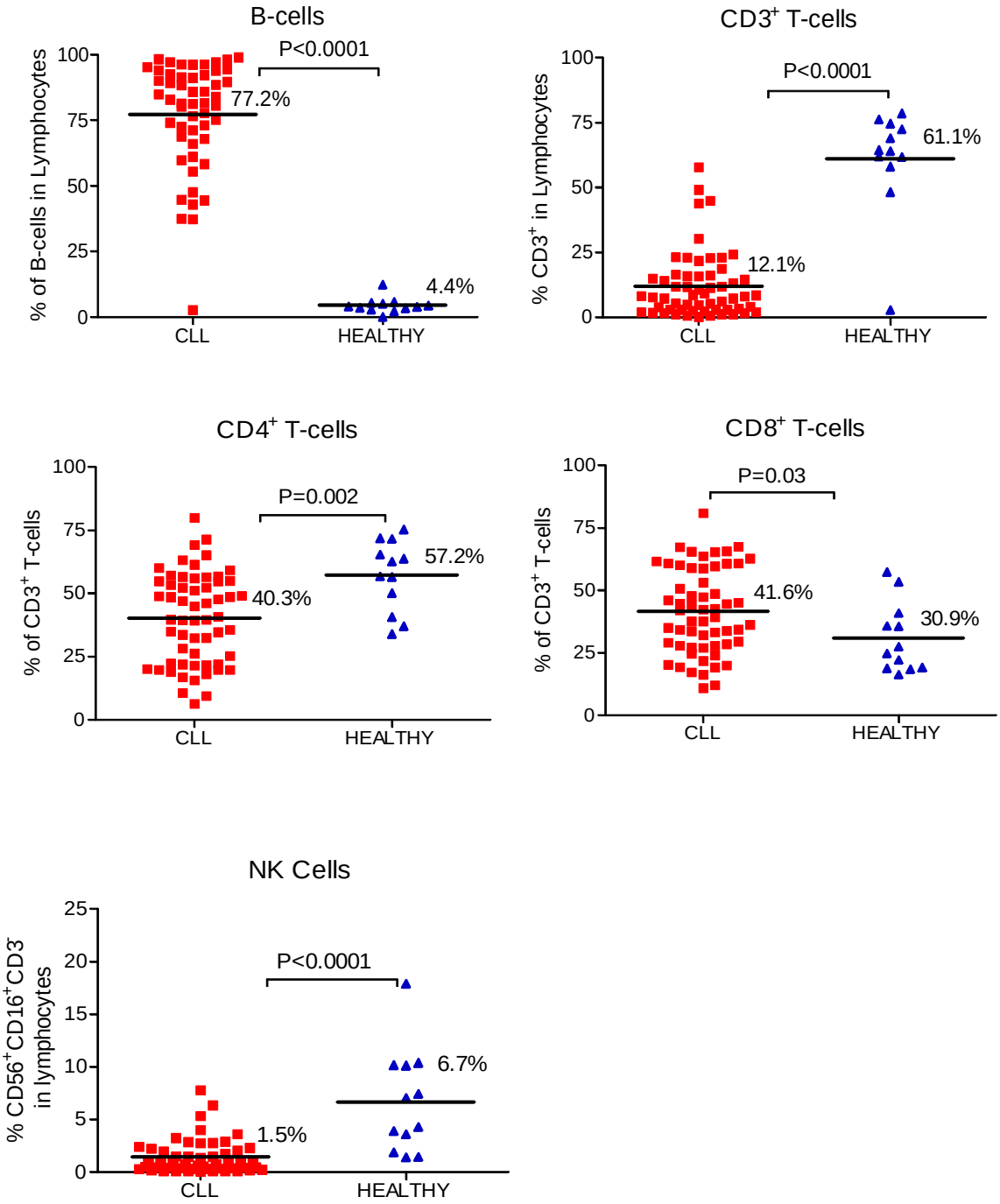


Figure 3.2. The frequency of peripheral blood lymphocyte populations in CLL patients (n=51-58) and healthy donors (n=12). The number of positive cells was measured using 8-colour flow cytometry (FAC Canto II). Lymphocytes were gated based on forward and side scatter profiles. The percentages of $CD19^+CD5^+$ (CLL patients) and $CD19^+$ (Healthy donors) B-cells, $CD3^+$ T-cells and $CD56^+CD16^+CD3^-$ (NK cells) within the lymphocyte gate are shown. $CD4^+$ and $CD8^+$ T-cells are measured within the lymphocyte/CD3 gate. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

3.2 T-cell subsets in CLL patients

In order to gain new insights into why CLL patients respond so poorly to immunological challenge, a comprehensive analysis of $CD4^+$ and $CD8^+$ T-cell subsets was performed comparing healthy donors (age range 44-77 yrs, mean: 64yrs) and CLL patients. In ageing and during persistent viral infections, studies have shown there is a decline in naïve T-cell numbers and a skewing of T-cells towards a memory phenotype with limited T-cell repertoire diversity (Nikolich-Zugich 2008). This process is coupled with the down regulation of the co-stimulatory receptor CD28 on the surface of T-cells and an increase in the expression of CD57, a marker associated with a senescent/exhausted phenotype (Brenchley et al. 2003, Joshi and Kaech 2008). High levels of $CD57^+$ T-cells have been observed in chronic viral infections such as HIV and have been associated with poor prognosis in solid cancers (Weekes et al. 1999, Focosi et al. 2010).

Using flow cytometry, T-cell subsets from CLL patients were separated based on their expression of the chemokine receptor CCR7; responsible for migration of T-cells to the lymph nodes from the peripheral blood and the surface marker CD45RO. T-cells were separated into naïve ($CCR7^+CD45RO^-$), central memory ($CCR7^+CD45RO^+$), effector memory ($CCR7^-CD45RO^+$) and highly differentiated effector cells (EMRA) ($CCR7^-CD45RO^-$). Within each subset CD28 and CD57 expression were analysed to identify cells with an antigen experienced/exhausted phenotype (Figure 3.3).

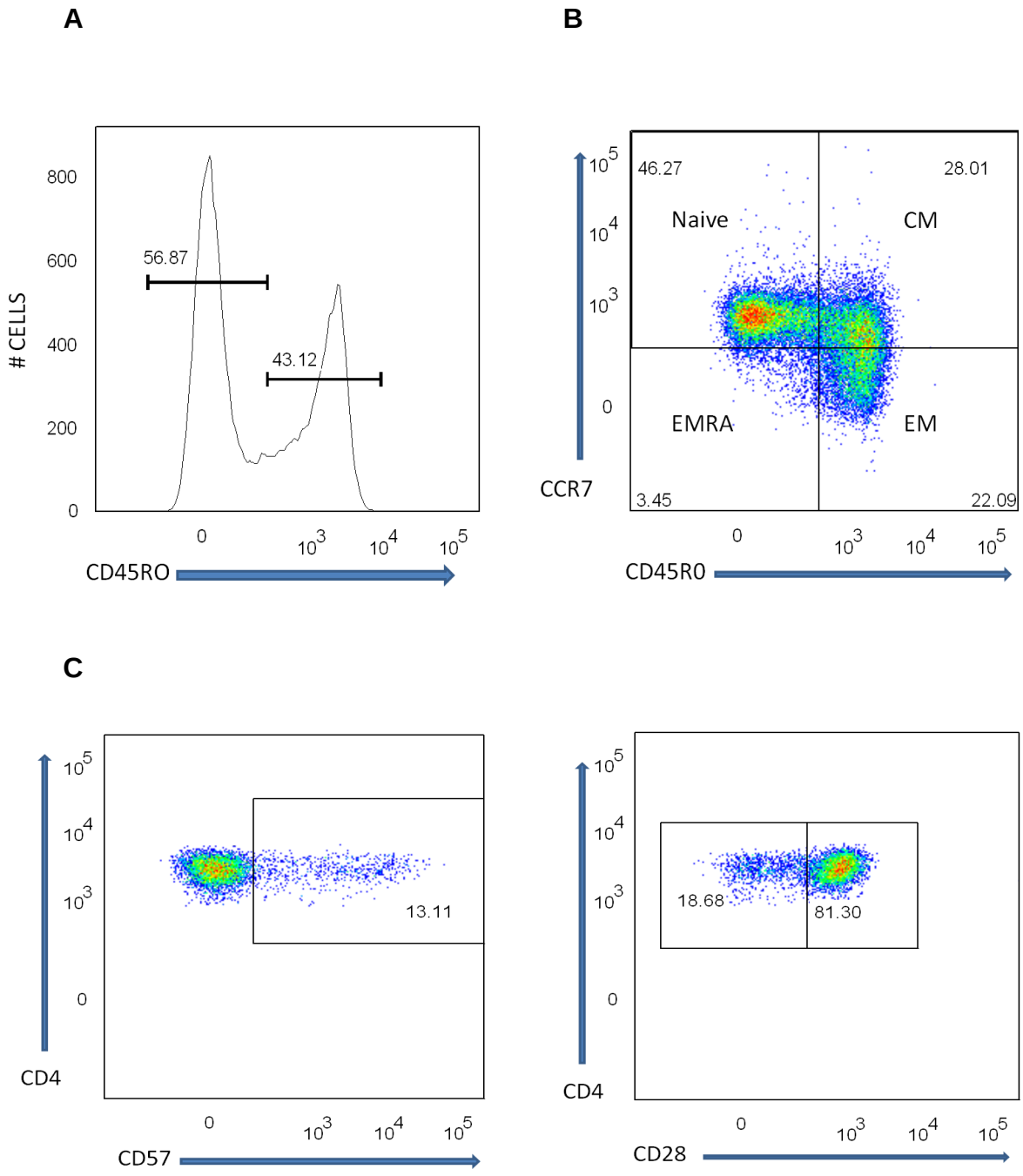


Figure 3.3. Gating strategy for the measurement of CD28 and CD57 expression within CD4$^+$ and CD8$^+$ T-cell subsets. The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. (A) Separation of CD4$^+$ T-cells into CD45RO$^+$ and CD45RO$^-$ subsets (B) Gating of Naïve, Central memory (CM), Effector memory (EM) and EMRA T-cells using both CCR7 and CD45RO markers within the CD4$^+$ T-cell population. (C) Within T-cell subsets the gating strategy for defining CD28$^-$ and CD57$^+$ cells.

3.2.1 $CD8^+$ T-cells

Within the $CD8^+$ population, T-cells were separated based on their expression of CD45RO (Figure 3.4). The $CD45RO^+$ population had a significantly higher percentage of $CD28^-$ (P=0.004) and $CD28^-CD57^+$ (P=0.009) T-cells in CLL patients compared to healthy controls. In contrast, analysis of the $CD45RO^-$ population showed no statistically significantly difference in $CD28^-$ or $CD28^-CD57^+$ T-cells between the CLL and healthy cohorts. Using CCR7 and CD45RO, $CD8^+$ T-cells were further sub-categorised into naïve, CM, EM and EMRA subsets (Figure 3.5). There was a significant decrease in naïve $CD8^+$ T-cells (P=0.004) in CLL patients when compared to healthy donors. The percentage of $CD8^+$ T-cells displaying a CM, EM and EMRA phenotype however were not statistically different in CLL patients compared to controls. Analysis of $CD28^-$ and $CD57^+$ T-cells within each $CD8^+$ T-cell subset showed there was a significant increase in $CD28^-$ (P=0.008) and $CD57^+$ (P=0.004) $CD8^+$ T-cells within the EM compartment of CLL patients compared to healthy donors. Increased percentages of $CD28^-$ and $CD57^+$ T-cells were also seen in the $CD8^+$ EMRA subset but this did not reach significance (Figure 3.6).

These results showed that there is a significant decrease in naïve $CD8^+$ T-cells and a trend towards increased EM $CD8^+$ T-cells in CLL patients compared to healthy-age matched controls. Analysis of $CD8^+$ T-cells revealed a significantly increase in $CD28^-$ and $CD57^+$ within the EM subset of CLL patients a phenotype previously associated with replicative senescence.

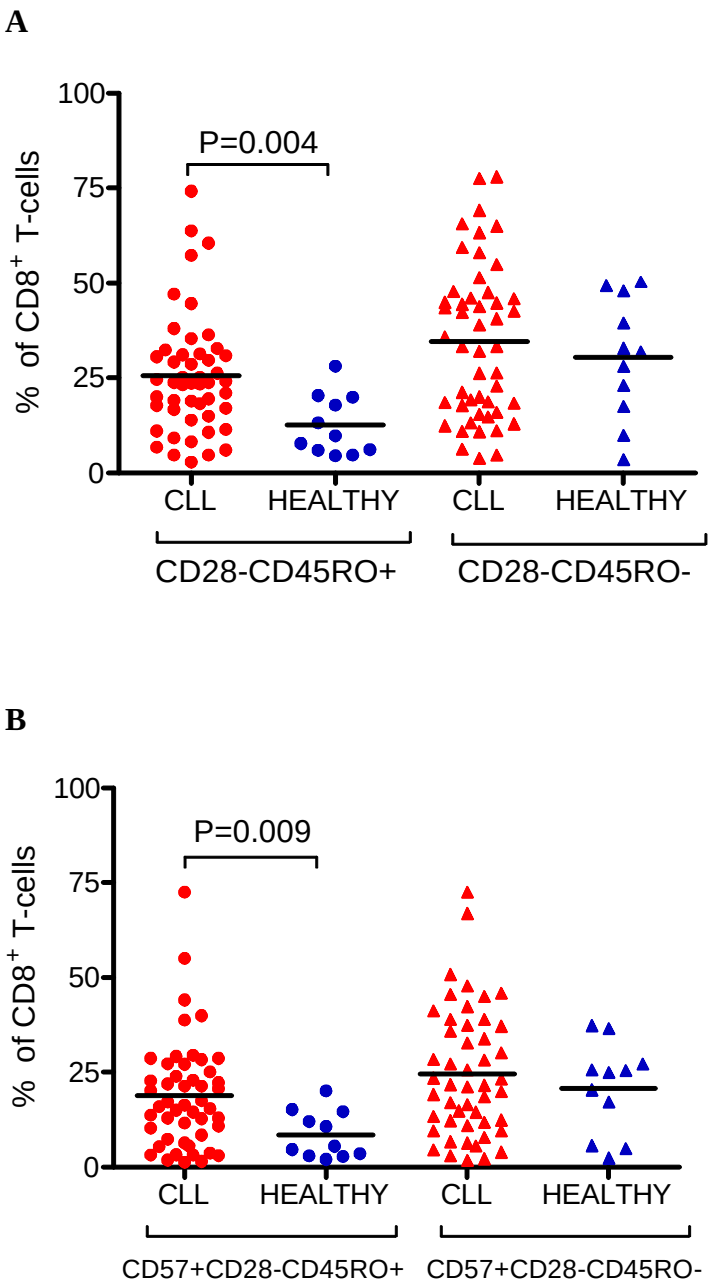


Figure 3.4. The expression of CD57, CD28 and CD45RO in $CD8^+$ T-cells from CLL patients (n= 49) and healthy donors (n=11). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. (A) The percentage of $CD28^-$ cells in the $CD45RO^+$ and $CD45RO^-$ subsets within the $CD8^+$ T-cell gate. (B) The percentage of cells negative for CD28 but positive for CD57 within both the $CD45RO^+$ and $CD45RO^-$ subsets in the $CD8^+$ T-cell gate. The mean within each group is shown. Statistical analysis (Mann-Whitney test) was carried out using GraphPad Prism.

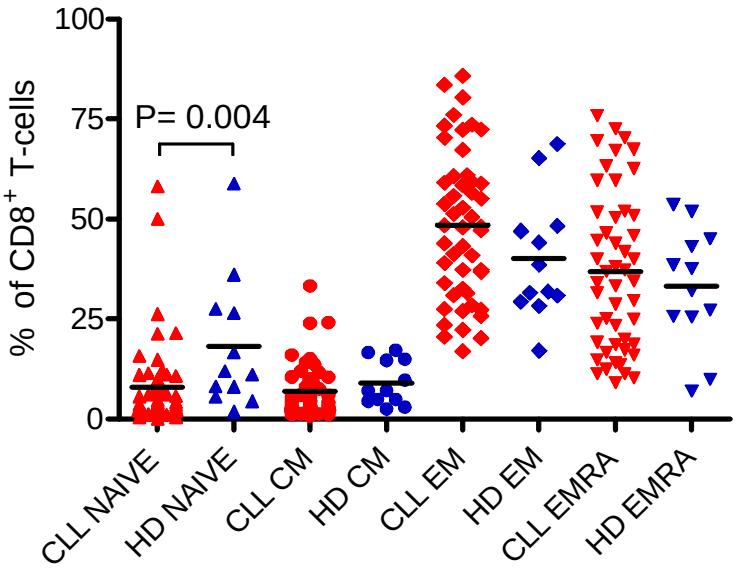
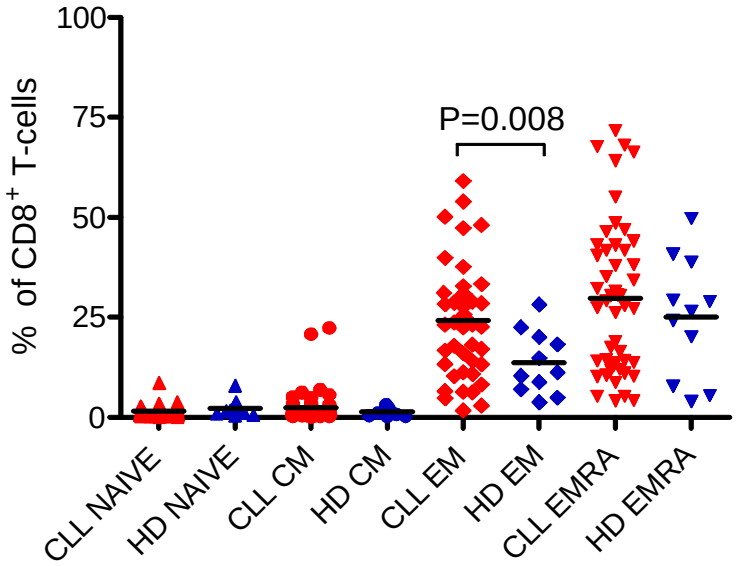


Figure 3.5. The expression of Naïve, Central memory, Effector memory, and Effector (EMRA) $CD8^+$ T-cells in CLL patients (n=49) and healthy donors (n=11). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. $CD8^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO, Naïve ($CCR7^+CD45RO^-$), Central memory (CM) ($CCR7^+CD45RO^+$), Effector memory (EM) ($CCR7^-CD45RO^+$) and Effector (EMRA) ($CCR7^-CD45RO^-$). CLL=CLL Patient, HD=Healthy age-matched donor. The mean within each group is shown. Statistical analysis (Mann-Whitney test) was carried out using GraphPad Prism.

CD28$^-$



CD57$^+$

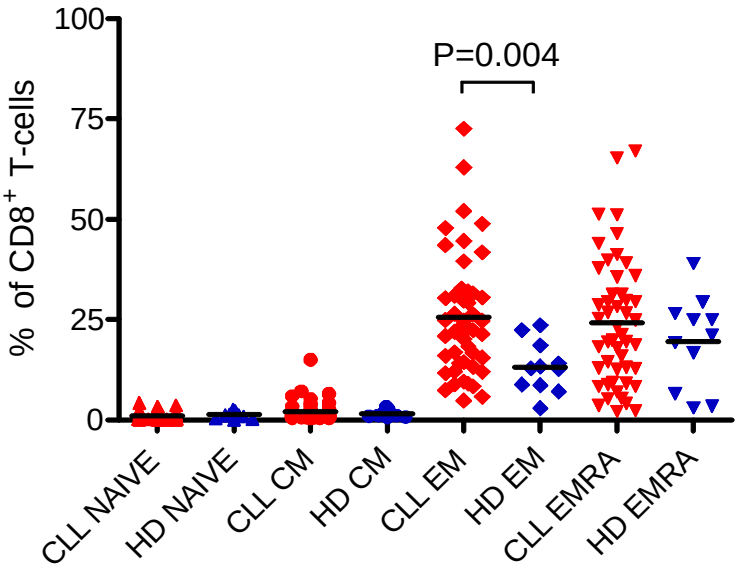


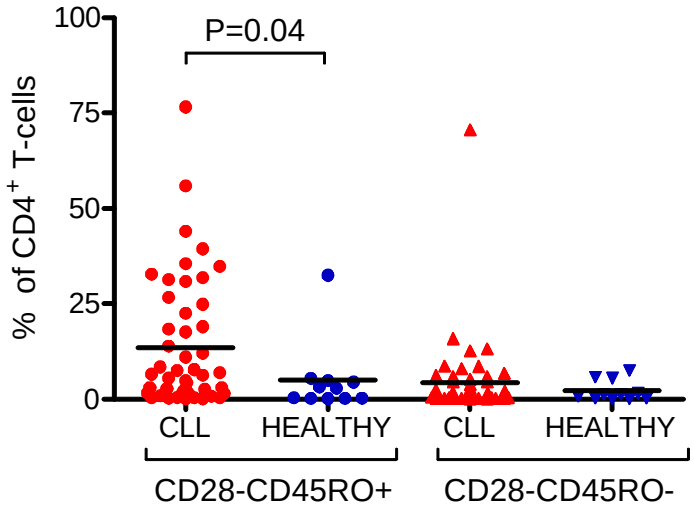
Figure 3.6. The frequency of CD28$^-$ and CD57$^+$ cells within CD8$^+$ T-cell subsets from CLL patients (n=49) and healthy donors (n=11). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. CD8$^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO, Naïve (CCR7$^+$CD45RO$^-$), Central memory (CM) (CCR7$^+$CD45RO$^+$), Effector memory (EM) (CCR7$^-$CD45RO$^+$) and Effector (EMRA) (CCR7$^-$ CD45RO$^-$). CLL=CLL Patient, HD=Healthy age-matched donor. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

3.2.2 $CD4^+$ T-cells

Within the $CD4^+$ T-cell population both $CD45RO^+$ and $CD45RO^-$ subsets were analysed from their expression of CD28 and CD57 (Figure 3.7). There was a significant increase in both $CD45RO^+CD28^-$ (P=0.04) and $CD45RO^+CD28^-CD57^+$ (P =0.04) cells within the $CD4^+$ compartment of CLL patients compared to healthy controls. Analysis of the $CD45RO^-$ $CD4^+$ T-cell compartment showed no significant difference in the percentage of $CD28^-$ or $CD28^-CD57^+$ cells. When the $CD4^+$ T-cells compartment was separated into naïve, CM, EM and EMRA subsets based on CCR7 and CD45RO expression a significant increase in the percentage of EM $CD4^+$ T-cells was observed in CLL patients compared to controls (P=0.04). In contrast a significant decrease in CM $CD4^+$ T-cells was seen in CLL patients (P=0.04, Figure 3.8). Analysis of $CD4^+$ T-cell subset revealed an increase in $CD28^-$ (P=0.02) and $CD57^+$ (P=0.008) cells within the EM $CD4^+$ T-cell compartment of CLL patients compared to healthy controls whilst the other compartments showed no statistically significant differences (Figure 3.9).

These results revealed a significant decrease in CM and a trend towards a decrease in naïve $CD4^+$ T-cells within CLL patients when compared to healthy age-matched controls. In contrast there was a significant increase in EM $CD4^+$ T-cells in CLL patients. In a similar fashion to the $CD8^+$ T-cell compartment, analysis of $CD57^+$ and $CD28^-$ cells revealed a significant increase in the EM compartment of $CD4^+$ T-cells in CLL patients.

A



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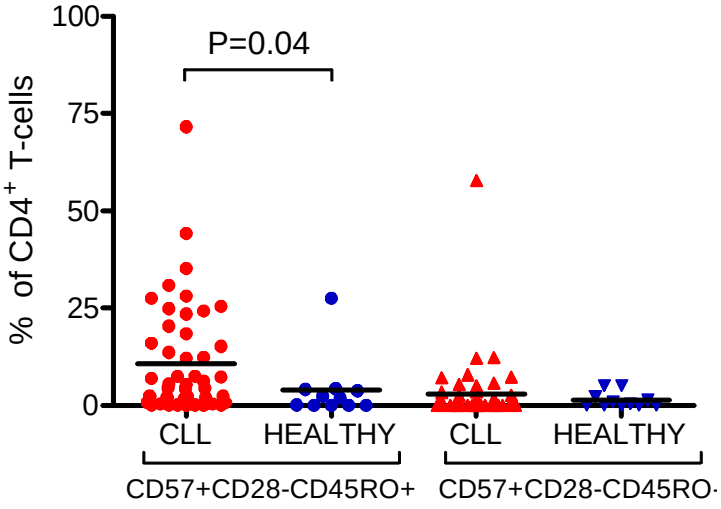


Figure 3.7. The expression of CD57, CD28 and CD45RO in $CD4^+$ T-cells in CLL patients (n= 49) and healthy donors (n=11). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. (A) The percentage of $CD28^-$ cells in the $CD45RO^+$ and $CD45RO^-$ subsets within the $CD4^+$ T-cell gate. (B) The percentage of cells negative for CD28 but positive for CD57 within both the $CD45RO^+$ and $CD45RO^-$ subsets in the $CD4^+$ T-cell gate. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

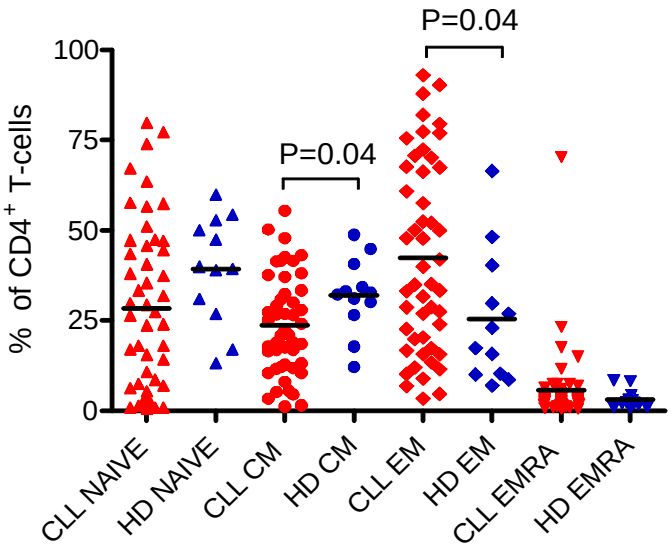
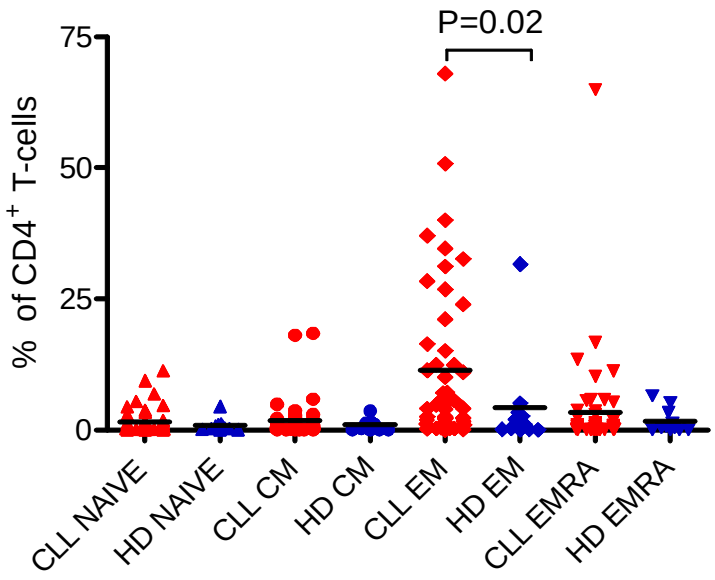


Figure 3.8. The expression of Naïve, Central memory, Effector memory, and Effector (EMRA) $CD4^+$ T-cells in CLL patients (n=49) compared to healthy controls (n=12). The number of positive cells was measured using 8-colour flow cytometry (FAC Canto II). Lymphocytes were gated based on forward and side scatter profiles. $CD8^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO: Naïve ($CCR7^+CD45RO^-$), Central memory (CM) ($CCR7^+$ $CD45RO^+$), Effector memory (EM) ($CCR7^-CD45RO^+$) and Effector (EMRA) ($CCR7^-CD45RO^-$). The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism. CLL=CLL Patient, HD=Healthy age-matched donor.

CD28$^-$



CD57$^+$

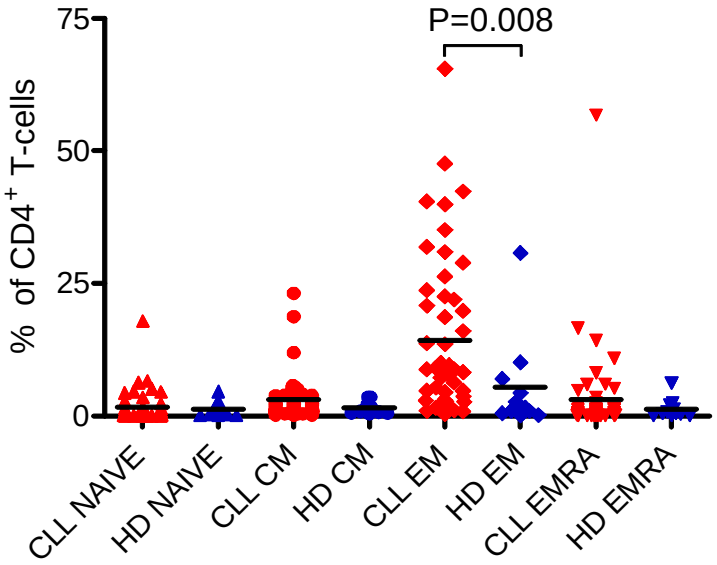


Figure 3.9. The frequency of CD28$^-$ and CD57$^+$ cells within the CD4$^+$ T-cell subsets from CLL patients (n=49) and healthy donors (n=12). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. CD4$^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO, Naïve (CCR7$^+$CD45RO$^-$), Central memory (CM) (CCR7$^+$CD45RO$^+$), Effector memory (EM) (CCR7$^-$CD45RO$^+$) and Effector (EMRA) (CCR7$^-$CD45RO$^-$). CLL= CLL Patient, HD= Healthy age-matched donor. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

3.3 The prognostic significance of T-cell subsets in CLL

Phenotypic markers identified on the malignant B-cell clone have proven highly successful in classifying patients likely to undertake rapid disease progression (Van Bockstaele, Verhasselt and Philippé 2009), yet little is known about the prognostic power of other immune cells including T-cells. In this study retrospective analysis was performed on T-cells using immunophenotyping data collected from over 100 CLL patients with the aim of identifying poor prognostic patients based on the presence of T-cell abnormalities.

3.3.1 Inversion of the CD4:CD8 ratio in a subset of CLL patients

Phenotypic analysis of the $CD3^+$ T-cell populations revealed that 47% of patients had a preferential expansion of $CD8^+$ T-cells compared to the $CD4^+$ T-cells (Figure 3.10A). Therefore the patient cohort was divided into patients with an inverted CD4:CD8 ratio (CLL^{IR}) and normal (non-inverted) ratio (CLL^{NR}) based on a CD4:CD8 ratio threshold of 1.0. This threshold was based on the median CD4:CD8 ratio of the whole patient cohort and also represented the ratio at which the percentage of $CD8^+$ T-cells outnumbered that of the $CD4^+$ T-cells (Figure 3.10B).

Absolute numbers of T-cells were measured in 25 CLL^{IR} and 25 CLL^{NR} patients and 10 healthy age-matched controls (Figure 3.11). Both CLL^{IR} and CLL^{NR} patients had increased numbers of $CD4^+$ and $CD8^+$ T-cells compared to healthy controls. Comparison CLL^{IR} and CLL^{NR} patients showed similar numbers of $CD4^+$ T-cells in both cohorts (CLL^{IR} 1289 vs CLL^{NR} 1317) but increased numbers of $CD8^+$ T-cells in the CLL^{IR} group (CLL^{IR} 1524 vs CLL^{NR} 842).

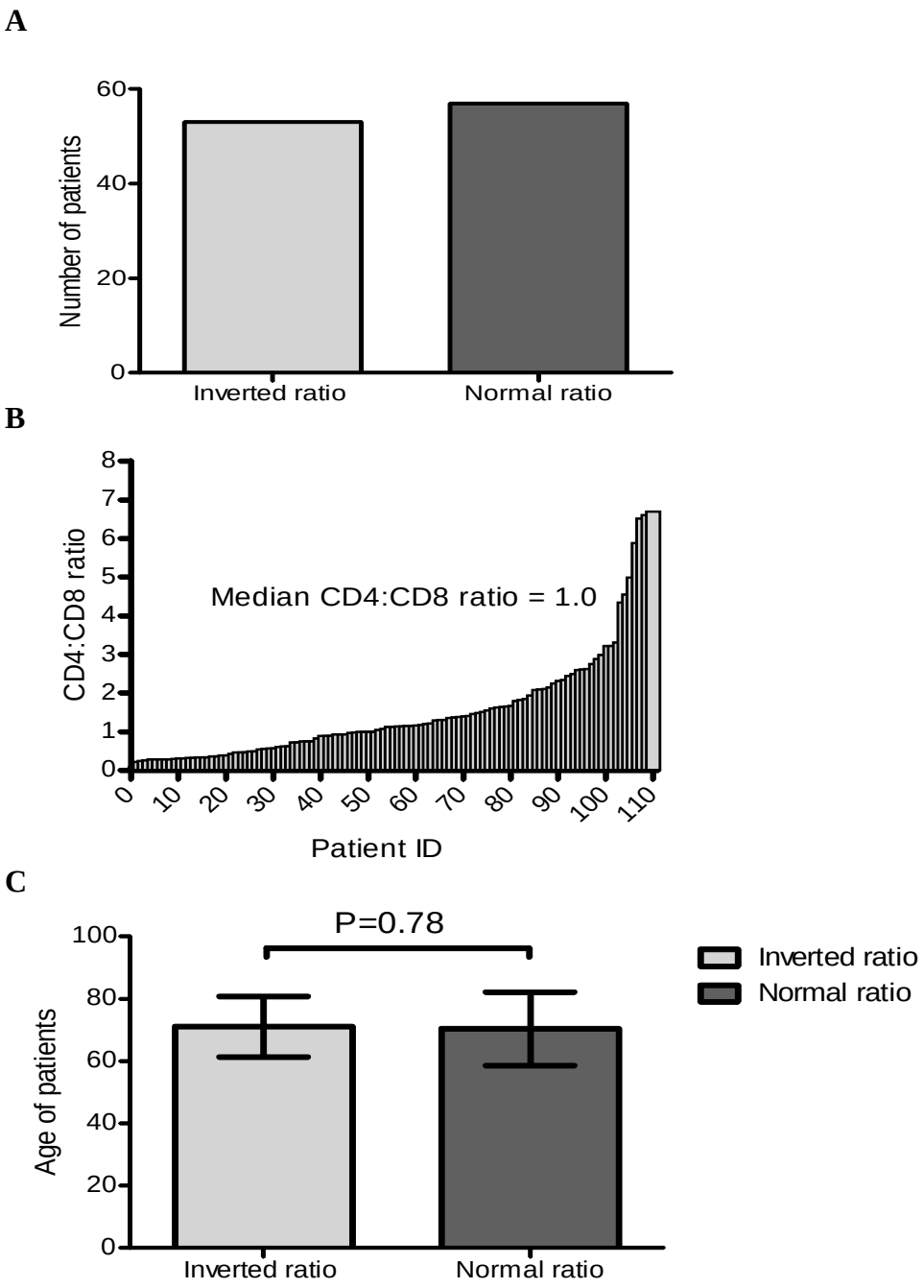


Figure 3.10. The incidence of normal and inverted CD4:CD8 ratios in CLL patients. (A) The number of patients within the cohort that had an inverted ratio (CD4:CD8 <1) and normal ratio (CD4:CD8 ≥1) of $CD4^+$ and $CD8^+$ T-cells. (B) The CD4:CD8 ratio was determined for the cohort of 110 CLL patients with a median ratio of 1.0. (C) The median age of CLL patients in the CLL^{IR} and CLL^{NR} groups. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

T lymphocyte subsets	CLL patient samples		Age-matched healthy donors (n=10)
	Normal CD4:CD8 (n=25)	Inverted CD4:CD8 (n=25)	
$CD4^+$ T-cells in CD3	1317± 564	1289 ± 441	713 ± 342
$CD8^+$ T-cells in CD3	842 ± 332	1524 ± 303	308 ± 183

Figure 3.11. Absolute numbers of $CD4^+$ and $CD8^+$ T-cells from normal and inverted ratio CLL patients and healthy age-matched controls.

3.3.2 Patients with an inversion of the CD4:CD8 ratio have an inferior prognosis

In order to determine whether like other cancers (Markowska et al. 1995, Sheu et al. 1999) the ratio of CD4:CD8 ratio had prognostic significance in CLL, the time to first treatment (TTFT) and progression-free survival of both the CLL^{IR} and CLL^{NR} patient groups were compared. Patients with an inverted ratio of CD4:CD8 were found to have a significantly shorter time to first treatment (P=0.03, Figure 3.11A) and progression-free survival (P= 0.005, Figure 3.11B) when compared to those patients with a normal CD4:CD8 ratio. Importantly the inferior prognosis seen in the CLL^{IR} group was independent of the age of patients as there was no significant difference between the median ages of both groups at the date of testing (Figure 3.10C)

3.3.3 T-cell subset analysis of CLL^{IR} and CLL^{NR} patient groups

In order to rationalise the poor prognosis associated with the inverted CD4:CD8 ratio group, detailed phenotypic analysis of T-cell subsets in both the CLL^{IR} and CLL^{NR} groups was performed. Using the markers CCR7 and CD45RO, T-cell subsets from both the CLL^{IR} and CLL^{NR} group were separated into naïve ($CCR7^{+}CD45RO^{-}$), central memory ($CCR7^{+}CD45RO^{+}$), effector memory ($CCR7^{-}CD45RO^{+}$) and highly differentiated effector cells (EMRA) ($CCR7^{-}CD45RO^{-}$). An increased frequency of $CD57^{+}CD27^{-}CD28^{-}$ T-cells has been shown during normal aging and in individuals with chronic viral infections and cancer (Merino et al. 1998). These $CD57^{+}CD27^{-}CD28^{-}$ T-cells have also been associated with a replicative senescence phenotype with an enhanced tendency to undergo apoptosis (Brenchley et al. 2003, Van den Hove et al. 1998). Therefore differences in the frequency of these $CD57^{+}CD27^{-}CD28^{-}$ T-cells within memory subsets of T-cells from CLL^{IR} and CLL^{NR} patient groups were examined, as well as for healthy age-matched donors.

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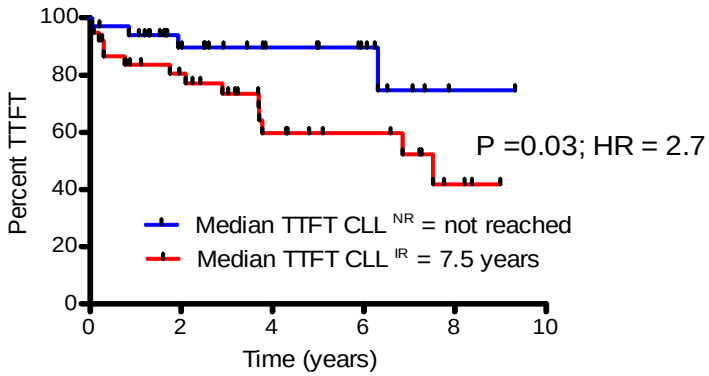

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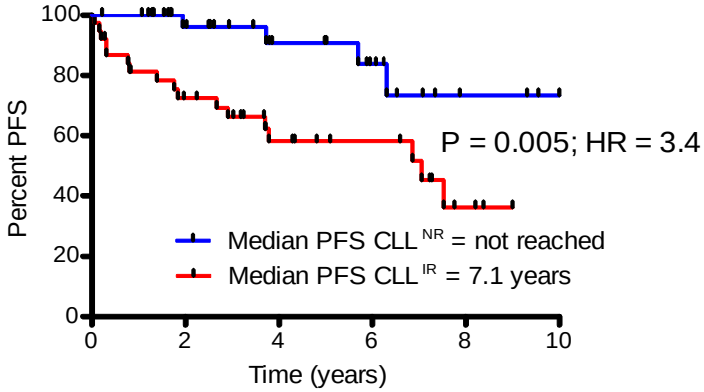
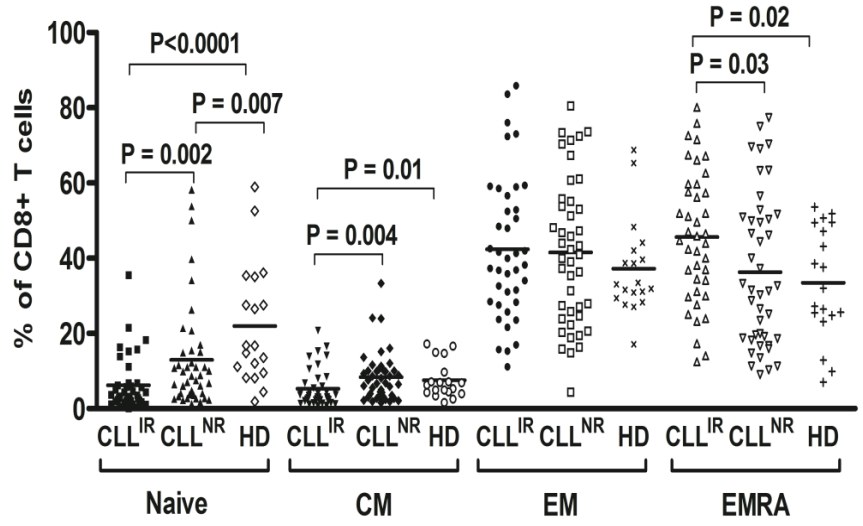


Figure 3.12. Kaplan-Meier curves for time to first treatment and progression-free survival for CLL^{IR} (n= 41) and CLL^{NR} patients (n=43). Kaplan-Meier analysis was used to assess the effect of a CLL patient's CD4:CD8 ratio on (A) time to first treatment (TTFT) and (B) progression-free survival (PFS). A log-rank test was used to test the difference between the two curves. (HR=Hazard ratio)

A



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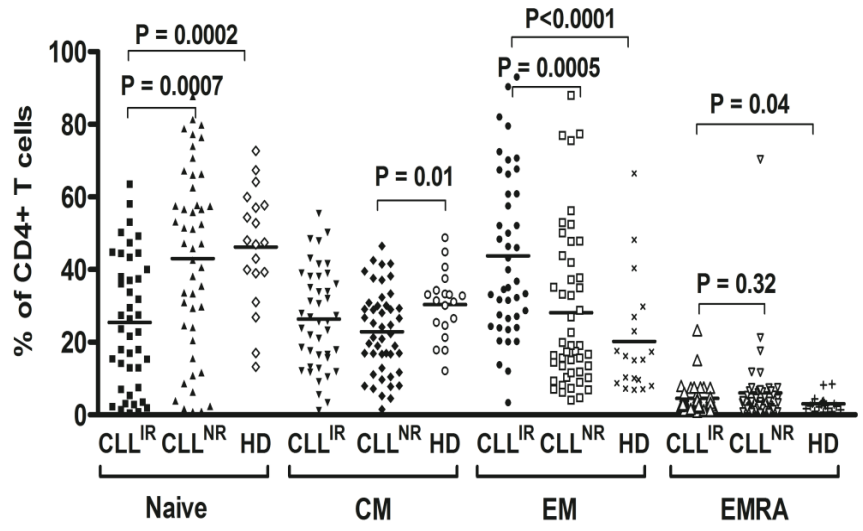


Figure 3.13. The percentage of Naïve, CM, EM and EMRA within the (A) $CD8^+$ and (B) $CD4^+$ T-cells populations of CLL^{IR} (n=43), CLL^{NR}(n=43) and healthy donors (n=19).
The number of positive cells was measured using 8-colour flow cytometry (FACA Canto II). Lymphocytes were gated based on forward and side scatter profiles. $CD8^+$ and $CD4^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO: Naïve ($CCR7^+CD45RO^-$), Central memory (CM) ($CCR7^+$ $CD45RO^+$), Effector memory (EM) ($CCR7^-CD45RO^+$) and Effector (EMRA) ($CCR7^-CD45RO^-$). The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism. CLL^{IR} = CLL Patient (CD4:CD8 ratio <1) CLL^{NR} = CLL patient (CD4:CD8 ratio ≥1) and HD = Healthy age-matched donor.

3.3.4 Patients with an inverted CD4:CD8 ratio have an expansion of $CD8^+$ T-cells with a highly differentiated effector phenotype

Analysis of T-cell subsets revealed a significant decrease in both naïve (P=0.002) and CM (P=0.004) $CD8^+$ T-cells in the CLL^{IR} group compared to the CLL^{NR} group. In contrast, there was a significant increase in EMRA $CD8^+$ T-cells in the CLL^{IR} group (P=0.03). Within the $CD4^+$ T-cell population there was also a significant decrease in naïve T-cells (P=0.0007) with a concomitant increase in EM T-cells (P=0.0005) but not EMRA T-cells (P=0.32) within the CLL^{IR} group in comparison to the CLL^{NR} group (Figure 3.13).

Overall these results identified a preferential decrease of naïve $CD4^+$ and $CD8^+$ T-cells in the CLL^{IR} group in comparison to the CLL^{NR} group. This change in naïve T-cells was associated with a significant increase in EMRA $CD8^+$ T-cells and EM $CD4^+$ T-cells in the CLL^{IR} group.

3.3.5 Increased frequency of $CD57^+$ $CD27^-CD28^-$ T-cells within the CLL^{IR} patient group

Within the $CD8^+$ T-cell compartment $CD57^+CD27^-CD28^-$ T-cells were significantly increased in the EM (P=0.019) subset and progressing towards significance in the EMRA subset (P=0.06) of CLL patients compared to healthy controls (Figure 3.14A). When the CLL patient cohort was separated into the CLL^{IR} and CLL^{NR} groups, significantly higher percentages of $CD8^+CD57^+CD27^-CD28^-$ T-cells were seen in the EM (P=0.0005) and EMRA (P=0.002) subsets in the CLL^{IR} compared to healthy donors. No significant difference in $CD8^+CD57^+CD27^-CD28^-$ T-cells was seen between the CLL^{NR} group and healthy group in both the EM and EMRA subsets (Figure 3.14B). The presence of $CD57^+CD27^-CD28^-$ T-cells was also assessed in the EM subset within $CD4^+$ T-cell population. There was a significant

increase in $CD4^+CD57^+CD27^-CD28^-$ T-cells within the EM subset from CLL patients compared to healthy controls (Figure 3.14C). When assessing both the CLL^{IR} and CLL^{NR} groups compared to healthy controls, only the CLL^{IR} patient group had a significantly higher percentage of $CD4^+CD57^+CD27^-CD28^-$ T-cells in the EM subset (P=0.01, Figure 3.14D). Due to the low frequency of $CD4^+$ T-cells displaying an EMRA phenotype in CLL patients, no comparative analysis was made of this population between the CLL^{IR} and CLL^{NR} groups.

Overall these results show an increase in $CD57^+CD27^-CD28^-$ T-cells within the EM/ EMRA $CD8^+$ T-cell compartment and EM $CD4^+$ T-cell compartment of CLL patients compared to healthy donors. These $CD57^+CD27^-CD28^-$ T-cells were preferential expanded in CLL patients from the CLL^{IR} group compared to the CLL^{NR} group.

3.3.6 $CD8^+CD57^+$ $CD27^-CD28^-$ T-cell expansions in the CLL^{IR} group are independent of patient CMV serostatus

A previous study reported the expansion of CMV-specific $CD8^+CD45RA^+CD27^-$ T-cells in CMV-seropositive CLL patients (Mackus et al. 2003). In order to determine whether CMV infection could be driving the $CD8^+CD57^+$ $CD27^-CD28^-$ T-cell expansions in the CLL^{IR} group, the CMV serostatus of 20 CLL^{IR} and 20 CLL^{NR} patients was determined by the local Health Laboratory Service, Cardiff, Wales. 17 out of 20 CLL^{IR} patients and 15 out of 20 CLL^{NR} patients were seropositive for CMV. Separation of CLL patients based on CMV serostatus revealed no significant differences in the percentage of $CD8^+$ T-cells or $CD57^+CD27^-CD28^-$ T-cells between both groups (Figure 3.14E).

These results suggest that CMV cannot be solely responsible for the inversion of the CD4:CD8 ratio or the increase in $CD57^{+}CD28^{-}CD27^{-}$ EM and EMRA T-cells observed in the CLL^{IR} group.

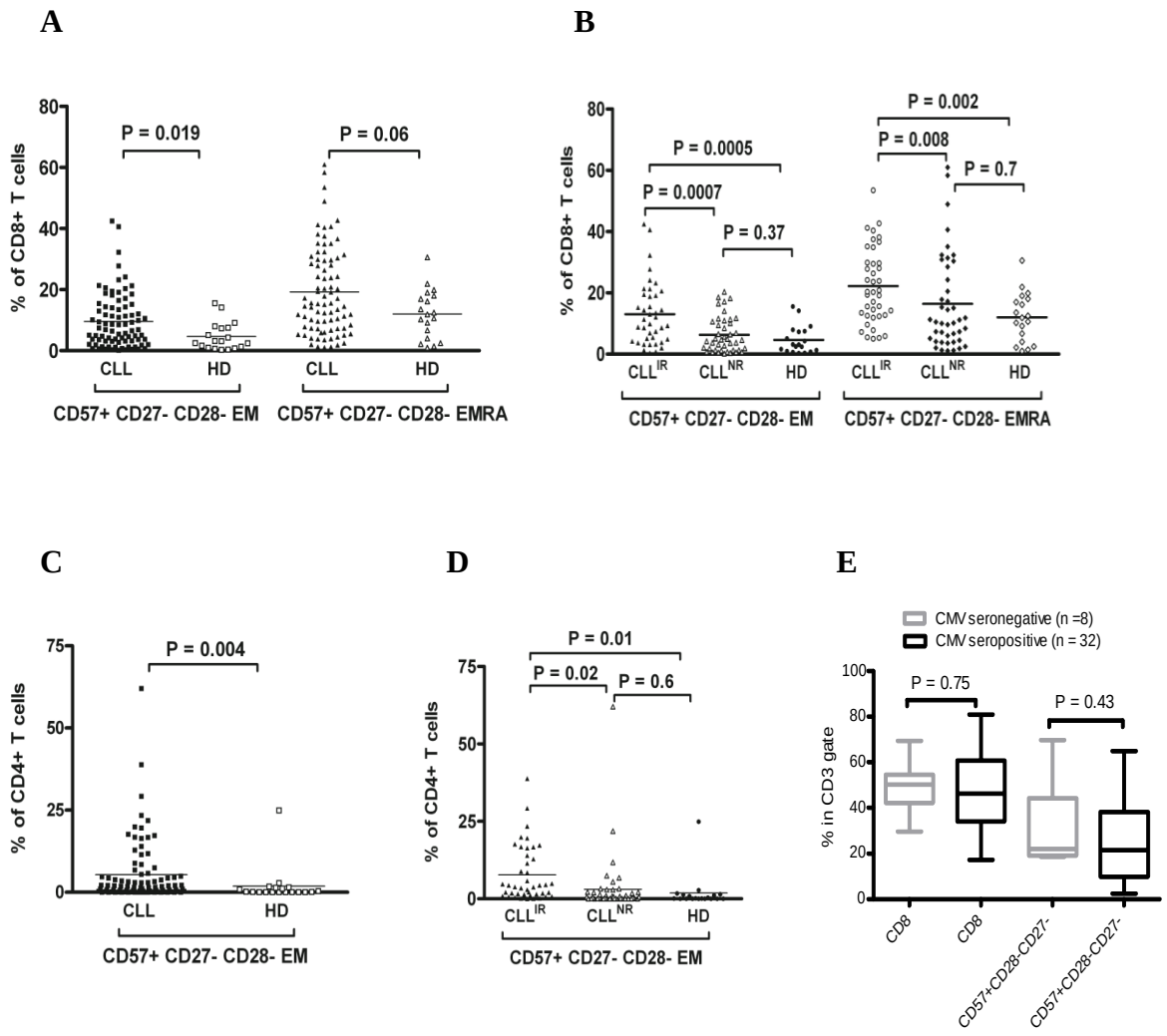


Figure 3.14. Expression of $CD57^{+}CD27^{-}CD28^{-}$ cells within the EM and EMRA T-cells from CLL^{IR} (n=40), CLL^{NR} (n=43) and healthy donors (n=19). (A) Percentage of $CD57^{+}CD27^{-}CD28^{-}$ EM and EMRA $CD8^{+}$ T-cells of the CLL patient cohort and age-matched healthy donors. (B) Percentage of $CD57^{+}CD27^{-}CD28^{-}$ EM and EMRA $CD8^{+}$ T-cells in the CLL^{IR}, CLL^{NR} patient cohorts and healthy donors. (C) Percentage of $CD57^{+}CD27^{-}CD28^{-}$ EM $CD4^{+}$ T-cells in CLL patients and healthy donors. (D) Percentage of $CD57^{+}CD27^{-}CD28^{-}$ EM in the CLL^{IR}, CLL^{NR} patient cohorts and healthy donors. The EMRA subset was not included for $CD4^{+}$ T-cells due to the low prevalence of this subset within the total $CD4^{+}$ T-cell subset. (E) The effect of CMV serostatus on the percentage of $CD8^{+}$ T-cells or the percentage of $CD57^{+}CD27^{-}CD28^{-}$ T-cells in the CLL patient cohort. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

3.3.7 Inversion of the CD4:CD8 ratio associated with increased $CD8^+PD\text{-}1^+$ expression

PD-1 is a negative regulator of TCR signalling which, like $CD57^+$ has been associated with replicative senescence (Miles et al. 2010, D'Souza et al. 2007). The expression of PD-1 in 77 out of 110 (70%) patients was investigated with the aim of determining whether this marker was differentially expressed between the CLL^{IR} and CLL^{NR} groups. There was a significant increase in the percentage of PD-1 expressing $CD8^+$ T-cells in the CLL^{IR} group when compared to the CLL^{NR} group (P=0.027, Figure 3.15A). This increase in PD-1 expression within the $CD8^+$ T-cell compartment was found to be independent of CMV serostatus (Figure 3.15B).

These results suggest that there is a preferential expansion of $PD\text{-}1^+$ $CD8^+$ T-cells within the CLL^{IR} group which is independent of patient CMV serostatus.

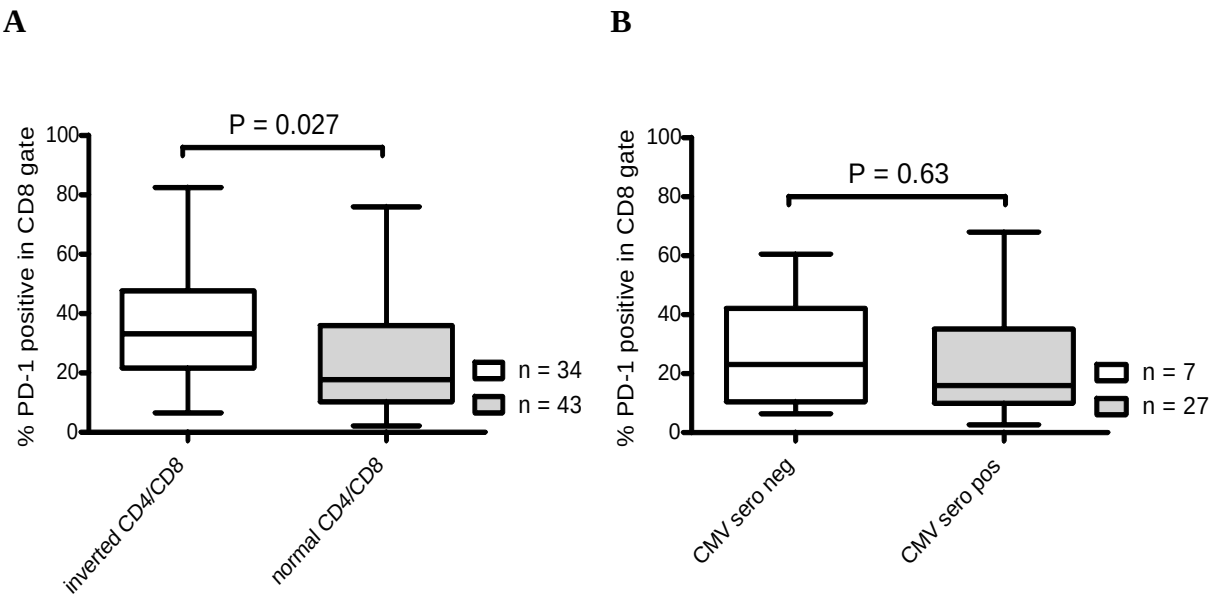


Figure 3.15. PD-1$^+$ expression on CD8$^+$ T-cells from CLL patients. (A) The percentage of CD8$^+$PD-1$^+$ T-cells in CLL patients with an inverted CD4:CD8 ratio and those with a normal CD4:CD8 ratio. (B) The percentage of CD8$^+$PD-1$^+$ T-cells in CMV-seronegative and CMV-seropositive CLL patient groups. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

3.3.8 CLL^{IR} is not associated with the percentage of regulatory T-cells within the CD4$^+$ compartment

A recent study showed an association between high levels of Tregs and shorter time to first treatment in CLL (Weiss et al. 2011). This study therefore looked at the frequency of regulatory T-cells within the CLL^{IR} group in order to determine if this could be responsible for the poor prognosis of this group (Figure 3.16). Within the total CLL cohort there was a significant increase in Tregs compared to healthy age matched controls (P=0.02, Fig. 3.16). However, upon separating the CLL patient cohort into CLL^{IR} and CLL^{NR} groups, there was no significant difference found in the percentage of Tregs between the CLL^{IR} group and healthy age-matched controls (P=0.07).

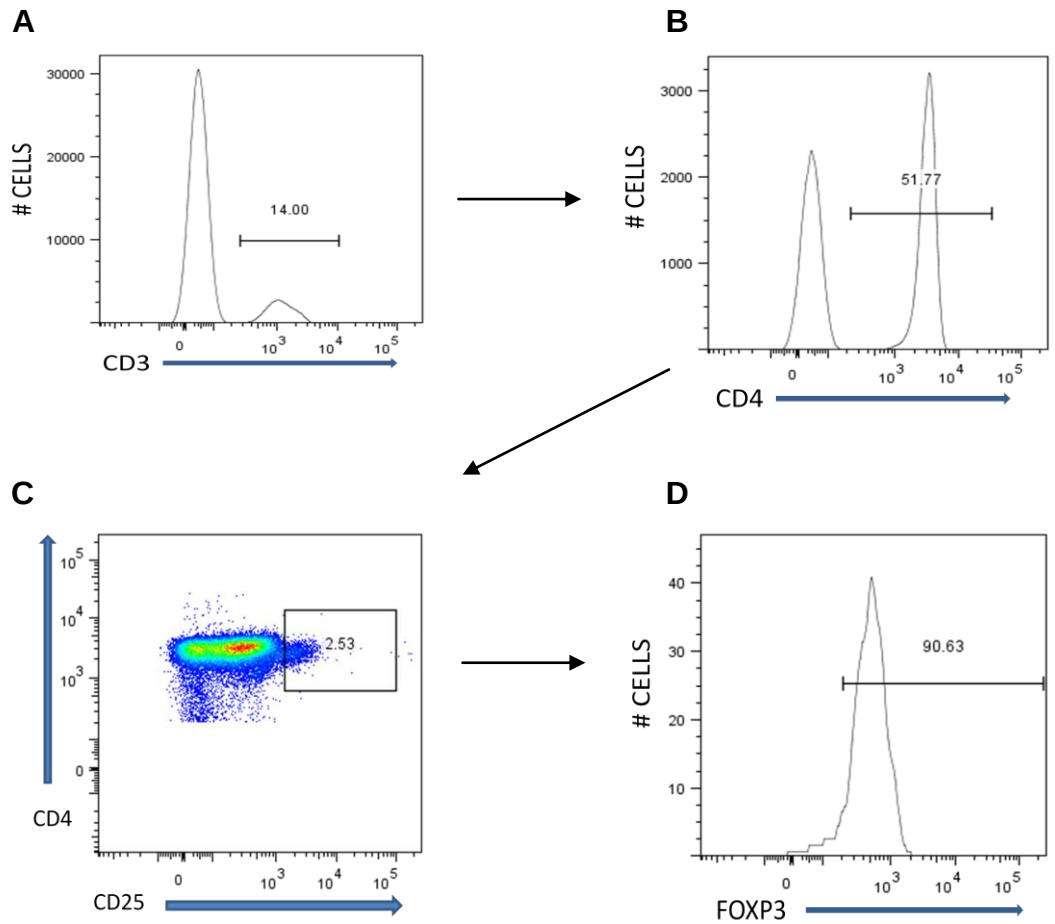


Figure 3.16. Gating strategy for the detection of $CD4^{+}CD25^{HIGH}FOXP3^{+}$ regulatory T-cells from the blood of CLL patients. The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. (A) $CD3^{+}$ cells were gated within the lymphocyte population, (B) $CD4^{+}$ cells within $CD3^{+}$ gate, (C) Gating of $CD4^{+}CD25^{HIGH}$ cells within $CD4^{+}$ gate and (D) $FOXP3^{+}$ cells within $CD4^{+}CD25^{HIGH}$ gate.

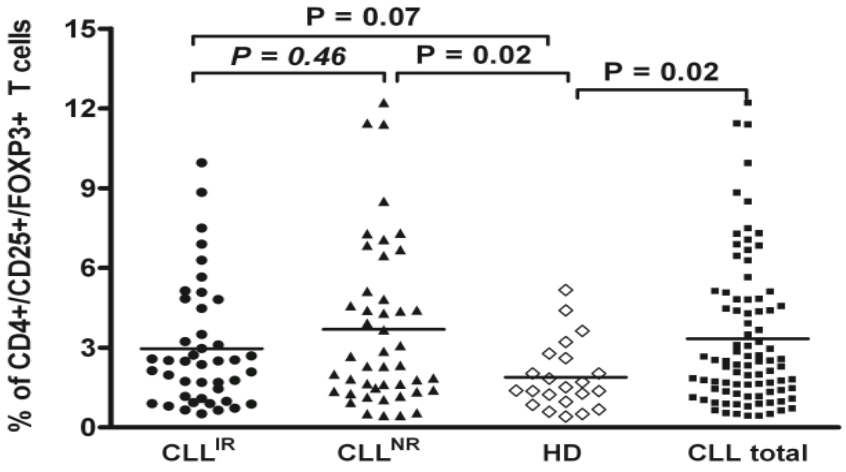


Figure 3.17. The frequency of $CD4^+CD25^{HIGH}FOXP3^+$ regulatory T-cells within the whole CLL patient cohort, the CLL^{IR} and CLL^{NR} subsets and healthy age-matched donors. Treg percentages were measured within the $CD4^+$ T-cell population of CLL patients and healthy age-matched donors. The mean within each group is shown. Statistical analysis (Mann-Whitney test) was carried out using GraphPad Prism. CLL^{IR} = CLL patient (CD4:CD8 ratio <1) CLL^{NR} = CLL patient (CD4:CD8 ratio ≥1) and HD = Healthy age-matched donor.

3.3.9 CLL^{IR} is not associated with tumour cell phenotype or Binet stage at diagnosis

In order to assess the prognostic significance of the inverted CD4:CD8 ratio further, CLL^{IR} and CLL^{NR} groups were analysed for their expression of established prognostic markers in CLL (Figure 3.18). This study found no significant difference in CD38 (P=0.8), ZAP-70 expression (P=0.15), *IGHV* mutational status (P=0.55) or Binet Stage (P=0.51) between the CLL^{IR} and CLL^{NR} groups. There was however a significant association between the inversion of the CD4:CD8 ratio and a shorter lymphocyte doubling time (LDT) (P=0.03).

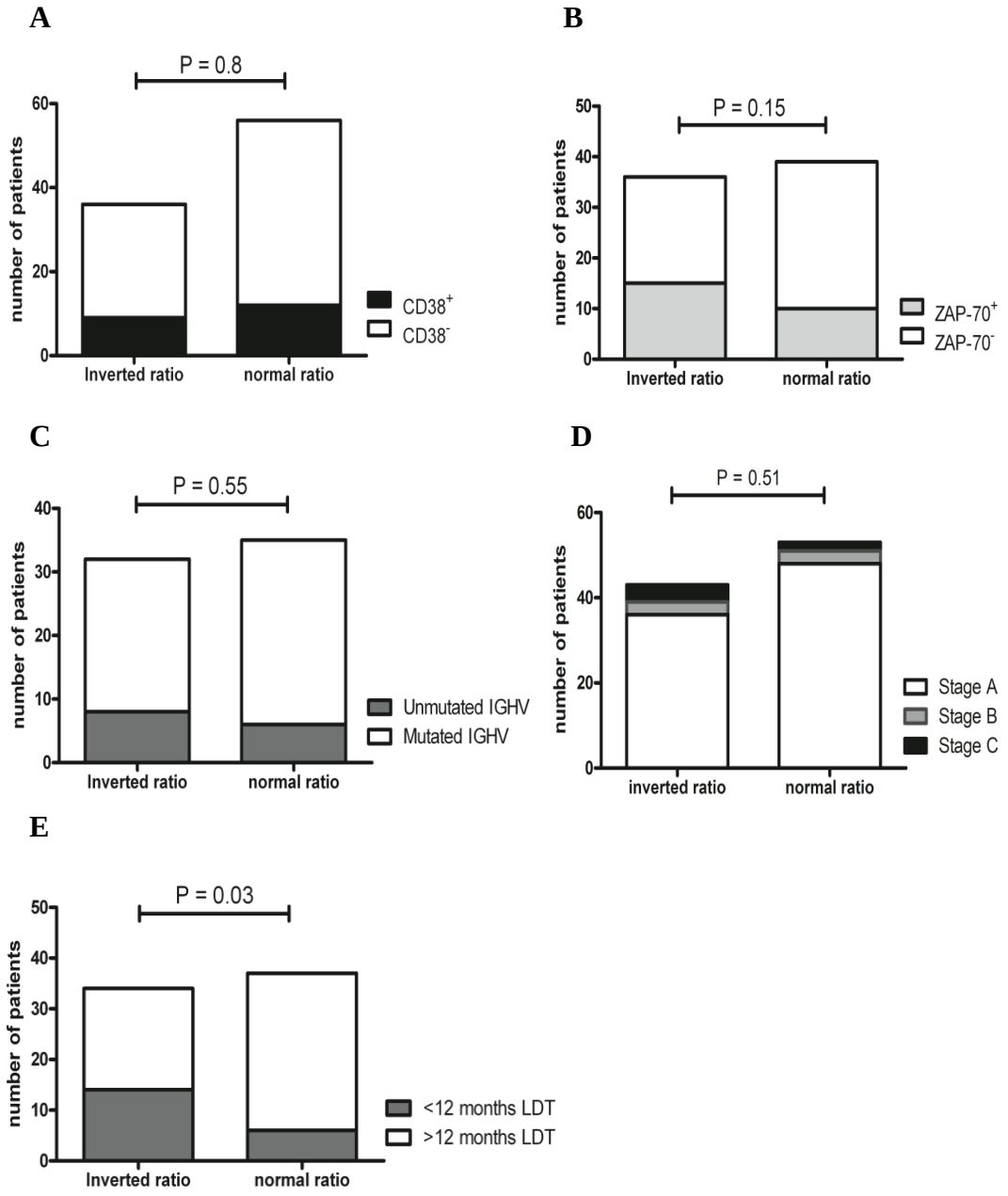


Figure 3.18. Correlation of CD4:CD8 ratio and CD38 expression, ZAP-70 expression, *IGHV* gene mutational status, Binet stage at diagnosis and lymphocyte doubling time. (A), percentage of CD38 expression in the CLL^{IR}, CLL^{NR} patients. (B), percentage of ZAP-70 expression in CLL^{IR}, CLL^{NR} patients. (C), percentage unmutated and mutated *IGHV* genes in CLL^{IR}, CLL^{NR} patients. (D), number of CLL^{IR} and CLL^{NR} patients in stage A, B and C. (E), lymphocyte doubling time of CLL^{IR} and CLL^{NR} patients. Statistical analyses were performed using a Chi-squared test (3 variables compared between groups) or a Fisher's exact test (2 variables compared between groups).

3.4 Discussion

This study performed immunophenotypic analysis of T-cells from treatment-naïve early stage CLL patients with the aim of (A) characterising their dysfunctional status and (B) to determine if any T-cell abnormalities in CLL had prognostic significance within the disease. General analysis of the lymphocyte population from CLL patients showed a significant decrease in T-cells ($CD4^+$ and $CD8^+$) and NK cells compared to healthy age-matched controls. As only percentages of cells were measured it is somewhat unsurprising they were decreased given the relative expansion within this population of the leukaemic B-cell clone. Previous studies have however shown that whilst the percentage of T-cells and NK cells in the peripheral blood decreased, the absolute number of both populations is frequently increased in CLL patients (Vuillier et al. 1988). Indeed absolute count data obtained from 50 CLL patients and 10 healthy donors in this study confirmed the expansion of T-cells in this study's patient cohort (Figure 3.11).

The analysis of both $CD4^+$ and $CD8^+$ T-cells in the cohort of CLL patients revealed further abnormalities in T-cell phenotype. This study showed an expansion of $CD28^-$ $CD45R0^+$ $CD4^+$ and $CD8^+$ T-cells, indicating an expansion of activated T-cells with a memory phenotype within CLL patients. This finding supports those of a previous study which showed an increased frequency of activated HLA-DR^+ T-cells in CLL patients (Tötterman et al. 1989). Subset analysis revealed reduced percentages of naïve T-cells and a trend towards increased EM and EMRA T-cells within the $CD8^+$ compartment. Similarly there was an expansion in EM and a trend towards a decrease in naïve T-cells within the $CD4^+$ compartment. This skewing of T-cells towards a memory phenotype has been reported previously in CLL and was found to be more pronounced in progressive patients compared to

stable patients (Tinhofer et al. 2009). These results confirm that T-cells are being chronically stimulated in CLL patients, which may contribute to the pathogenesis of the disease.

Phenotypic analysis of T-cell subsets revealed an increase in $CD28^-$ EM T-cells in CLL patients. This reduction in CD28 expression may impair the ability of T-cells from CLL patients to receive activation signals from antigen presenting cells, including leukaemic B cells, which are known to display reduced levels of CD80 and CD86 (Dazzi et al. 1995). The study also showed that CD28 negativity was associated with an increase in CD57 expression on T-cells in CLL patients. Down regulation of CD28 and an increase in CD57 has been reported previously (Brenchley et al. 2003, Serrano et al. 1997, Weekes et al. 1999, Van den Hove et al. 1998) where it has been associated with reduced proliferative potential and an increased tendency to undergo apoptosis. Subset analysis of $CD4^+$ and $CD8^+$ T-cells revealed that this expansion of $CD28^-$ and $CD57^+$ cells was predominantly increased in the EM and EMRA compartments. $CD4^+CD57^+$ and $CD8^+CD57^+$ T-cells expansions have been reported previously in CLL patients where they were shown to have considerable oligoclonality (Serrano et al. 1997). The cause of these oligoclonal expansions remains unresolved, however several groups have suggested CMV reactivation may play a role in driving $CD4^+$ and $CD8^+$ effector memory T-cell expansions in CLL (Mackus et al. 2003, Walton et al. 2010, Pourgheysari et al. 2010). Alternatively, other groups have pointed to this expansion as evidence for an anti-tumour T-cell response (Goddard et al. 2001, Rezvany et al. 2000). If indeed T-cell activation is being regulated by the CLL clone, studying these alterations in T-cell populations may be important for understanding the pathogenesis of the disease.

As part of the comprehensive analysis of T-cells in CLL patients this study showed that 52% of our patient cohort had a preferential expansion of $CD8^+$ T-cells, leading to an inversion in the normal CD4:CD8 ratio. Patients with an inverted CD4:CD8 ratio have been

reported previously in CLL patients (Platsoucas et al. 1982) but this study showed for the first time that patients with an inverted ratio had a shorter time to first treatment conferring an inferior prognosis compared to normal ratio patients. This was independent of age and other established prognostic markers in CLL including CD38 and ZAP-70 expression, *IGHV* gene mutation status and Binet stage at diagnosis. The prognostic significance of CD4:CD8 ratios have been reported in solid cancers (Hernberg et al. 1997, Sheu et al. 1999) whilst in CLL the ratio of naïve to EM $CD4^+$ T-cells has been shown to be an indicator of aggressive disease (Tinhofer et al. 2009). A recent study by Gonzalez-Rodriguez *et al* reported somewhat contradictory results to the findings presented here. They suggested that higher CD8 counts corresponded with higher medium survival times in CLL patients (Gonzalez-Rodriguez et al. 2010). These results were however based on the ratio of T-cells to the number of malignant B-cells and therefore not directly comparable to results presented in this study. In addition, disease progression in CLL is usually associated with an increase in B-cell expansions. Therefore it is somewhat unsurprising that patients with a low T-cell to B-cell ratio had a poorer prognosis.

In keeping with other reports this study showed an increase in Tregs in CLL patients (Giannopoulos et al. 2008). As Tregs have been showed to increase with disease progression and have been associated with decreased T-cell responses in CLL (Giannopoulos et al. 2008), the percentage of Tregs was analysed within both the CLL^{IR} and CLL^{NR} groups. This study revealed no correlation between Treg percentages and the invertion of the CD4:CD8 ratio suggesting Tregs are not responsible for the inferior prognosis of this group. There was however a positive correlation between the inversion of the CD4:CD8 ratio and LDT. LDT has been recently shown to be the most important determinant of TTFT in Stage A patients (Pepper et al. 2012).

The reasons behind the inversion of the CD4:CD8 ratio in a subset of CLL patients remains unclear; however absolute T-cell count analysis suggests that this inversion is caused by the preferential expansion of the $CD8^+$ T-cell compartment rather than a relative reduction in $CD4^+$ T-cells. Several studies have indicated that expansions of T-cells in CLL patients might be due to chronic antigen stimulation from persistent viral infections such as CMV (Pourgheysari et al. 2010, Walton et al. 2010, Mackus et al. 2003). It is possible that sub-clinical CMV reactivation in these immunocompromised individuals may drive chronic antigen stimulation and exhaustion of the T-cell compartment. However, in this study the preferential expansion in $CD8^+$ T-cells occurred in both CMV seropositive and seronegative CLL patients, indicating that CMV cannot be solely responsible for the inversion of the CD4:CD8 ratio or the increase in $CD57^+CD28^-CD27^-$ EM and EMRA T-cells. These results are supported by a previous study that showed $CD8^+$ T-cell expansions in CMV seronegative CLL patients, with CMV serostatus having no effect on the overall survival of CLL patients (Pourgheysari et al. 2010). Further analysis revealed an increase in $CD8^+$ T-cells expressing PD-1 in the inverted ratio group, a marker associated with replicative senescence. Importantly this increase in PD-1 expression was independent of CMV serostatus. A logical extension to this work would be to use CMV tetramer technology to determine the number and phenotype of CMV-specific $CD8^+$ T-cells in CLL patients and healthy donors. This would allow us to rule out CMV stimulation as the sole cause of this expanded $CD8^+$ T-cell population in the CLL^{IR} group including those cells displaying $CD57^+CD27^-CD28^-$ EM and EMRA phenotypes.

It is clear that CLL patients' exhibit increased T-cell numbers compared to healthy age-matched controls regardless of CMV serostatus. It is possible that other infectious antigens may be driving T-cell expansions or possibly that the tumour itself may support T-cell

proliferation through the release of cytokines or through the presentation of tumour antigens. T-cell responses to autologous CLL cells have been documented *in vitro* which most likely represent responses against multiple tumour antigens rather than a single immunodominant epitope (Gitelson et al. 2003). Several candidate tumour antigens have been identified in CLL including survivin, fibromodulin and MDM2 which are capable of expanding functional autologous tumour specific T-cells (Mayr et al. 2005, Schmidt et al. 2003, Mayr et al. 2006). If a tumour-specific response is responsible for the expansion of $CD8^+$ T-cells seen in the CLL^{IR} group it would seem counter intuitive that these patients would have an inferior prognosis when compared to patients with a normal CD4:CD8 ratio. It is however possible that these expanded $CD8^+$ T-cells are functionally tolerised and thus unable to elicit an anti-tumour response. Tolerisation of T-cells has been shown to follow an initial period of T-cell proliferation (Redmond and Sherman 2005). Furthermore high frequencies of tolerised TAA-specific $CD8^+$ T-cells have been reported in cancer patients with impaired cytokine responses to mitogens and an inability to lyse tumour cell targets (Lee et al. 1999). The increased number of $CD8^+$ T-cells found in the CLL^{IR} group may be due to a loss of immunological control in these patients whereby $CD8^+$ T-cells are expanding in response to chronic stimulation from the tumour but ultimately become anergic. Indeed the increase in antigen experienced $CD8^+$ T-cells within the CLL^{IR} group, which are $CD28^-CD27^-CD57^+$ and $PD\text{-}1^+$ indicates that there are a large proportion of effector cells that are highly differentiated and have a low proliferative potential in these patients. This may contribute to a reduced ability of memory responses to protect against reinfection or for established anti-tumour memory T-cell responses to respond to the growth of the tumour. Furthermore the relative reduction in naïve T-cells may lead to depletion in the TCR repertoire diversity within the naïve T-cell pool making it more difficult to mount immune responses to new infections or novel tumour antigens.

Alternatively the poor prognosis of the CLL^{IR} patient group may be explained by the presence of $CD57^+$ T-cells. $CD8^+CD57^+$ T-cells have been shown to secrete a lectin-binding soluble factor capable of inhibiting the cytolytic function of $CD8^+CD57^-$ T-cells and increasing the risk of infection (Autran et al. 1991). It is also possible that the expanded $CD8^+$ T-cells are capable of driving the growth of tumour cells. $CD8^+CD57^+$ T-cells can secrete high levels of IFN-γ and TNF-α upon activation which has been shown to promote the survival of CLL cells *in vitro* (Chong et al. 2008, Buschle et al. 1993, di Celle et al. 1994).

Given that the poor prognosis of the inverted ratio group is associated with an expansion of a potentially tolerised $CD8^+$ T-cells population over expressing PD-1, it would be interesting to know if this PD-1 positive population has specificity for the CLL clone. It would also be of great interest to evaluate PD-1 signalling blockade as a potential therapeutic strategy to break T-cell tolerance within the disease. Blocking antibodies to PD-1 have already been shown to reverse the exhaustive phenotype of HIV-specific $PD\text{-}1^+$ $CD8^+$ T-cells allowing the recovery of proliferative and cytotoxic responses (Trautmann et al. 2006). In addition PD-1-PDL-1 signalling blockade has been shown to provide clinical benefit in phase 1 clinical trials of patients with solid and haematological malignancies (Berger et al. 2008, Brahmer et al. 2010).

The results of this chapter show that there is a clear perturbation in the normal lymphocyte populations in CLL patients, including a reduction in the percentages of T-cells and an increase in the frequency of regulatory T-cells. Within the CLL patient cohort we also identified a subset of patients that display an inversion in the CD4:CD8 T-cell ratio. These patients had a preferential expansion in $CD8^+$ T-cells displaying a highly differentiated and replicative senescent phenotype. Importantly this group of patients had shorter lymphocyte

doubling times, representing patients with more progressive disease who are likely to require earlier treatment.

CHAPTER 4

Investigating the role of immunosuppressive molecules in CLL

Immunosuppressive molecules, including PD-1 and CD200, have been implicated in the suppression of T-cell responses during chronic infections as well as in solid and haematological malignancies (Zhang, Gajewski and Kline 2009a, Gao et al. 2009). Sustained signalling via these molecules can result in T-cell exhaustion including suppression of T-cell proliferation, cytokine release and effector function, whilst facilitating immune escape of cancer cells (Norde et al. 2012). Furthermore immunosuppressive molecules may reduce the efficacy of current therapeutic strategies aimed at promoting T-cell responses in haematological malignancies including vaccination, allo-HSCT and adoptive T-cell therapy (Berger et al. 2008).

This study has previously described a cohort of CLL patients with inferior prognosis based on their inversion in CD4:CD8 T-cell ratio. Such patients were found to have a preferential expansion of $CD8^+$ T-cells skewed towards a highly differentiated effector memory phenotype with increased expression of PD-1. PD-1 expression has been associated with a replicative senescent phenotype and may contribute to T-cell dysfunction in CLL patients thereby impairing immune regulation of the leukaemic clone (Grzywnowicz et al. 2012). The aims of this chapter were to further investigate the expression of immunosuppressive molecules including PD-1 and CD200, and to evaluate their functional and clinical significance in CLL patients.

4.1 Immunosuppressive molecules in CLL

Immunosuppressive molecules such as CD200 and PD-1 have been shown to be upregulated during chronic infections and in cancer (Dorfman and Shahsafaei 2010, Caserta et al. 2012, Zhang et al. 2009a, Day et al. 2006). CD200 is a cell surface glycoprotein found on B, T and NK cells that is known to play a role in regulating immune responses. As CD200 does not possess a cytoplasmic domain with signalling motifs, inhibitory signals are believed to be delivered to CD200R expressing cells including myeloid cells (macrophages and neutrophils) and T-cells upon CD200:CD200R engagement (Gorczynski et al. 1999, Wang et al. 2010). CD200 upregulation has already been demonstrated in haematological malignancies including AML and MM, where it is associated with inferior prognosis (Moreaux et al. 2006). In accordance with previous work (El Desoukey et al. 2012, Wong et al. 2010, Dorfman and Shahsafaei 2010, Rawstron et al. 2010) the expression of CD200 was analysed on the surface of CLL cells in this study including analysis of T-cells and NK cells. The expression of its receptor (CD200R) was measured on T-cells in a smaller cohort of CLL patients to demonstrate the potential for CD200-CD200R inhibition of T-cells in CLL.

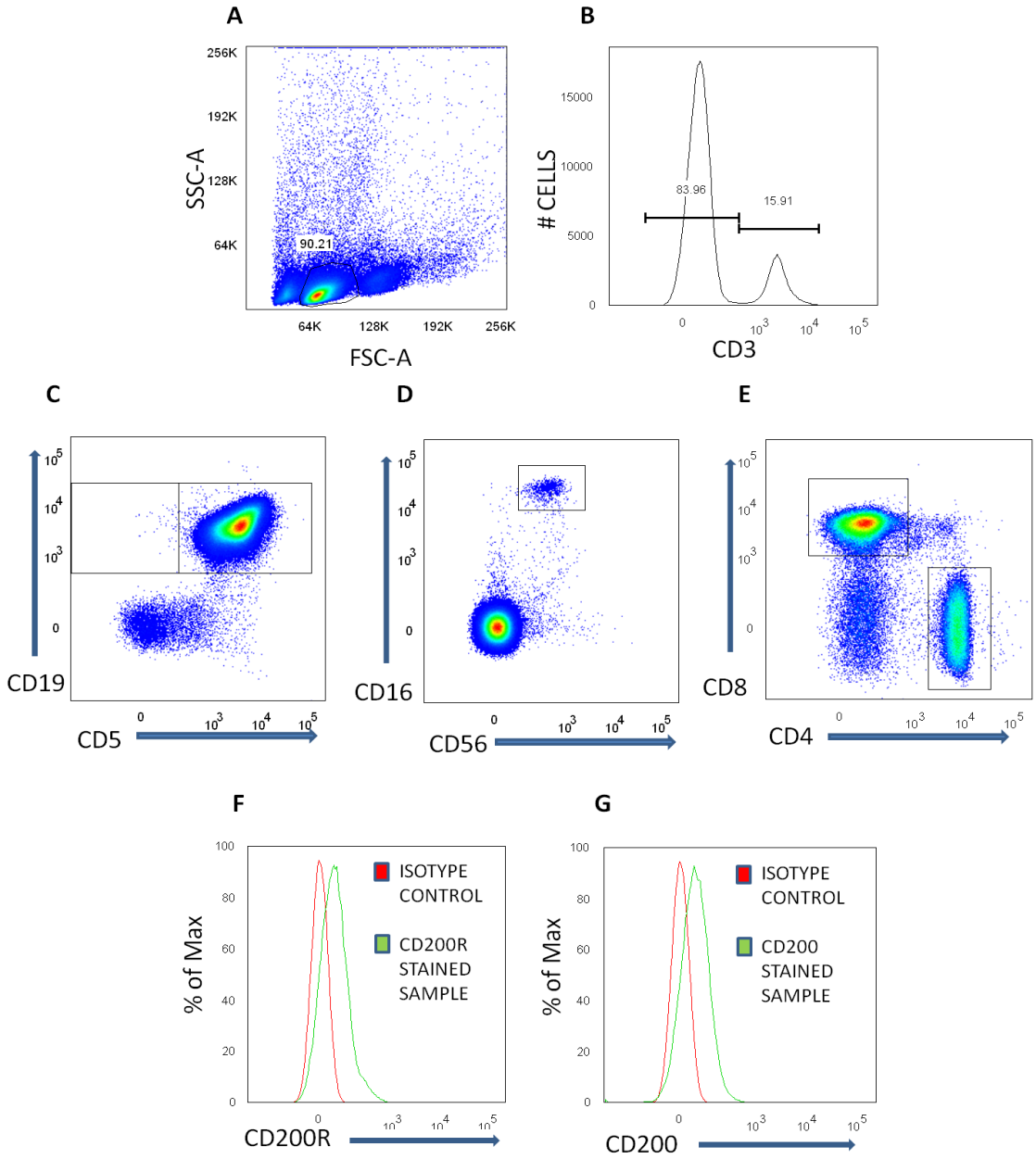


Figure 4.1. Gating strategy for the identification of B, T and NK cells from CLL patients and the measurement of CD200 and CD200R expression. Lymphocytes were gated based on forward and side scatter and separated on cell surface expression of CD3 (A-B). Within the CD3 negative population $CD19^{+}CD5^{+}$ B-cells (C) and $CD16^{+}CD56^{+}$ NK cells (D) were gated. In the CD3 positive population both $CD4^{+}$ and $CD8^{+}$ T-cells were gated (E). CD200 was measured within in B, T and NK cells and CD200R in T-cells as a percentage above an isotype control antibody (F-G). The numbers of positive cells were determined using flow cytometry and analysis was performed using Flowjo software.

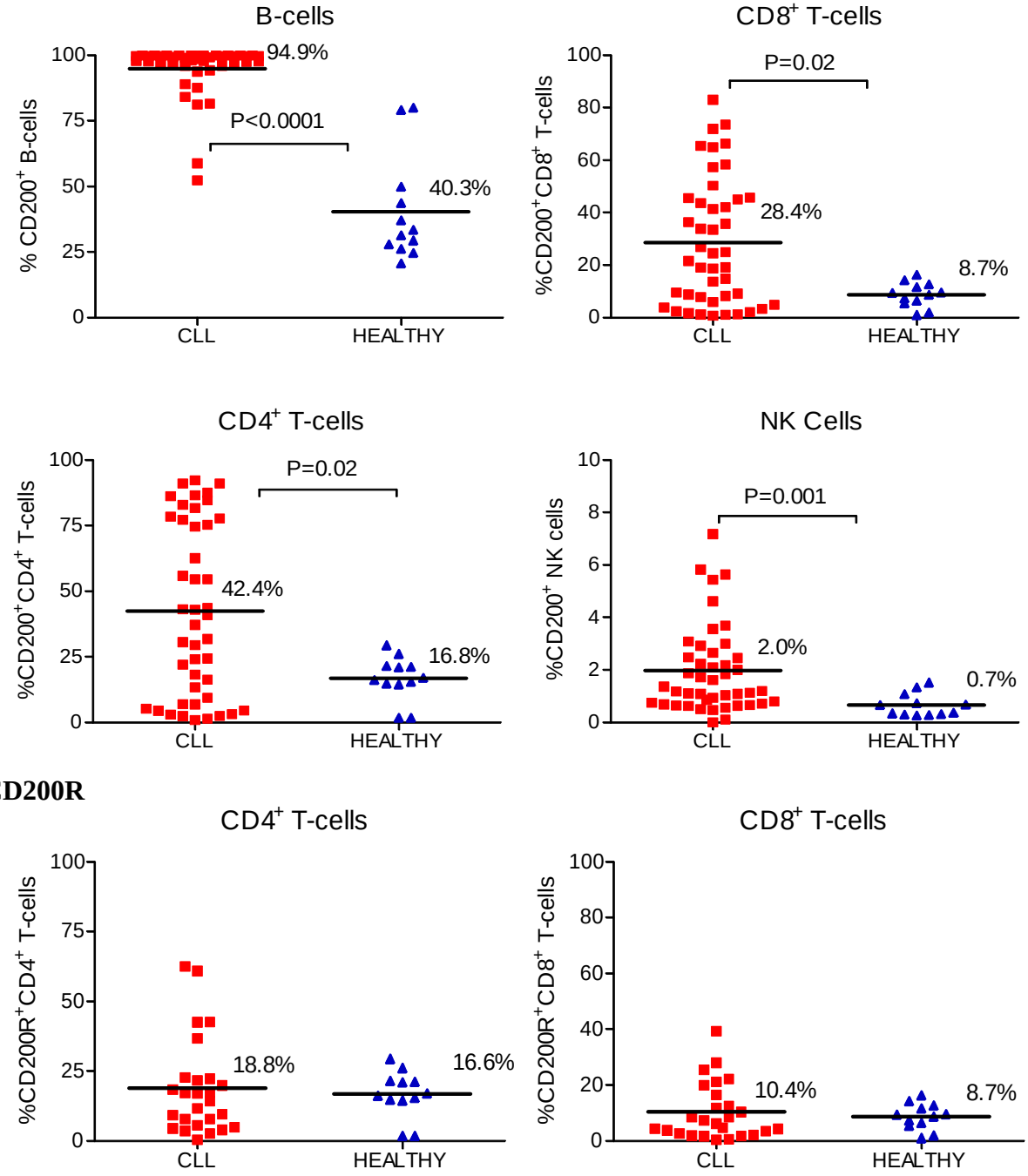
4.1.1 CD200 and CD200R expression in CLL patients

CD200 expression was measured on $CD19^+CD5^+$ B-cells, T-cells and NK cells from the blood of CLL patients and healthy age-matched donors (Figure 4.1). The percentage of $CD200^+CD19^+CD5^+$ B-cells from CLL patients was significantly higher than healthy controls (Mean: 94.9% VS 40.3% P<0.0001). Analysis of T-cells and NK cells showed significant increases in the percentages of CD200 expressing $CD8^+$ T-cells (28.4% vs. 8.7%, P=0.02), $CD4^+$ (42.4% vs. 16.8%, P=0.02) and NK cells (Mean 2.0% vs. 0.7%, P=0.001). Measurement of the receptor for CD200 (CD200R) on the surface of T-cells from CLL patients and healthy donors showed there was no statistically significant difference between both groups (Figure 4.2: $CD4^+$ 18.8% vs. 16.6%, $CD8^+$ 10.4% vs. 8.7% respectively).

Analysis of T-cells revealed a significant correlation between the percentage $CD4^+CD200^+$ T-cells and $CD8^+CD200^+$ T-cells in CLL patients (P<0.0001). A similar correlation was shown between the percentage of $CD4^+CD200R^+$ T-cells and $CD8^+CD200R^+$ T-cells in CLL patients (P<0.0001). In addition no significant correlations were observed between the percentages of CD200 and CD200R expressing T-cells within the $CD4^+$ T-cell population (P=0.14) or $CD8^+$ T-cell population (P=0.33) in CLL patients. No correlations were seen between the percentages of $CD4^+$ (P=0.57) or $CD8^+$ T-cells (P=0.50) expressing CD200 and CLL cells expressing CD200R in CLL patients (Figure 4.3).

Collectively the data presented here shows that CD200 is over expressed in CLL patients including on B, T and NK cells when compared to healthy donors. Moreover there was a strong correlation between the percentage of $CD4^+$ and $CD8^+$ T-cells expressing either CD200 or CD200R in CLL patients. There however appeared to be no link between CD200R expression on T-cells and CD200 expression on either T cells or CLL cells.

CD200



CD200R

Figure 4.2. CD200 (n=44) and CD200R (n=26) expression on lymphocytes from CLL patients compared to healthy age-matched controls (n=12). The numbers of positive cells were measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. CD200 expression was measured on $CD19^+CD5^+$ (CLL patients) and $CD19^+$ (Healthy donors) B-cells, $CD3^+$ T-cells ($CD4^+$ and $CD8^+$) and $CD56^+CD16^+CD3^-$ (NK cells) and CD200R expression was measured on $CD4^+$ and $CD8^+$ T-cells. Both CD200 and CD200R expression was measured as a percentage of expressing cells above that of an isotype control. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

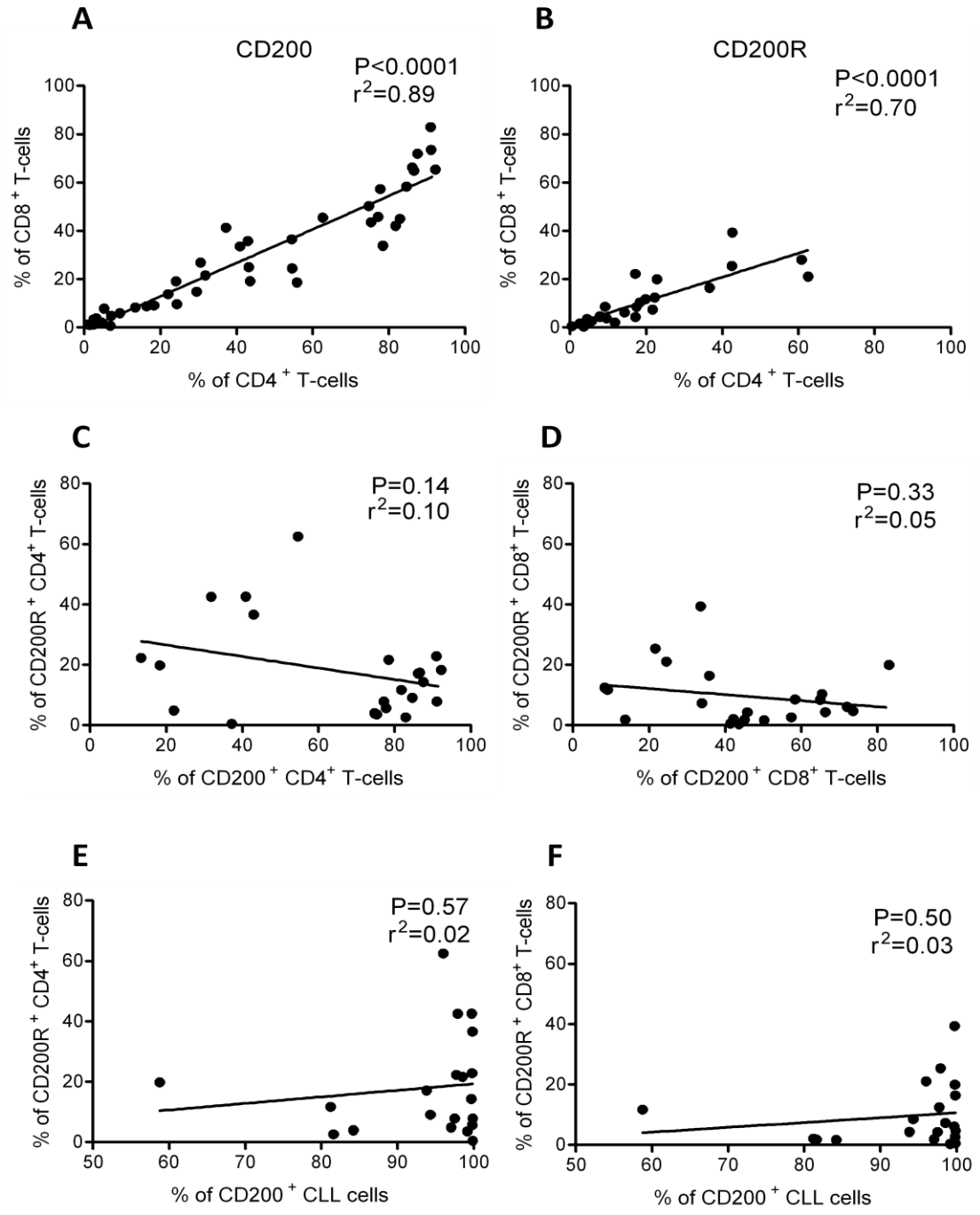


Figure 4.3. Analysis of CD200 and CD200R expression on T-cells and tumour cells in CLL patients. Linear regression plots comparing the percentage of CD200 (A) or CD200R (B) expressing $CD8^+$ T-cells against the percentage of CD200 or CD200R expressing $CD4^+$ T-cells for each patient. (C) Linear regression plots comparing the percentage of CD200R vs. CD200 expressing $CD4^+$ T-cells within the CLL patient cohort. (D) Linear regression plots of the percentage of CD200R vs. CD200 expressing $CD8^+$ T-cells within the CLL patient cohort. Linear regression plot comparing the percentage of $CD200^+$ $CD4^+$ (E) or $CD8^+$ (F) T-cells vs. CD200 expressing CLL cells within the CLL patient cohort.

4.1.2 Programmed death-1 (PD-1) and its ligand (PDL-1)

PD-1 is a co-inhibitory molecule expressed on activated B and T-cells and has been shown to promote immune tolerance including T-cell anergy (Tsushima et al. 2007). Interaction of PD-1 with its respective ligand (PDL-1) found on leukocytes and non-haematopoietic cells have been shown to inhibit TCR signalling (Sheppard et al. 2004). PD-1-PDL-1 ligation promotes the recruitment of the phosphatases SHP-1 and SHP-2 to the cytoplasmic tail of PD-1 that subsequently interfere with the phosphorylation of ZAP-70 (Fife et al. 2009). PDL-1 expression on tumour cells has been shown to suppress PD-1 expressing tumour-reactive T-cells in solid cancers and may suppress T-cells in haematological malignancies including CLL and AML (Zhang et al. 2008, Brusa et al. 2013, Berthon et al. 2010). In addition, tumour expression of PDL-1 has also been associated with inferior clinical outcome in several solid cancers including gastric and ovarian cancer (Hino et al. 2010).

PDL-1 engagement with PD-1 has not only been shown to interfere with TCR signalling but may also deliver signals to PDL-1-expressing cells (Keir et al. 2008). Soluble PD-1 has been shown to induce IL-10 production from $CD4^+$ T-cells in rheumatoid arthritis (Dong et al. 2003). In addition PDL-1 on T-cells is able to bind CD80 on APCs mediating T-cell inhibition (Park et al. 2010). Here both PD-1 and its ligand PDL-1 were analysed on the surface of T-cells and tumour cells from CLL patients and compared to a cohort of healthy age-matched controls (Figure 4.4).

4.1.3 PD-1 and PDL-1 expression in CLL patients

PD-1 expression was significantly increased on $CD19^+CD5^+$ B-cells (15.3 vs. 2.0%, P=0.005), $CD4^+$ T-cells (21.7% vs. 6.3%, P=0.0005) and $CD8^+$ T-cells (18.6 vs. 7.5%, P=0.04) from CLL patients compared to healthy aged-matched controls (Figure 4.5). Analysis of PDL-1 showed it was decreased on $CD19^+CD5^+$ B-cells (53.8% vs. 72.6%, P=0.02) but increased on $CD4^+$ T-cells (34.8 vs. 24.2%, P=0.002) and $CD8^+$ T-cells (31.7% vs. 24.5%, P=0.01) from CLL patients compared to healthy controls.

Analysis of T-cells showed that there was a significant correlation between the percentage of $CD4^+PD\text{-}1^+$ T-cells and $CD8^+PD\text{-}1^+$ T-cells in CLL patients (P<0.0001). A similar correlation was seen when comparing $CD4^+PD\text{-}L1^+$ and $CD8^+$ $PDL\text{-}1^+$ T-cells (P<0.0001). Furthermore significant correlations were observed between the percentages of PD-1 and PDL-1 expressing $CD4^+$ T-cells (P=0.0004) and $CD8^+$ T-cells (P=0.03) in our patient cohort. No correlation was seen between the percentages of $CD4^+$ (P=0.41) or $CD8^+$ T-cells (P=0.39) expressing PD-1 and CLL cells expressing PDL-1 in CLL patients (Figure 4.6).

These findings show that PD-1 is over expressed on T-cells from CLL patients suggesting T-cells may be subject to PD-1-PDL-1 mediated suppression in this disease. In addition PDL-1 was upregulated on T-cells from CLL patients demonstrating the potential for an alternative mechanism of T-cell inhibition through PDL-1 ligation on T-cells.

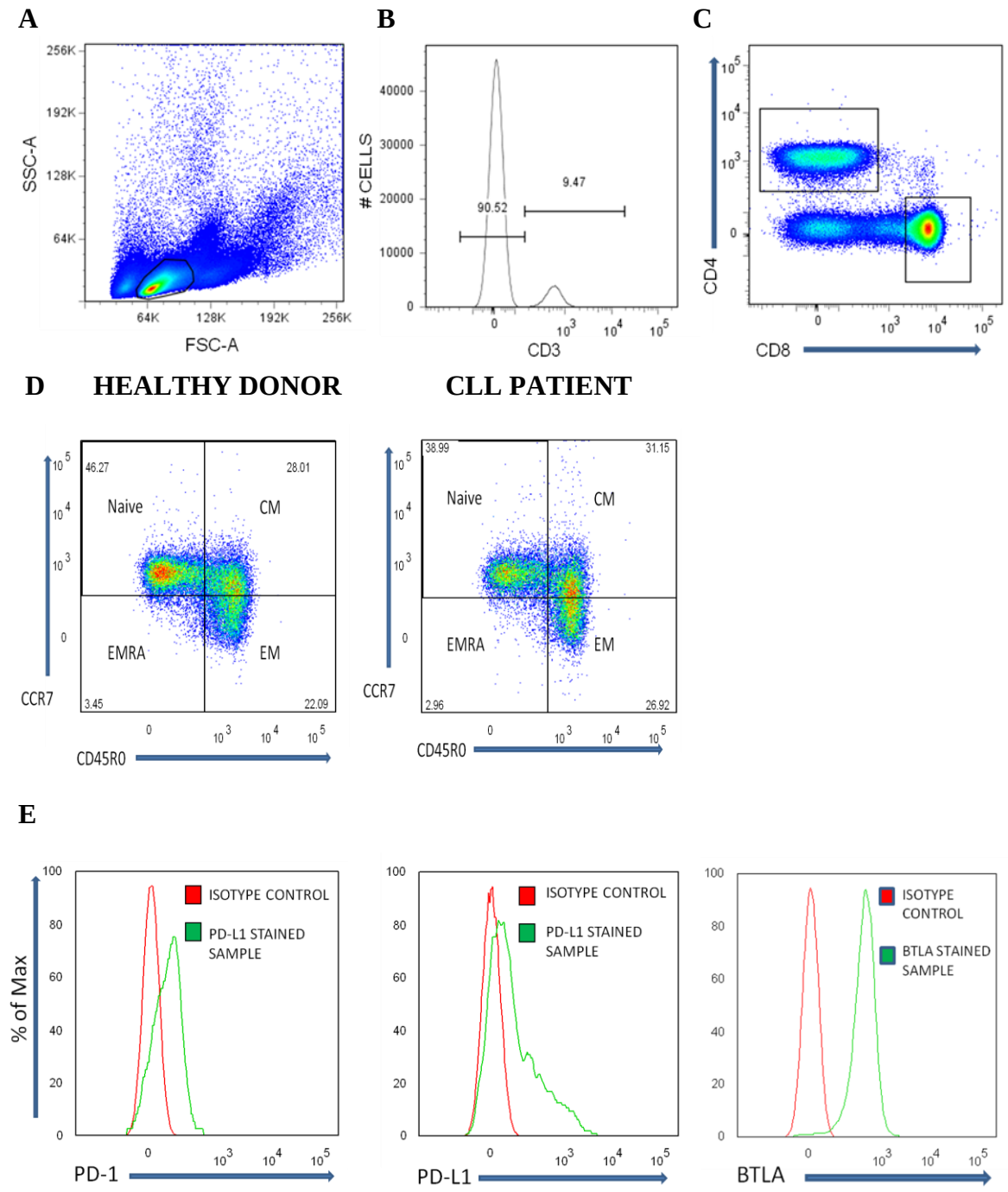


Figure 4.4. Gating strategy for measurement of PD-1, PDL-1 and BTLA within CD4$^+$ and CD8$^+$ T-cell subsets. The numbers of positive cells were measured using 8-colour flow cytometry (FACS Canto II). (A) Lymphocytes were gated based on forward and side scatter profiles. (B) Separation of T-cells within lymphocyte population based on CD3 positivity. (C) Gating of CD4$^+$ and CD8$^+$ T-cells within CD3$^+$ population. (D) Gating of Naïve, Central memory (CM), Effector memory (EM) and EMRA T-cells based on CCR7 and CD45RO expression within the CD4$^+$ T-cell population. (D) Expression of PD-1, PDL-1 and BTLA within each subset of T-cells (Naïve, CM, EM and EMRA) was measured as a percentage above an isotype control antibody.

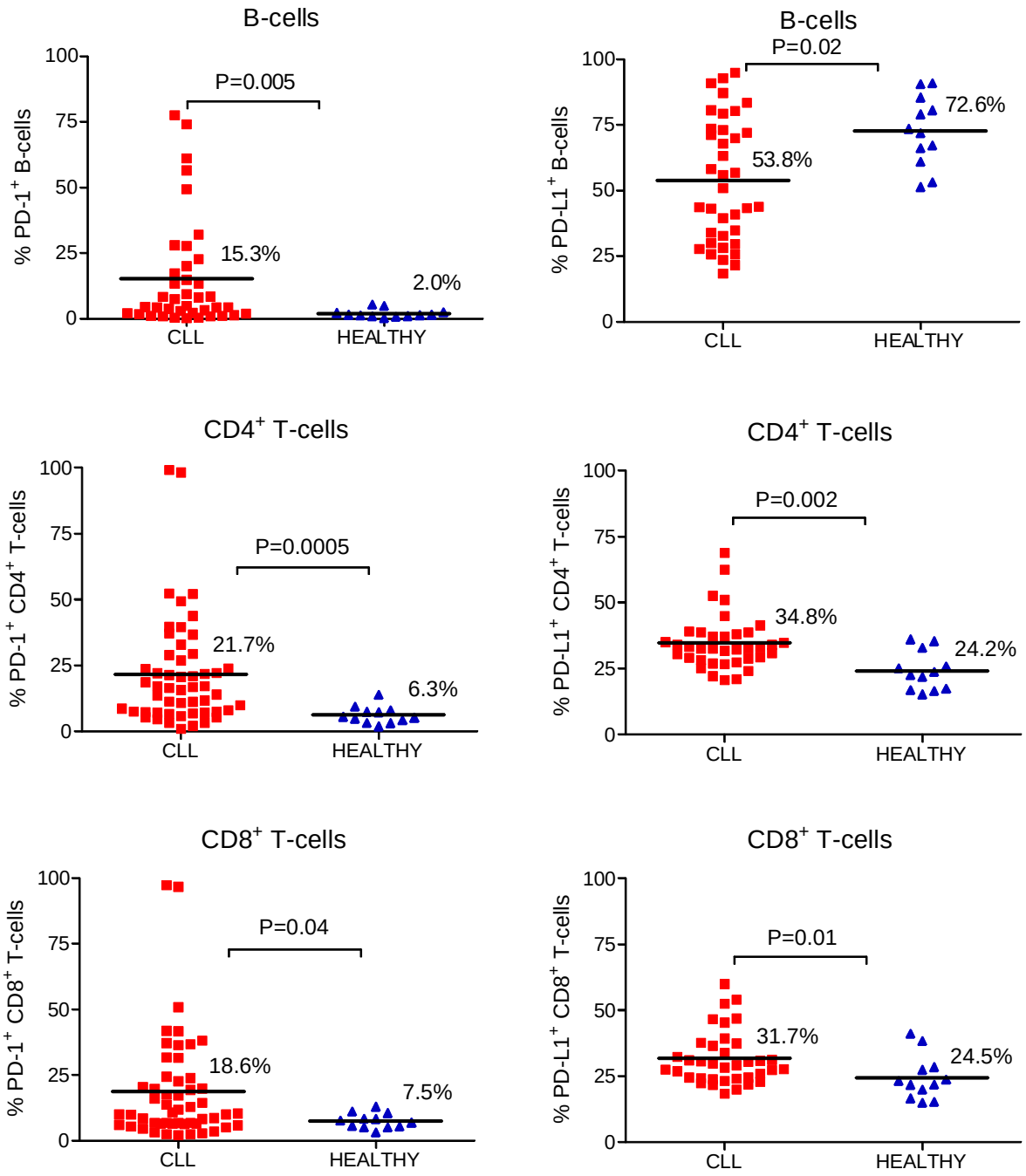


Figure 4.5. PD-1 (n=52) and PDL-1 (n=37) expression on lymphocytes from CLL patients compared to healthy age-matched controls (n=12). The numbers of positive cells were quantified using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. PD-1 and PDL-1 expression was measured on $CD19^+CD5^+$ (CLL patients) and $CD19^+$ (Healthy donors) B-cells, $CD4^+$ T-cells and $CD8^+$ T-cells. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism.

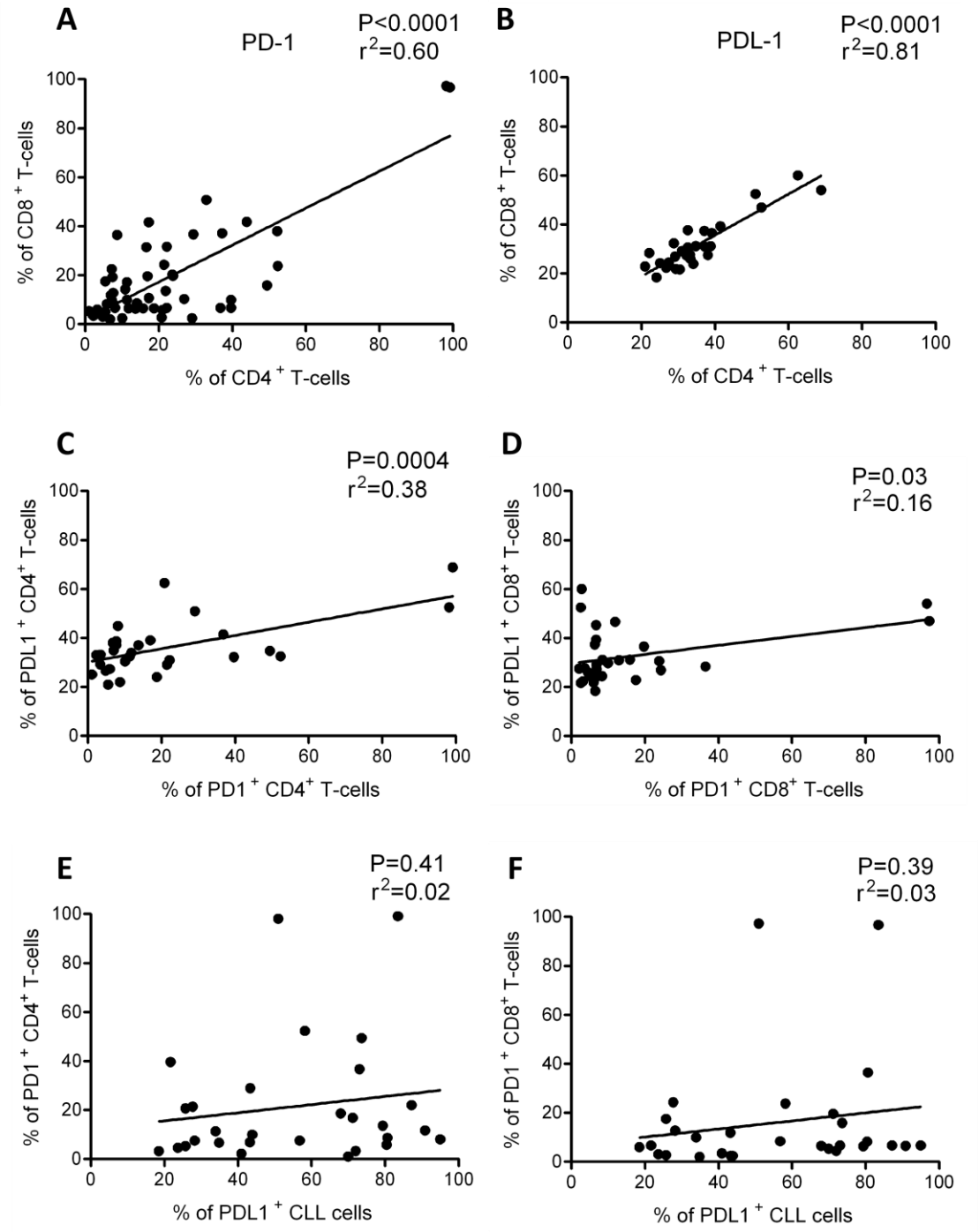


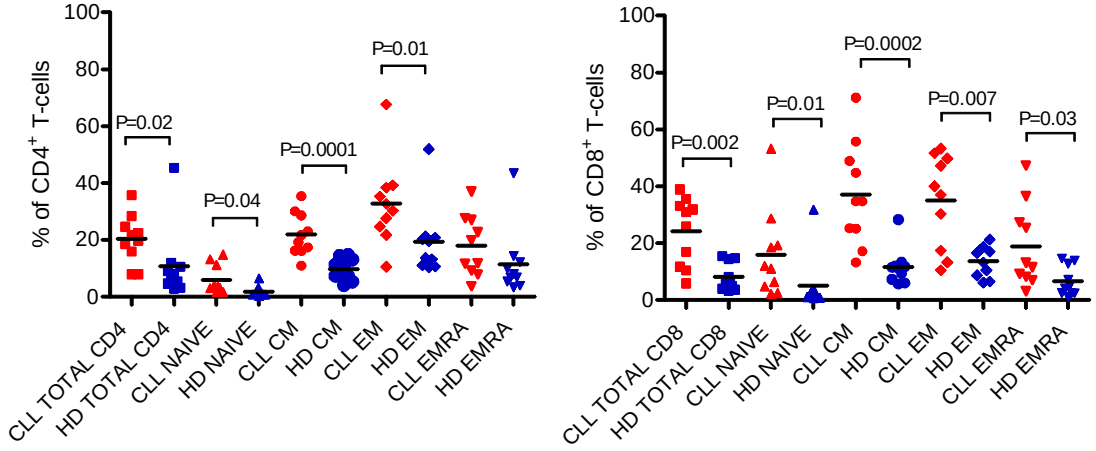
Figure 4.6. Analysis of PD-1 and PDL-1 expression on T-cells and tumour cells in CLL patients. Linear regression plots comparing the percentage of PD-1 (A) or PDL-1 (B) expressing $CD8^+$ T-cells against the percentage of PD-1 or PDL-1 expressing $CD4^+$ T-cells for each patient. (C) Linear regression plots comparing the percentage of PDL-1 vs. PD-1 expressing $CD4^+$ T-cells within the CLL patient cohort. (D) Linear regression plots of the percentage of PDL-1 vs. PD-1 expressing $CD8^+$ T-cells within the CLL patient cohort. Linear regression plot comparing the percentage of PD-1^+ $CD4^+$ (E) or $CD8^+$ (F) T-cells vs. PDL-1 expressing CLL cells within the CLL patient cohort. (A-B (n=52), C-F (n=29)).

4.1.4. PD-1 and PDL-1 expression in T-cell memory subsets

In order to determine if the increase in PD-1 and PDL-1 expression seen on T-cells from CLL patients was due to an increase in expression in a particular subset of T-cells, CCR7 and CD45RO were used to subcategorise T-cells into Naïve, CM, EM and EMRA phenotypes in a cohort of 10 CLL patients and 10 healthy donors (Figure 4.6). Analysis of PD-1 showed it was predominately expressed in the CM and EM T-cell subsets in the $CD4^+$ and $CD8^+$ T-cell compartments. In contrast PDL-1 was predominately expressed in the Naïve and CM T-cell subsets in both the $CD4^+$ and $CD8^+$ T-cell compartments. Comparison of CLL patients and healthy donors showed that PD-1 positive cells were significantly increased in nearly all $CD8^+$ T-cell and $CD4^+$ T-cell subsets except the $CD4^+$ EMRA population in CLL patients. Analysis of PDL-1 positive cells showed they were significantly increased on every $CD4^+$ T-cell subsets, but only on the Naïve and CM subsets of the $CD8^+$ T-cell population in CLL patients. PDL-1 positive cells within the $CD8^+$ EM and EMRA populations of CLL patients were not statistically different to healthy controls (Figure 4.7).

These findings show that PD-1 is predominately expressed on previously activated T-cells displaying an antigen experienced phenotype including CM and EM T-cells. PDL-1 however is predominately expressed on naïve and CM T-cells. Comparison of CLL patients and healthy donors suggest that PD-1 and PDL-1 are upregulated on multiple T-cell subsets, suggesting the potential for immunosuppression through these molecules.

PD-1



PDL-1

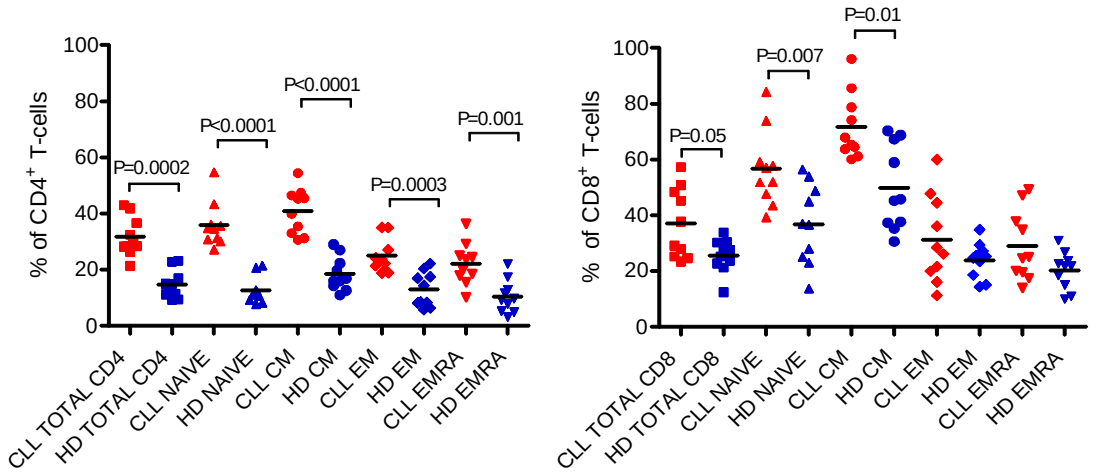


Figure 4.7. The expression of PD-1 and PDL-1 on Naïve, Central memory, Effector memory, and Effector (EMRA) CD8$^+$ and CD4$^+$ T-cells in CLL patients (n=10) compared to healthy age-matched controls (n=10). The numbers of positive cells were quantified using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. CD8$^+$ and CD4$^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO, Naïve (CCR7$^+$CD45RO$^-$), Central memory (CM) (CCR7$^+$CD45RO$^+$), Effector memory (EM) (CCR7$^-$CD45RO$^+$) and Effector (EMRA) (CCR7$^-$CD45RO$^-$). PD-1 expression was then measured on CD4$^+$ and CD8$^+$ T-cells subsets. The ligand for PD-1, PDL-1 was also measured on CD4$^+$ T-cell subsets and CD8$^+$ T-cell subsets. The mean within each group is shown. Statistical analysis (Mann-Whitney U test) was carried out using GraphPad Prism. CLL=CLL Patients, HD=Healthy donors.

4.1.5 B and T lymphocyte attenuater (BTLA) expression on T-cells in CLL

In order to determine if the upregulation of PD-1 seen in T-cell subsets was part of a more generalised upregulation of co-inhibitory molecules, the expression of BTLA on T-cells from CLL patients was also measured (Figure 4.4). BTLA was identified in 2003 as a co-inhibitory molecule with similarities to CTLA-4 and PD-1 (Watanabe et al. 2003). Loss of PD-1 or BTLA function results in a predisposition to experimentally-induced autoimmune encephalomyelitis in mouse models, suggesting that both molecules play a crucial role in maintaining T-cell tolerance (Watanabe et al. 2003, Salama et al. 2003). Analysis of both $CD4^+$ and $CD8^+$ T-cell from CLL patients showed no difference in BTLA expression when compared to healthy age-matched donors (Figure 4.8). Separation of T-cells into subsets, based on CCR7 and CD45RO expression, revealed no differences in the percentage of BTLA expression in naïve, CM or EMRA $CD4^+$ or $CD8^+$ T-cells between both groups. There was however a significant increase in BTLA expression in the EM $CD4^+$ T-cells compartment ($P=0.02$) and a trend towards an increase in the $CD8^+$ T-cell compartment ($P=0.07$).

The data presented here suggests unlike PD-1, BTLA is highly expressed on T-cells from CLL patients and healthy age-matched controls. In addition no significant differences in BTLA expressing T-cells were observed in CLL patients compared to heathy age matched controls suggesting it may not have a role in suppression of T-cells in the disease.

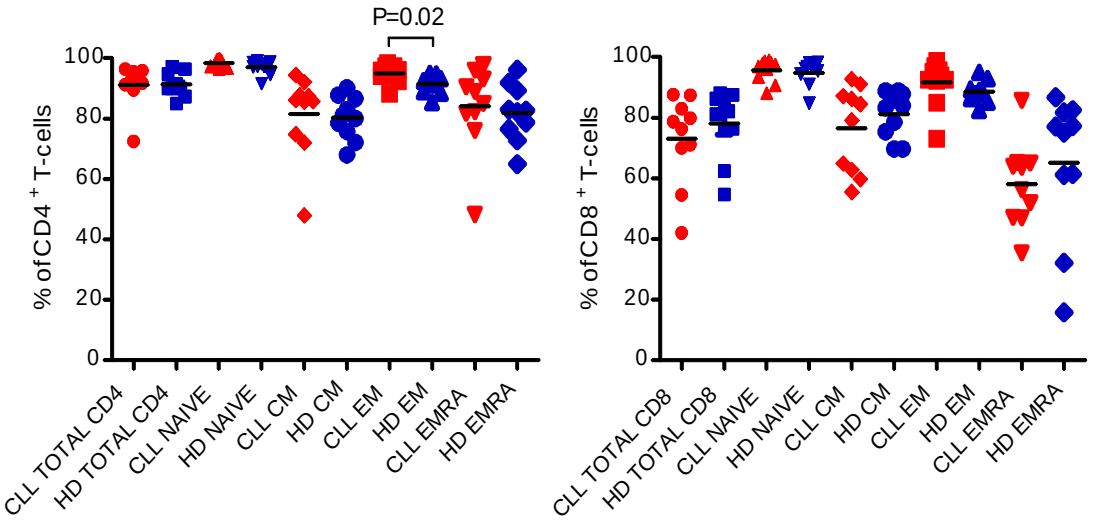


Figure 4.8. The expression of BTLA on Naïve, Central memory, Effector memory, and Effector (EMRA) CD8$^+$ and CD4$^+$ T-cells in CLL patients (n=10) compared to healthy age-matched controls (n=10). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. CD8$^+$ and CD4$^+$ T-cell subsets were gated based on their expression of CCR7 and CD45RO, Naïve (CCR7$^+$CD45RO$^-$), Central memory (CM) (CCR7$^+$CD45RO$^+$), Effector memory (EM) (CCR7$^-$CD45RO$^+$) and Effector (EMRA) (CCR7$^-$CD45RO$^-$). BTLA expression was then measured on CD4$^+$ and CD8$^+$ T-cells subsets. The mean within each group is shown. Statistical analysis (Mann-Whitney test) was carried out using GraphPad Prism. CLL=CLL Patients, HD=Healthy donors.

4.1.6 PD-1 expression has no effect on TTFT and PFS in CLL patients

In order to determine if the presence of PD-1 in CLL patients had an effect on prognosis, PD-1 expression was measured on T-cell and PDL-1 on CLL cells was analysed and compared to time to first treatment and progression free survival. No correlation was found between the percentage of CD4$^+$ and CD8$^+$ T-cells expression PD-1 and either TTFT or PFS. Similiarly no correlation was found between the percentage of PDL-1$^+$ CLL cells and TTFT and PFS (Figure 4.9).

Overall these results suggest that PD-1-PDL-1 signalling may not have any effect on the prognosis of CLL patients.

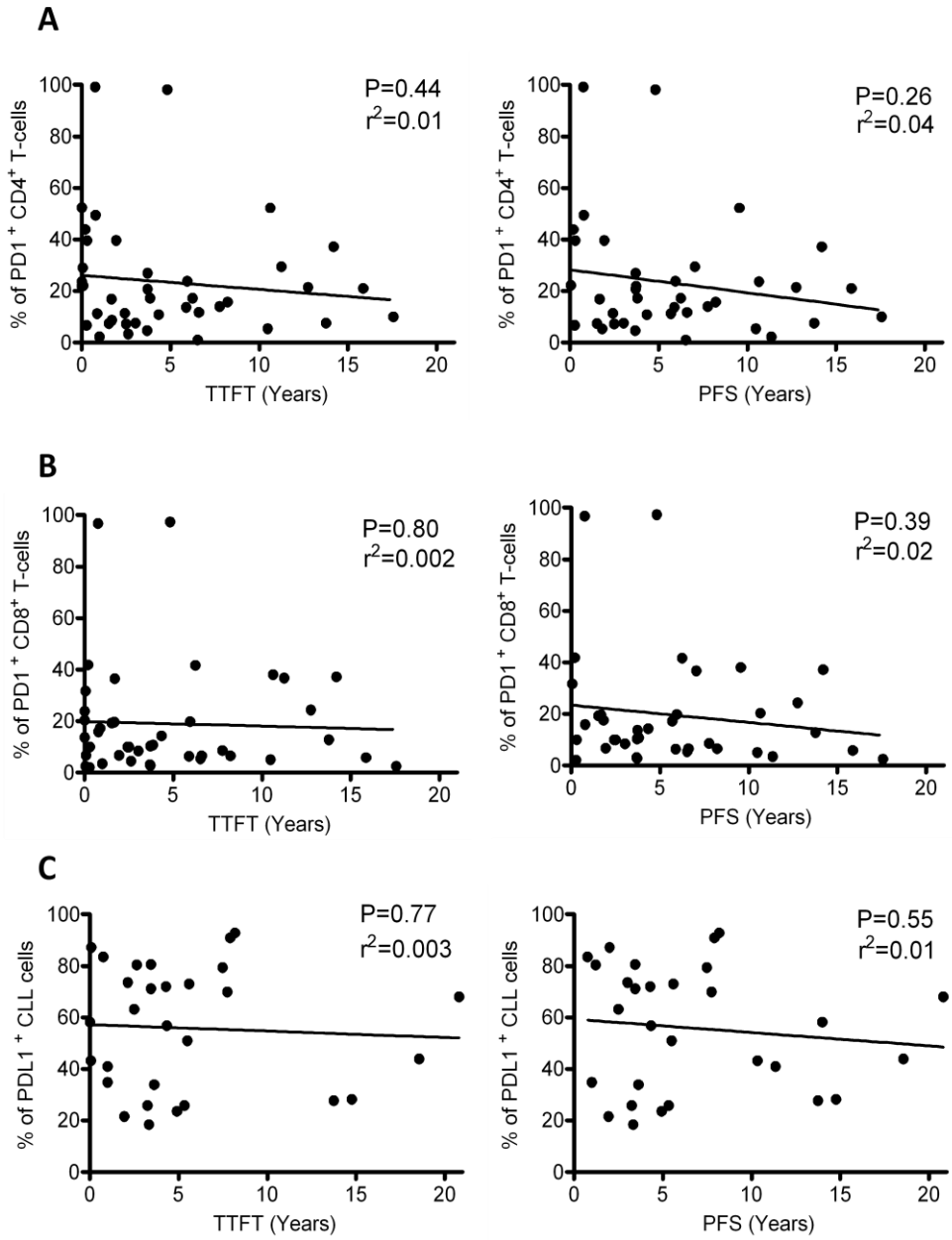


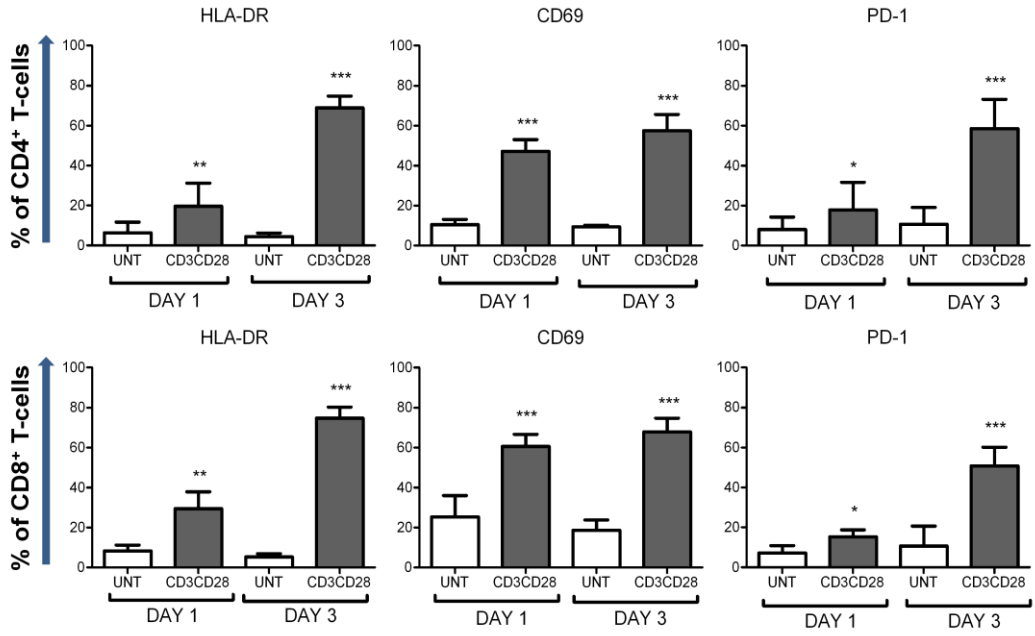
Figure 4.9. Analysis of the percentage of PD-1$^+$ T-cells and PDL-1$^+$ CLL cells vs time to first treatment (TTFT) and progression free survival (PFS) in CLL patients. Linear regression plots comparing the percentage of PD-1$^+$ T-cells (CD4$^+$ (A) and CD8$^+$ (B)) and PDL-1$^+$ CLL cells (C) against TTFT and PFS of CLL patients

4.2 PD-1 is upregulated following activation and appears to be a late activation marker

Several studies have shown that PD-1 can be induced on T-cells upon activation (Hokey et al. 2008, Barber et al. 2006). In order to see whether PD-1 was also upregulated upon T-cell activation in CLL, PBMC cultures were stimulated with CD3/CD28 beads and measured for PD-1 expression and other activation markers. The expression of HLA-DR, CD69 and PD-1 on $CD4^+$ and $CD8^+$ T-cells were found to be significantly increased in PBMC cultures at both Day 1 and Day 3 following CD3/CD28 bead stimulation (Figure 4.10A). Furthermore, significantly higher percentages of both $CD4^+$ and $CD8^+$ T-cells were found to express CD69 after CD3/CD28 stimulation at day 1 compared to HLA-DR or PD-1 confirming CD69 as an early activation marker. In contrast, by day 3 the percentages of both $CD4^+$ and $CD8^+$ T-cells expressing HLA-DR were significantly higher than those expressing CD69 (CD4: P= 0.02 and CD8: P= 0.04). PD-1 expression was also found to be expressed on significantly fewer $CD8^+$ T-cells compared to HLA-DR at day 3 (P= 0.01) (Figure 4.10B).

These findings show that PD-1 expression can be induced upon T-cell activation in CLL and is upregulated late in comparison to activation markers including HLA-DR and CD69.

A



B

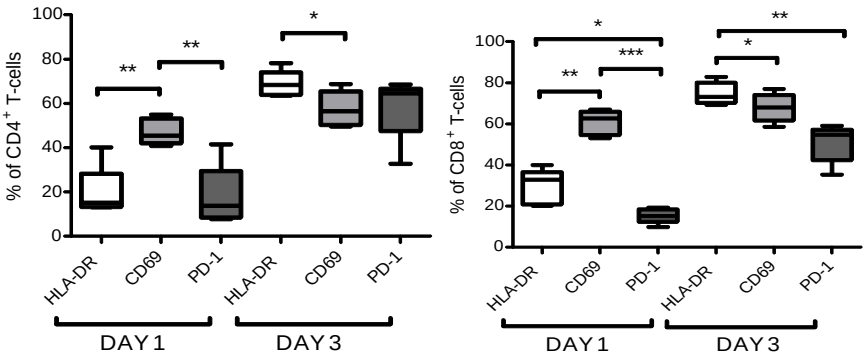


Figure 4.10. The expression of HLA-DR, CD69 and PD-1 on T-cells following activation with CD3/CD28 beads (n=5). The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. $CD4^+$ and $CD8^+$ T-cells were measured within the $CD3^+$ gate. (A) HLA-DR, CD69 and PD-1 expression was measured upon both $CD4^+$ and $CD8^+$ T-cell populations. The mean and standard deviation within each group is shown. (B) Comparative analysis of PD-1, HLA-DR and CD69 expression on $CD4^+$ and $CD8^+$ T-cells following CD3/CD28 stimulation. Activation marker expression was measured at both day 1 and day 3 timepoints. Statistical analysis was performed using a paired *t*-test. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$).

4.3 CLL cell activation and upregulation of PDL-1 in response to T-cell activation.

In order to determine if T-cell activation in PBMC cultures had any effect on bystander tumour cells, CLL cells were analysed for the expression of activation markers and immunosupressive molecules PDL-1 and CD200 in PBMC cultures treated with CD3/CD28 beads (Figure 4.11). CLL cells showed a significant increase in $CD69^{+}$ expression in PBMC cultures treated with CD3/CD28 beads at day 1 and day 3. This was accompanied by a significant upregulation of PDL-1 on CLL cells at day 1 and day 3 after CD3/CD28 bead treatment. In contrast, no increase in the percentage of CD200 expressing CLL cells was seen under the same conditions

Collectively the data presented here shows that CLL cells are capable of responding to T-activation, upregulating both activation markers and PDL-1.

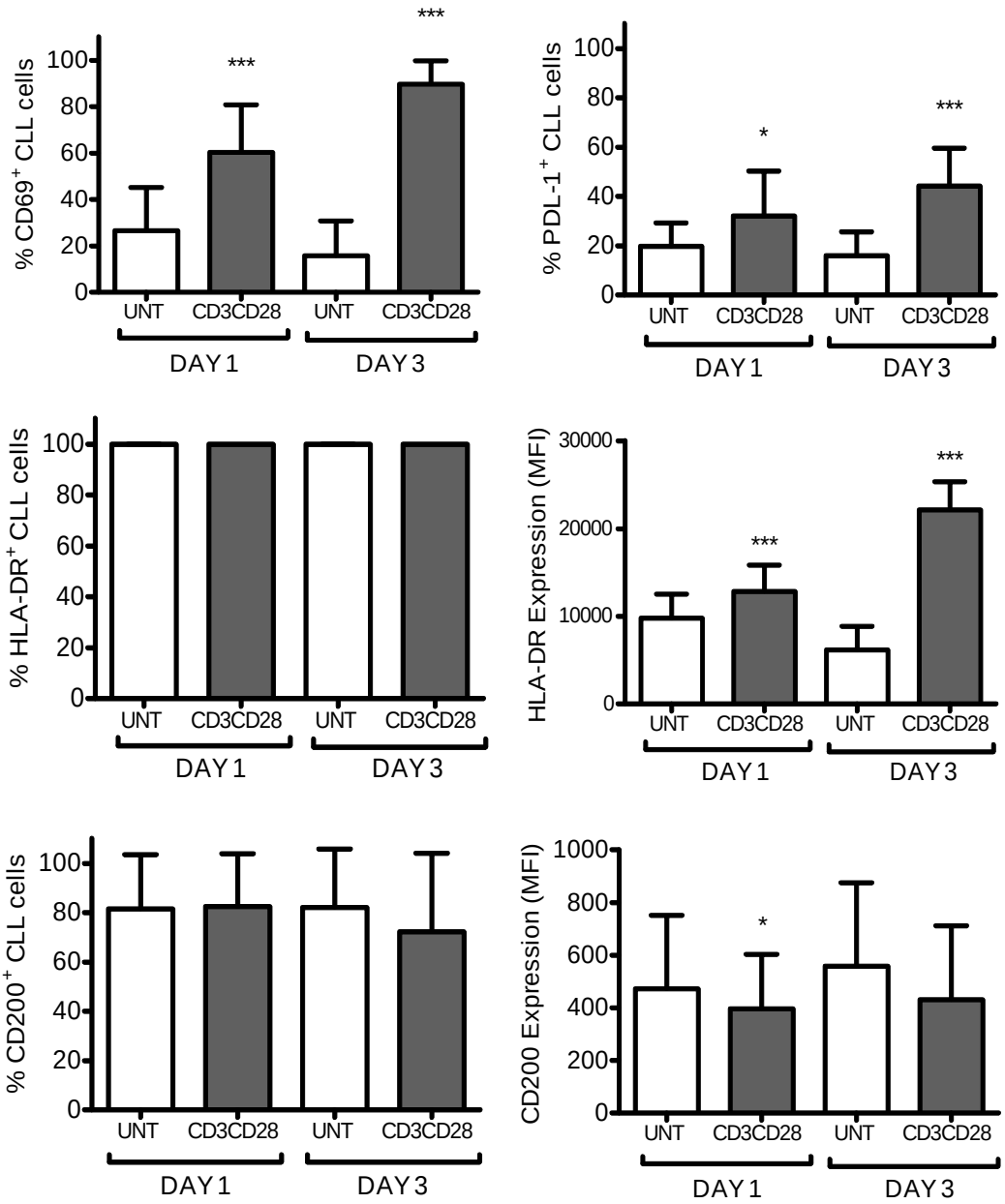


Figure 4.11. The expression of CD69, HLA-DR, PDL-1 and CD200 on CLL cells from PBMC cultures treated with CD3/CD28 beads. The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). Lymphocytes were gated based on forward and side scatter profiles. Expression of PDL-1, CD200, HLA-DR and CD69 were measured on CLL cells defined by expression of CD19 and CD5. The mean and standard divation within each group is shown. Statistical analysis was performed using a paired *t*-test. *= (P<0.05), **= (P<0.01) and ***= (P<0.001). Significance was calculated based on values from CD3/CD28 treated cultures compared to untreated cultures at each time point

4.4 PDL-1 upregulation on CLL cells in response to T-cell activation can be induced by soluble mediators including the cytokines IL-4.

To determine whether soluble factors were responsible for the upregulation of PDL-1 on CLL cells, supernatants from cultures previously treated with CD3/CD28 beads were added to CLL B cells purified by negative selection (Figure 4.12). Incubation of CLL cells with supernatants from CLL PBMC cultures treated with CD3/CD28 beads induced a significant upregulation in CLL cell CD69, HLA-DR and PDL-1 expression compared to controls.

Given that conditioned media was able to induce the expression of PDL-1 on CLL cells, further experiments were carried out to establish which cytokines might be responsible for the activation and upregulation of PDL-1 on CLL cells (Figure 4.13). Both IL-4 ($P=0.005$) and TNF-α ($P=0.03$) were found to significantly increase the level of the activation marker CD69 on the surface of CLL cells. There was also a significant increase in the percentage of PDL-1^+ CLL cells after incubation with IL-4 (5ng/ml) ($P=0.05$) and a trend towards an increase with TNF-α (10ng/ml) ($P=0.09$) and IFN-γ (10ng/ml) ($P=0.09$). In addition, 4 out of 5 patients showed increased percentages of PDL-1-expressing CLL cells in the presence of IL-10 (5ng/ml) but this did not reach significance ($P = 0.11$). Interestingly, there was a significant decrease in PDL-1 expression on CLL cells after the addition of IL-12 (5ng/ml) ($P=0.04$). In contrast, no changes in the percentage of PDL-1-expressing CLL cells were observed after the addition of IL-2 (10 IU/ml) or IL-8 (10ng/ml).

These findings show that CLL cells can respond to T-cell derived cytokines including IL-4 upregulating activation markers and PDL-1 expression.

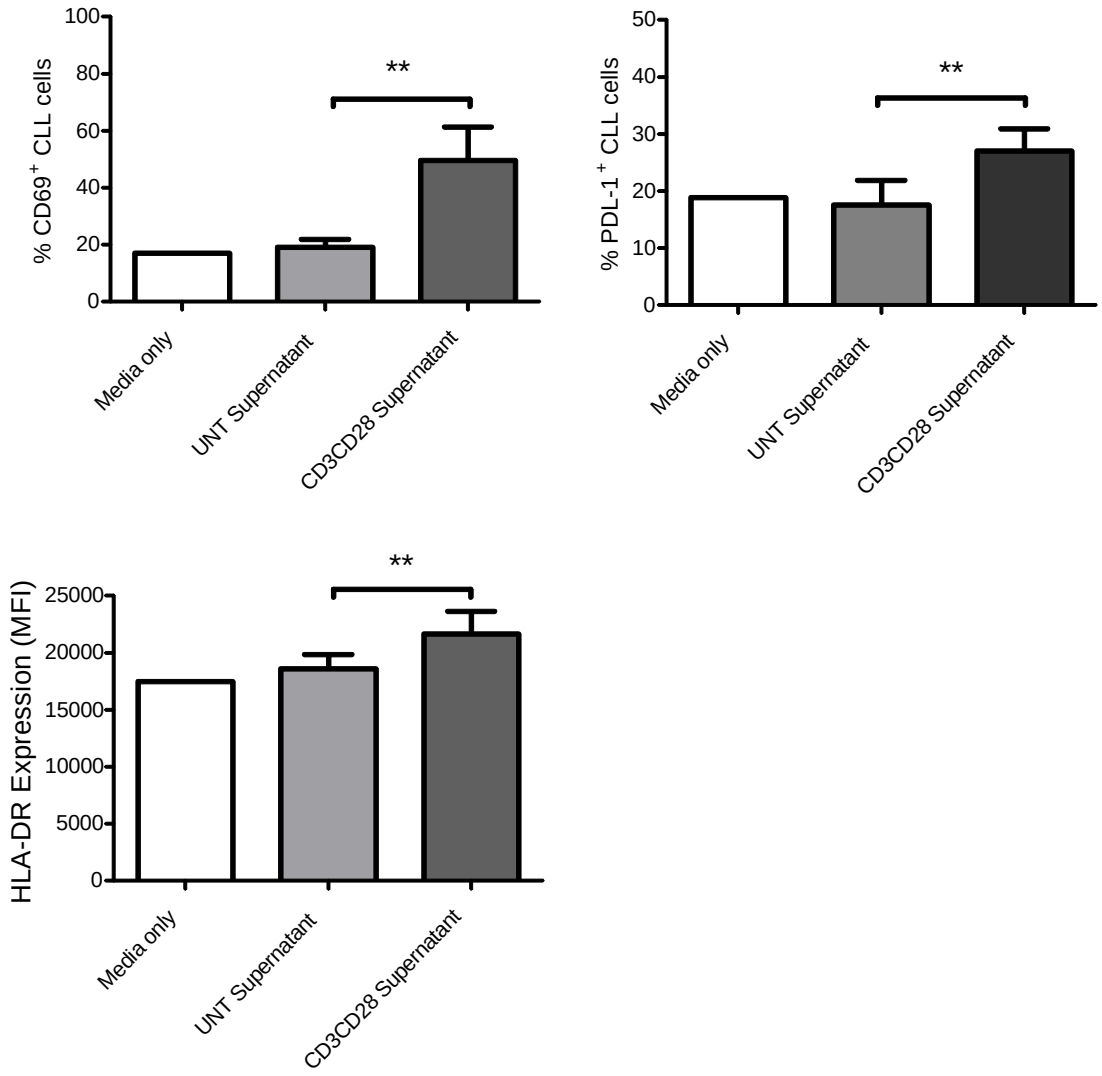


Figure 4.12. Expression of activation markers CD69 and HLA-DR and PDL-1 on CLL cells cultured with conditioned media from PBMC cultures treated with CD3/CD28 beads. Purified B-cells were incubated with conditioned media from PBMC cultures of 5 patients treated with or without CD3/CD28 beads. Lymphocytes were gated based on forward and side scatter profiles. CLL cells were differentiated based on there expression of CD19 and CD5. The mean and standard deviation within each group is shown. The data shown represents one of three independently performed experiments. Statistical analysis was performed using a paired *t*-test. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$).

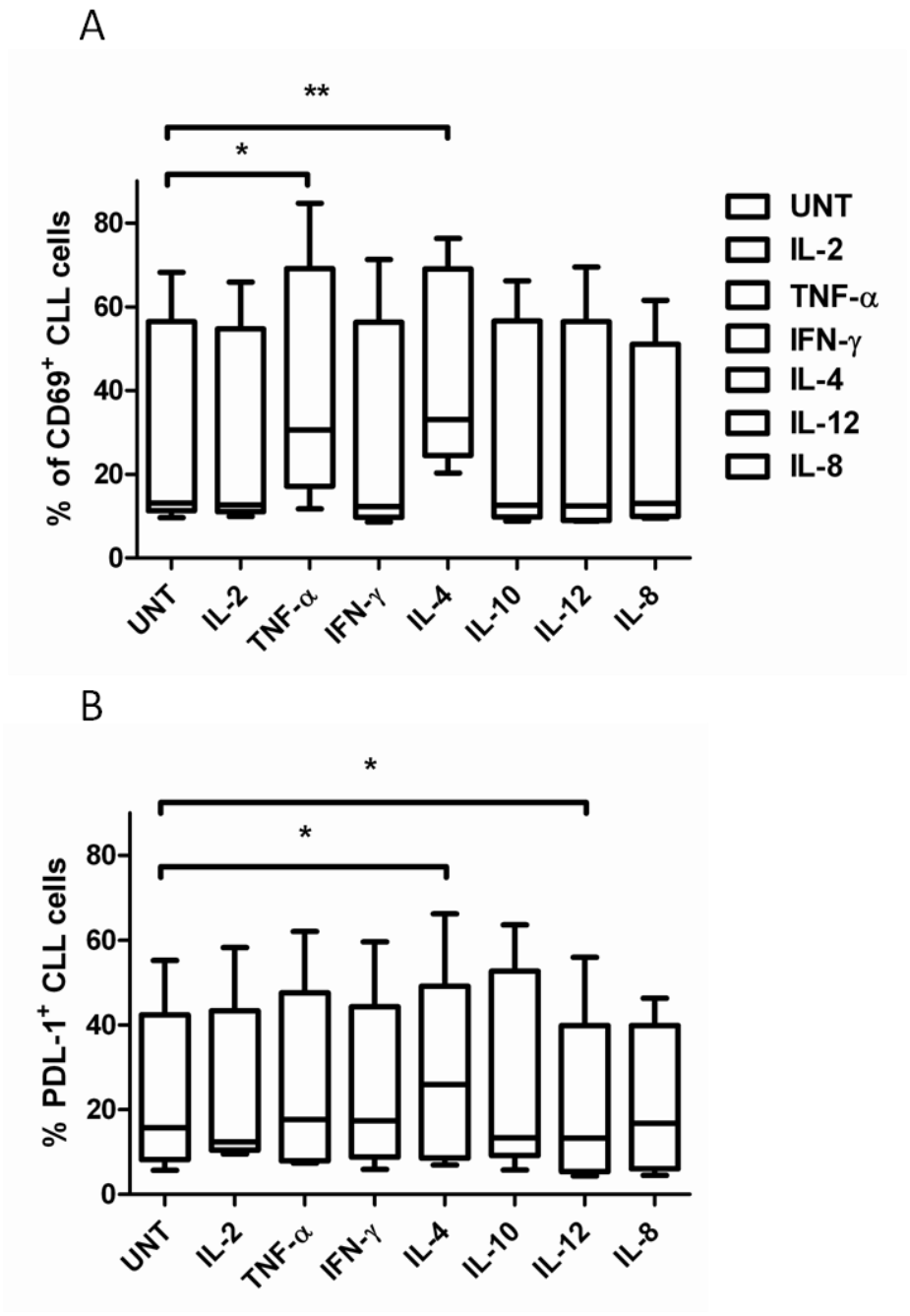


Figure 4.13. CLL cell activation and PDL-1 expression in response to cytokine stimulation. Purified B-cells were incubated for 24h in the presence of absence of various cytokines (IL-4 (5ng/ml), TNF-α (10ng/ml), IFN-γ (10ng/ml), IL-10 (5ng/ml), IL-12 (5ng/ml), IL-2 (10 IU/ml) and IL-8 (10ng/ml). The percentage of $CD69^+$ and $PDL\text{-}1^+$ CLL cells was measured by flow cytometry. CLL cells were gated based on their co-expression of CD19 and CD5 expression. PDL-1 and CD69 expression was then measured within the $CD19^+CD5^+$ gate by calculating the percentage of positive cells above isotype controls. Statistical analysis was performed using a paired *t*-test. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$).

4.5 Increased PDL-1 expression on CLL cells after CD40L stimulation

The lymph node microenvironment is thought to play a key role in promoting the survival and proliferation of CLL cells through tumour cell interactions with accessory cells including T-cells and $CD14^+$ nurse-like cells (Burger and Gandhi 2009). A recent study showed that $PD\text{-}1^+$ T-cells locate close to proliferating CLL cells within the lymph node suggesting PD-1:PDL-1 signalling may have an important role in promoting CLL cell survival and potentially T-cell dysfunction (Yallop et al. 2010). In order to investigate the expression and regulation of immunosuppressive molecules in these areas, CLL PBMCs were co-cultured *in vitro* with a CD40L transfected fibroblast cell line. CD40L trasfected fibroblast cells provide CLL cells with pro-survival and activation signals thought to be similiar to signals received by CLL cells in the lymph node microenvironment. Co-culture of CLL PBMCs on CD40L transfected fibroblasts induced an upregulation of activation markers CD69 and HLA-DR and immunosuppressive molcules PDL-1 and CD200 on CLL cells when compared to liquid culture and non transfected fibroblast controls (Figure 4.14).

These results suggest that CD40L stimulation can directly upregulate PDL-1 and CD200 *in vitro*. In turn this suggests that CD40L interactions in the lymph node microenvironment could contribute to high expression of immunosuppressive molecules on CLL cells.

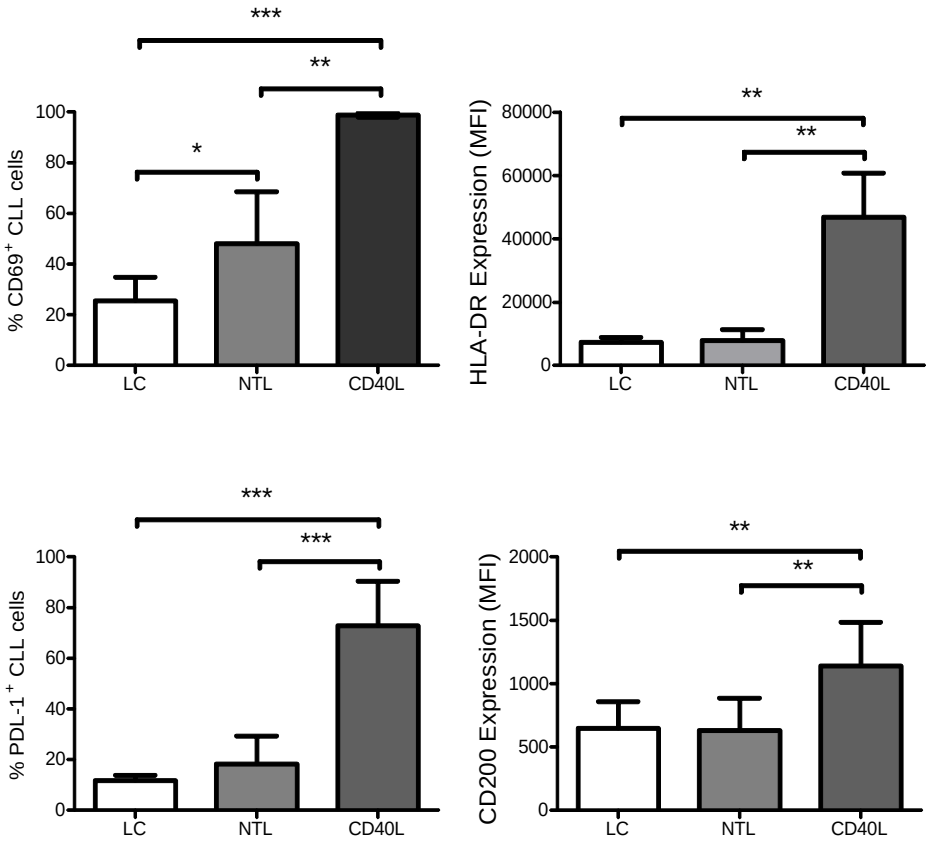


Figure 4.14. Expression of CD69, HLA-DR, CD200 and PDL-1 on CLL cells after co-culture with NTLs or CD40(TL)s. PBMCs from CLL patients were incubated in liquid culture (LC) or co-cultured on non-transfected fibroblast cells (NTL) or fibroblast cells transfected with CD40L (CD40L(TL)). The expression of CD69, HLA-DR, PDL-1 and CD200 on CLL cells was measured after 24h by flow cytometry. CLL cells were gated based on their expression of CD19 and CD5 expression. Statistical analysis was performed using a paired *t*-test. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$).

4.6 Activation of CLL cells does not induce PD-1 or PDL-1 expression on bystander T-cells

Given that T-cell activation could induce PDL-1 expression on bystander CLL cells, this study looked at whether CLL cell activation could promote PD-1 expression on T-cells. T-cells from CLL PBMC cultures incubated in the presence of CD40L transfected fibroblast cells were analysed for their expression of activation markers CD69 and HLA-DR and

immunosuppressive molecules PD-1 and PDL-1 (Figure 4.15). Analysis of T-cells in these PBMC cultures showed that there was no increase in $CD69^+$ or HLA-DR^+ $CD4^+$ T-cells in response to CLL stimulation with CD40L. In contrast, there was a significant increase in the percentage $CD8^+$ T-cells expressing CD69 and HLA-DR markers (CD69: P = 0.02 and HLA-DR: P = 0.02 respectively).

Analysis of both $CD4^+$ and $CD8^+$ T-cells showed no increase in PD-1 expression following CD40L stimulation of CLL cells in PBMC cultures compared to contols. As PDL-1 may also play a role in suppressing T-cells in CLL, PDL-1 expression on T-cells was also measured following CD40L stimulation of CLL cells. The percentage PDL-1^+ T-cells remained relatively unchanged between PBMC incubated in liquid culture and those co-cultured on CD40L(TL).

These data demonstrate that CLL cell activation by itself is unable to promote PD-1 or PDL-1 expression on T-cells. It does however provide evidence that CD40L stimulated CLL cells have an activating effect on $CD8^+$ but not $CD4^+$ T-cells..

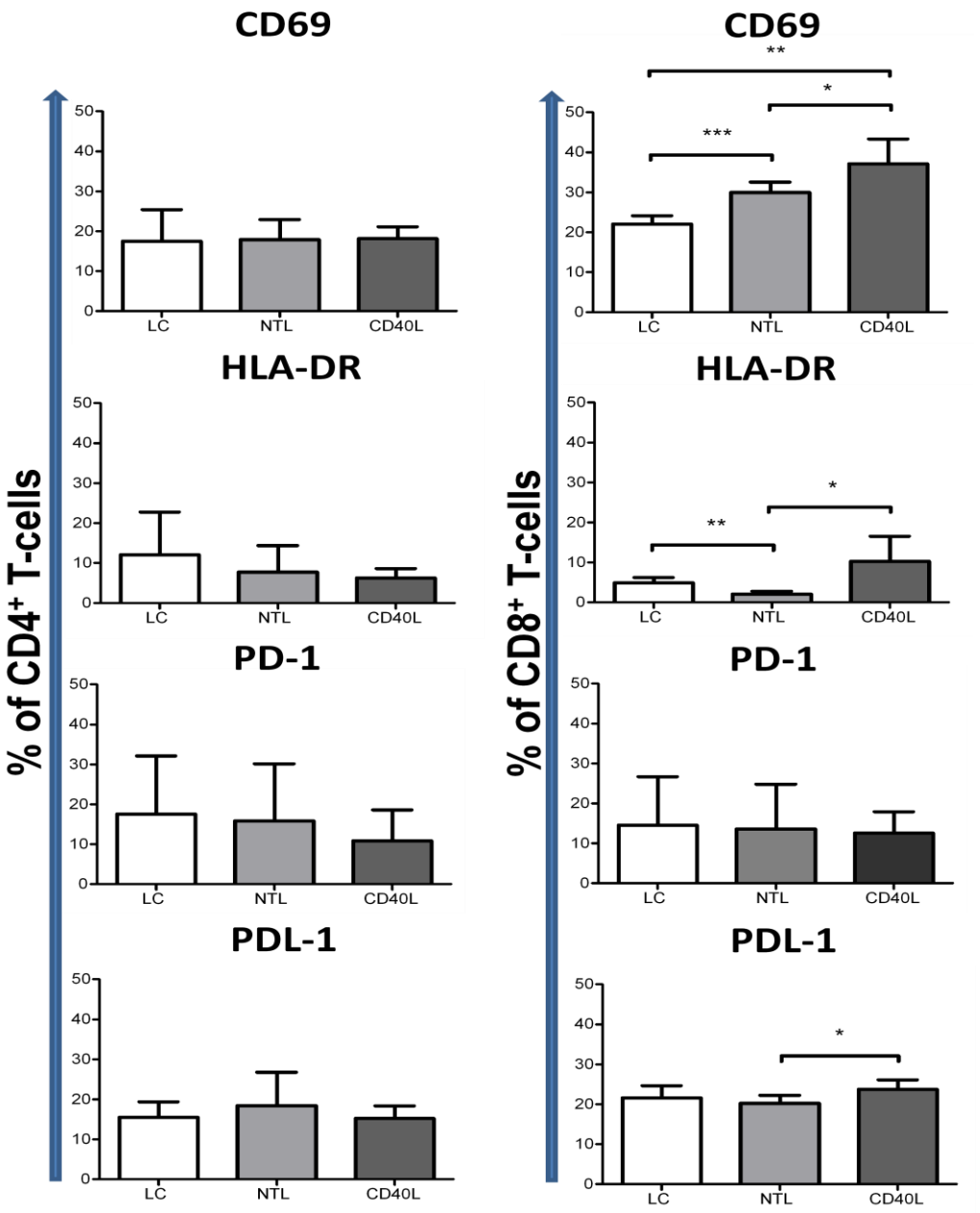


Figure 4.15. Expression of activation markers CD69 and HLA-DR and immunosuppressive molecules PD-1 and PDL-1 on T-cells from CLL PBMC co-cultured with a CD40L transfected cell line. PBMCs from CLL patients were incubated in liquid culture (LC) or co-cultured on non transfected fibroblast cells (NTL) or fibroblast cells transfected with or CD40L (TL(CD40L)). The expression of CD69, HLA-DR, PDL-1 and PD-1 on T-cells was measured after 24h by flow cytometry. T-cells were gated based on their expression of CD3 and either CD4 or CD8 surface markers. PD-1, HLA-DR, PDL-1 and CD69 expression was then measured on $CD4^+$ and $CD8^+$ T-cells by calculating the percentage of positive cells above isotype controls. Statistical analysis was performed using a paired *t*-test. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$).

4.7 PD-1 and CD200 blockade does not enhance the activation of T-cells from PBMC cultures treated with CD3/CD28 beads

Based on the immunosuppressive effects of CD200 and PD-1/PDL-1 in other experimental systems (Tsushima et al. 2007, Zhang et al. 2009a, Pallasch et al. 2009, Kretz-Rommel et al. 2008) blocking antibodies against CD200 and PDL-1 were assessed for their ability to enhance T-cell responses in CLL PBMC cultures treated with CD3/CD28 beads (Figure 4.16). Neither PD-1 or CD200 blockade had any effect on the expression of the activation marker CD69 on $CD4^+$ and $CD8^+$ T-cells following CD3/CD28 stimulation (Figure 4.16 A-B). Analysis of $CD4^+$ and $CD8^+$ T-cell intracellular IFN-γ expression after CD3/CD28 stimulation showed no increase after PDL-1 blockade. In contrast, CD200 blockade led to a significant reduction in intracellular IFN-γ production in $CD8^+$ T-cells (P=0.05) and a trend towards a reduction in $CD4^+$ T-cells (P=0.07) when compared to isotype control cultures (Figure 4.16 C-D). Surface CD107 expression was investigated as a marker of $CD8^+$ T-cell cytotoxic granule release. There was no significant change in $CD8^+$ T-cell CD107 expression following CD3/CD28 stimulation in the presence of PD-1 or CD200 blocking antibodies when compared to isotype controls (Figure 4.16 E).

These findings suggest that PD-1-PDL-1signalling blockade in CLL PBMC cultures does not effect T-cell activation or IFN- γ expression. CD200 blockade however significantly inhibited IFN- γ expression in $CD8^+$ T-cells.

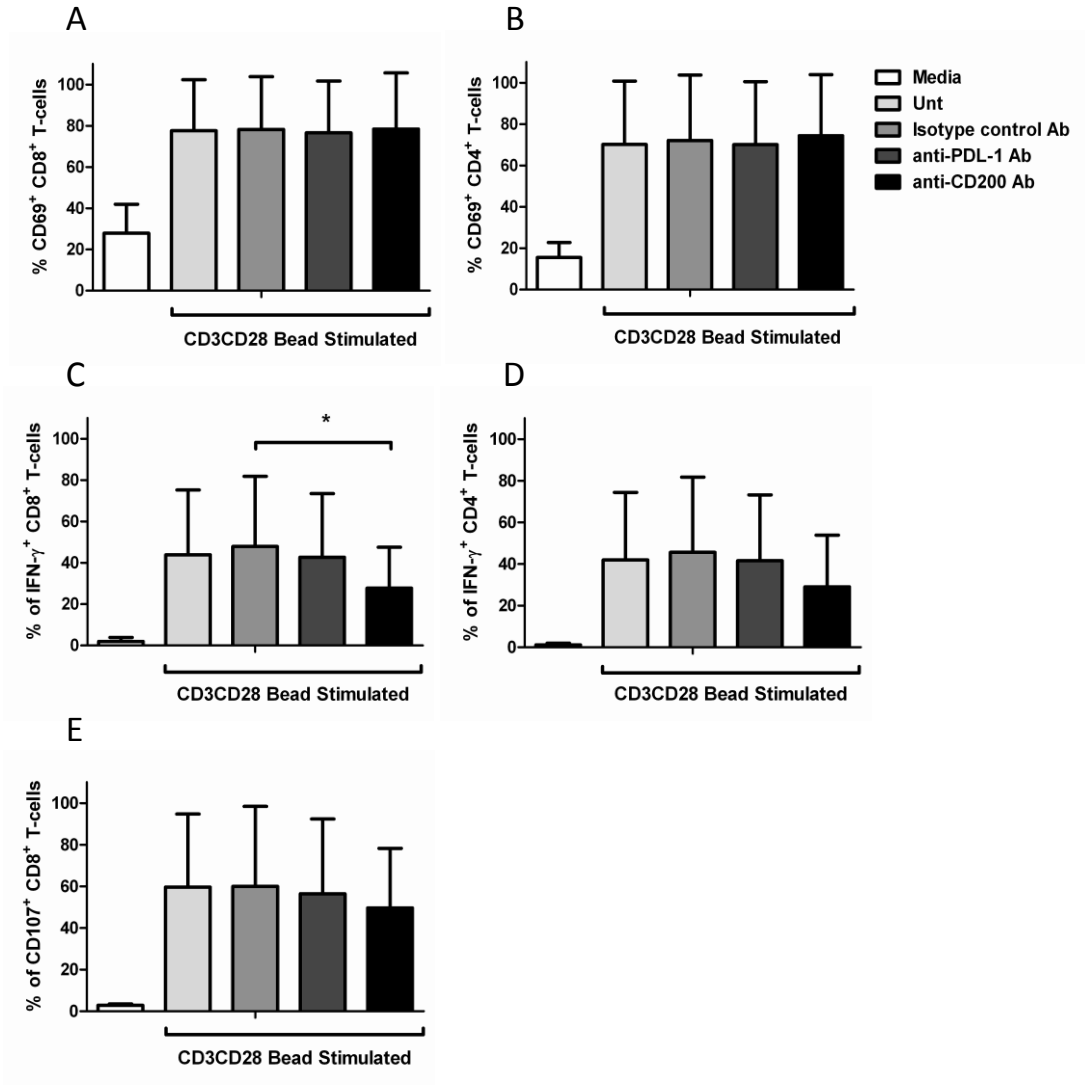


Figure 4.16. T-cell responses to CD3/CD28 stimulation in the presence of PDL-1 and CD200 blocking antibodies. T-cells from CLL PBMC cultures were stimulated with CD3/CD28 beads and incubated in the presence or absence of a PD-1 or CD200 blocking antibody for 48h. The expression of the activation marker CD69 (A-B) and intracellular expression of IFN-γ (C-D) were measured on $CD4^+$ and $CD8^+$ T-cells. In addition, surface expression of CD107 was measured on $CD8^+$ T-cells (E). Statistical analysis was performed using a paired *t*-test. *= (P<0.05), **= (P<0.01) and ***= (P<0.001).

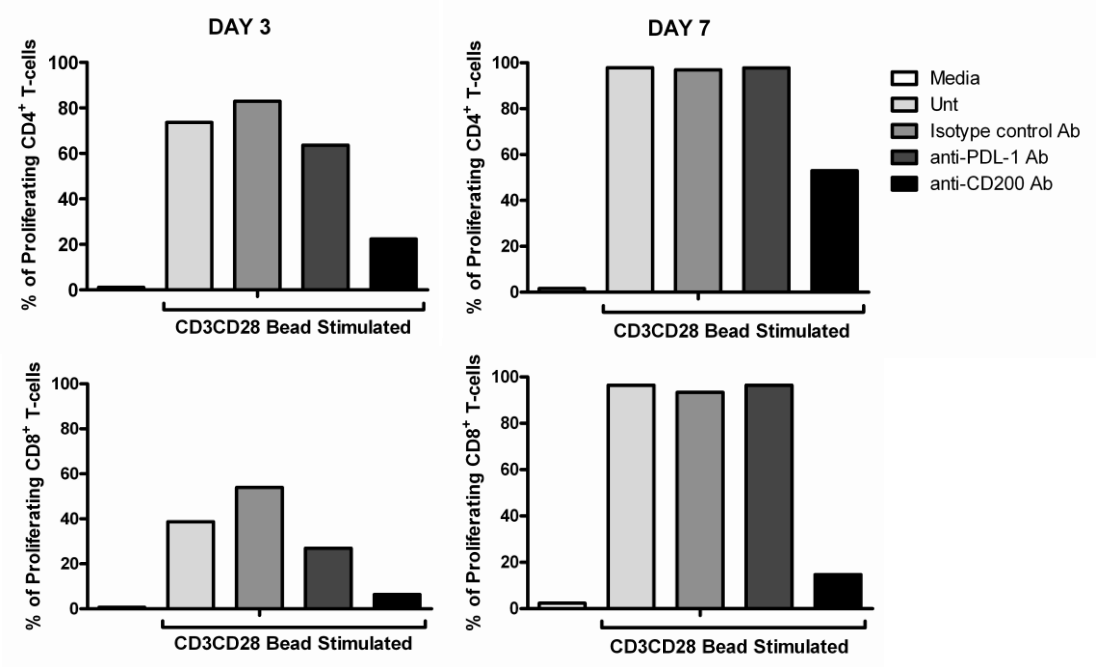


Figure 4.17. Proliferation of T-cells in response to CD3/CD28 stimulation in CLL PBMC cultures treated with a CD200 or PDL-1 blocking antibody. CFSE labelled CLL PBMC were stimulated with CD3/CD28 beads and incubated for 3 or 7 days in the presence or absence of CD200 or PDL-1 blocking antibodies. The percentage of proliferating T-cells was measured based on the percentage of CFSE dim T-cells in blocking antibody treated cultures.

4.8 PD-1 blockade and CD200 blockade have differential effects on T-cell proliferation

To further investigate the effects of PDL-1 and CD200 on T-cells in CLL, T-cell proliferation was assessed as a more stringent measure of T-cell activation. CLL PBMC were loaded with CFSE, stimulated with CD3/CD28 beads and incubated in the presence of PDL-1 and CD200 blocking antibodies (Figure 4.17). At day 3 both $CD4^+$ or $CD8^+$ T-cells show reduced proliferative responses to CD3/CD28 beads in the presence of a PDL-1 or CD200 blocking antibody when compared to an isotype control. By day 7 no difference in the percentage of proliferating $CD4^+$ or $CD8^+$ T-cells were observed in cultures receiving the

PDL-1 blocking antibody compared to the isotype control. In contrast reduced levels of proliferating $CD4^+$ and $CD8^+$ T-cells were seen after 7 days of incubation with a CD200 blocking antibody in comparison to an isotype control antibody.

These data indicates that PD-1-PDL-1 interactions are not involved in regulating T-cell proliferative responses or other T-cell functions in this CLL PBMC system. However, blockade of CD200-CD200R interactions appears to affect at least two T-cell functions (proliferation and IFN-γ secretion) in $CD8^+$ T-cells.

4.9 Discussion

CLL cells possess multiple mechanisms of escaping T-cell mediated immunity including the secretion of immunosuppressive cytokines, recruitment of regulatory T-cells and reduced capacity to act as APCs (Riches et al. 2010). In the last decade another immunosuppressive mechanism has come to light, the discovery of co-inhibitory molecules. Expressed on both tumour cells and immune effector cells, these molecules have the ability to suppress anti-tumour immunity and may hamper current immunotherapeutic strategies for the treatment of haematological malignancies including transplantation, adoptive T-cell therapy and vaccination (Norde et al. 2012). In this study the expression, regulation and function of the immunoinhibitory molecules PD-1 and CD200 were investigated in CLL patients.

CD200 has been shown to be over expressed in a number of cancers where CD200:CD200R signalling is thought to have a role in regulating anti-tumour immunity including down regulation of Th1 type immune responses (Moreaux et al. 2006, Coles et al. 2011). In accordance with other studies CD200 was shown to be over expressed on $CD19^+CD5^+$ B-cells from CLL patients compared to healthy age-matched controls

(McWhirter et al. 2006). Furthermore this study showed that CD200 is over expressed on other cell types in CLL including T-cells and NK cells. The significance of CD200 on these cell types remains to be determined but their presence shows the potential for CD200-CD200R signalling in CLL, involving multiple immune effector cells. CD200 signalling is mediated through the binding to its receptor CD200R found on monocytes/macrophages and T-lymphocytes (Rygiel and Meyaard 2012). The expression of CD200R was measured on T-cells from CLL patients and found to be slightly but not significantly increased in comparison to healthy donors. This suggests that CD200 over expression on tumour cells may be the main factor driving CD200-CD200R signalling to T-cells in CLL. The CD200-CD200R inhibitory pathway may also act through accessory cells such as DC and monocytes shifting Th1 to Th2 cytokine profiles and indirectly effecting T-cell effector function (Gorczynski et al. 1999). It would be interesting to analyse the expression of CD200R on these cell types in CLL patients to determine if CD200-CD200R signalling may be contributing to their immunosuppressed state.

PD-1 is an immunoinhibitory molecule which, following ligation to its ligand PDL-1, negatively regulates T-cell activation (Sheppard et al. 2004). The role of PD-1 in CLL is unknown but phenotypic analysis carried out in this study showed that there was a significant increase in PD-1 expression on $CD19^{+}CD5^{+}$ B-cells from CLL patients, which confirmed results obtained from previous studies (Xerri et al. 2008, Grzywnowicz et al. 2012). This study extended these observations by showing that PD-1 is increased on both $CD4^{+}$ and $CD8^{+}$ T-cells from CLL patients. In depth analysis of T-cell subsets revealed PD-1 expression was predominately increased in EM and CM $CD4^{+}$ and $CD8^{+}$ T-cell compartments. These results reveal the existence a large subset of antigen experienced T-cells in CLL patient that may be subject to tolerisation involving PD-1-PDL-1 signalling. PD-1 expression has already been

associated with exhaustion/tolerisation of T-cells during chronic viral infections (Day et al. 2006). Increased expression of PD-1 has been observed on virus-specific $CD8^+$ T-cells in HIV patients and on $CD8^+$ TEM/TEMRA during chronic HCV infection where it has been shown to correlate with viral load (Trautmann et al. 2006, Shen et al. 2010).

As PD-1 signalling is dependent on interactions with its ligand PDL-1, its expression was measured on B and T-cells from CLL patients. Interestingly this study showed a significant decrease in PDL-1 on $CD19^+CD5^+$ B-cells from CLL patients compared to healthy controls. This result was quite unexpected given that PDL-1 has been shown to be upregulated in solid tumours including breast cancer when compared to normal tissue (Keir et al. 2008). A previous report in 44 CLL patients however documented similar results to this study, showing PDL-1 expression on CLL cells was slightly decreased when compared with healthy donors albeit not significantly (Grzywnowicz et al. 2012). Despite this, PDL-1 expression was detected on over 50% of CLL cells from patients in this study and given the abundance of CLL cells compared to T-cells in CLL patients, the potential for PDL-1-PD-1 signalling would appear to be higher in CLL patients than healthy donors.

Several studies have reported the potential for bi-directional signalling upon PD-1 and PDL-1 interactions. PD-1 engagement with PDL-1 on DCs inhibited DC activation and increased IL-10 production (Kuipers et al. 2006). Similarly increased production of IL-10 has been reported from $CD4^+$ T-cells expressing PDL-1 in the presence of soluble PD-1 (Dong et al. 2003). IL-10 is a cytokine that has previously been shown to inhibit T-cell activation and proliferation promoting T-cell tolerance (Akdis and Blaser 2001). The observation that PDL-1 is significantly increased on T-cells derived from CLL patients suggests that this negative regulatory pathway may also play a role in T-cell inhibition in CLL.

The PD-1 and CD200 pathways have emerged as potentially important pathway utilised by tumour cells to avoid detection by the immune system. Understanding the mechanisms that regulate the expression of these immunoinhibitory molecules may reveal effective strategies to block such pathways in CLL. PD-1 was shown to be upregulated on CLL patient T-cells after activation and appears to be a late activation marker when compared to HLA-DR and CD69. These results support the initial finding that PD-1 is predominantly expressed on T-cells displaying an antigen-experienced memory phenotype. A previous report suggested that T-cell PD-1 expression in response to acute activation is a transient effect and that these T-cells may differ from truly tolerised T-cells that continuously express PD-1 (Grosso et al. 2009). Further characterisation of PD-1^+ T-cells from CLL patients, including a systematic analysis of exhaustion markers and telomere lengths, may help to differentiate between recently activated T-cells and truly exhausted T-cells in CLL patients.

In CLL PD-1^+ T-cells are found at higher frequencies in the lymph nodes than peripheral blood where they localise close to proliferating CLL cells (Yallop et al. 2010). Such interactions may be crucial for mediating the suppression of T-cells at these sites but may also play an important role in supporting the survival and proliferation of CLL cells. This study investigated the expression of immunosuppressive molecules on CLL cells in a co-culture system in order to mimic the pro-survival conditions of a CLL lymph node. CLL cells incubated in these pro-survival co-culture conditions upregulated both PDL-1 and CD200 expression. These results indicate that PD-1-PDL-1 and CD200-CD200R signalling pathways may be highly prevalent in pro-survival microenvironmental niches.

Given that lymph node germinal centres represents an important area for T-cell recognition and activation in response to antigen, this study further investigated whether T-cell activation altered the phenotype of bystander CLL cells. CD3/CD28 stimulation of T-

cells led to an activation of bystander tumour cells and an upregulation of PDL-1 expression but not CD200 on the CLL cell surface. Further investigation revealed that CLL activation was driven by the T-cell derived cytokines TNF-α and IL-4, whilst PDL-1 expression was driven by IL-4 and possibly IFN-γ, TNF-α and IL-10. An interesting additional experiment for this study would be to use blocking antibodies for different cytokines or their receptors to confirm the ability of these cytokines to activate and upregulate PDL-1 on CLL cells *in vitro*.

Overall these results indicate that CLL cells are capable of reacting to T-cell activation by upregulating immunoinhibitory molecules such as PDL-1; possibly to suppress tumour specific T-cell recognition, activation and lysis of CLL cells. Indeed, a recent study showed that CD200 and PDL-1 may play a key role in synapse dysfunction between leukaemic cells and T-cells, impairing CTL effector function (Ramsay et al. 2012). In addition, PD-1-PDL-1 signalling may play an important role in germinal centre B-cell survival, whereby B-cell PDL-1 engagement with PD-1 on follicular helper T-cells promotes IL-4 and IL-21 production aiding B-cell survival (Good-Jacobson et al. 2010). Increased numbers of IL-4 producing T-cells have been previously documented in CLL with IL-4 shown to enhance CLL cell survival *in vitro* (Scrivener et al. 2003, Mainou-Fowler and Prentice 1996). Collectively, these studies indicate that the PD-1-PDL-1 signalling pathway may have a multi-functional role in the pathogenesis of CLL not only aiding T-cell suppression but also potentially supporting the survival of the CLL clone.

Therapeutic strategies including the use of blocking antibodies and si-RNA knockdown of immunoinhibitory molecules are currently under investigation to enhance both anti-viral and anti-tumour immunity (Norde et al. 2012). Previous work in chronic viral infections, including HIV, have demonstrated the potential for PD-1-PDL-1 signalling blockade to enhance the proliferative and cytokine responses of HIV-specific PD-$1^{+}$$CD8^{+}$ T-cells *in vitro*

(Trautmann et al. 2006, Barber et al. 2006). PD-1 blocking antibodies has also shown efficacy in murine leukaemia models and in phase 1 clinical trials in solid cancers and haematological malignancies (Zhang et al. 2009a, Brahmer et al. 2010, Berger et al. 2008). Although CLL patients have increased percentages of PD-1^+ T-cells this study showed that PDL-1 blockade did not enhance T-cell effector function or proliferative responses in CLL PBMC cultures stimulated with CD3/CD28 beads. This result is not completely unexpected given that a previous study by Salih *et al* showed no enhancement of allogeneic T-cell responses to leukaemia cells after PDL-1 blockade (Salih et al. 2006). In addition a phase 1 clinical trial of a PD-1 blocking antibody in patients with advanced haematological malignancies, including several CLL patients, showed no increase in activated ($CD69^+$) lymphocytes or serum pro-inflammatory cytokines including IFN-γ and TNF-α following PD-1 blockade. This was despite describing clinical benefit in 33% of patients (Berger et al. 2008). The failure to enhance T-cell responses following PDL-1 blockade in the current *in vitro* system may be due to several factors. Firstly, it is possible that immunosuppression mediated through PD-1 signalling may involve multiple immune and non-immune cells which may not be represented in the *in vitro* model. Secondly, success of PDL-1 blockade may require prior conditioning regimens to reduce the CLL tumour burden and remove suppressive cell populations including Tregs and myeloid-derived suppressor cells. In the clinical trial documented above, all CLL patients received lymphodepleting therapy, including irradiation and chemotherapy, prior to administering of the PD-1 blocking antibody (Berger et al. 2008). Lastly blockade of the PD-1-PDL-1 signalling pathway may not be sufficient by itself to break the suppression of T-cell responses in CLL patients. Exhausted T-cells have been shown to upregulate several different immunoinhibitory molecules, thus the efficacy of PD-1 may be improved through combination therapy with other blocking antibodies targeting immunosuppressive molecules such as CTLA-4, LAG-3 and TIM-3.

Alternatively PDL-1 blocking antibodies may be better utilised to improve the efficacy of other treatment modalities aimed at promoting anti-tumour T-cell responses in cancer patients including vaccines, bi-specific antibody therapy and adoptive T-cell therapy.

When the effect of a CD200 blocking antibody on T-cell responses was assessed in CLL PBMC cultures stimulated with CD3/CD28 beads, CD200 blockade inhibited T-cell effector function and proliferation. These data may indicate that CD200 is acting as a co-stimulatory molecule in CLL rather than a co-inhibitory molecule. These results are somewhat contradictory to those previously published by Pallash *et al* which showed an enhancement of T-cell proliferation in response to CD40L stimulated CLL cells and an anti-CD200 blocking antibody (Pallasch et al. 2009). The disparity between these results may be due to difference in the ability of each blocking antibody to antagonize CD200-CD200R signalling. In addition CD200 is known to be upregulated on activated T-cells, which may make them susceptible to ADCC mechanisms in the presence of certain CD200 blocking antibodies. A report by Kretz-Rommel *et al* demonstrated the potential for CD200 blocking antibodies to induce ADCC of activated human T-cells *in vitro*. Interestingly when they engineered the C region of the antibody from IgG1 to a hybrid IgG2/G4 to minimise ADCC and CDC effector functions, no lysis of activated T-cells was observed (Kretz-Rommel et al. 2008). Analysis of cell counts from this study's blocking experiments revealed reduced cell numbers in cultures receiving the CD200 blocking antibody compared to those receiving the isotype control. Thus it is possible that ADCC of activated T-cells expressing CD200 may be occurring in cultures receiving the CD200 blocking antibody in this study. These observations suggest that caution should be used when selecting or engineering blocking antibodies targeting immunosuppressive molecules, in order to minimise ADCC of T-cells

critical for anti-tumour immunity as well as normal cells expressing CD200 including neural cells and glomerular cells (Wright et al. 2001).

Unfortunately due to time and funding constraints it was impossible to perform a dose response with the blocking antibodies or time course experiments. The results of such experiments could provide important information as to whether such antibodies could enhance T-cell responses in CLL. It may also be interesting to alter the experimental set-up to assess the effects of immunosuppressive molecule blockade. It is possible that the PD-L1 blocking antibody used was not capable of effectively blocking PD-1-PDL-1 signalling in this system and therefore other PDL-1 blocking antibodies should be tested. In addition the CD3/CD28 stimulus given in this study may be optimal and independent on interactions with CLL cells, therefore preventing enhancement of T-cell responses upon blockade of PD-1. However, the results obtained in this chapter suggest that T-cell proliferative and IFN-γ responses to CD3/CD28 beads can be at least be affected by CD200 blockade. It may be better to assess the effects of PD-1 blockade on T-cells sub-optimally activated with an agonist anti-CD3 antibody and titrated doses of an anti-CD28 antibody. In addition PD-1 T-cells from CLL patients could be flow sorted and used in experiments rather than trying to activate and block PD-1 using a mixed population of T-cells in CLL PBMC cultures; which may mask any enhancement of $PD\text{-}1^{+}$ T-cell responses. Ultimately, the effects of PD-1 blockade on T-cell responses in CLL may be more appropriately tested *in vivo*, either in an animal model or in clinical trials.

In summary, this chapter documents the over expression of the immunosuppressive molecules PD-1 and CD200 in CLL patients. Furthermore, it demonstrates the potential for immunosuppressive molecules and their ligands to be upregulated following activation of both T-cells and CLL cells. Blockade of PD-1-PDL-1 signalling however failed to enhance

T-cell responses *in vitro* whilst CD200-CD200R blockade actually suppressed the response of T-cells to CD3/CD28 stimulation. Finally this work highlights the need for caution when selecting blocking antibodies targeting immunosuppressive molecules for therapeutic use in CLL.

CHAPTER 5

Breaking T-cell tolerance in CLL patients using a bi-specific antibody targeting CD3 and CD19

CLL cells are known to possess multiple immunosuppressive mechanisms aimed at preventing T-cell activation including defects in immune synapse formation, cytokine production and abnormal co-stimulatory and co-inhibitory molecule expression (Riches et al. 2010). These factors have made it a challenging disease for the successful application of T-cell immunotherapy. Despite this, several therapeutic strategies aimed at promoting tumour-specific T-cell responses *in vivo* have been developed including, vaccine therapies with dendritic cells pulsed with CLL cell lysates and adoptive T-cell therapy including CD3/CD28 activated T-cells and T-cells genetically-modified to express chimeric antigen receptors for CD19 (Hus et al. 2005, Porter et al. 2011, Foster, Brenner and Dotti 2008). The widespread application of such therapies has however been limited due to several factors including technical challenges of *ex vivo* expansion/genetic modification of cells and the cost of preparing such 'personalised' therapies. Unlike pharmacologically available drugs, which can be processed at one site and supplied to many patients, such 'personalised' therapy may require local production at specialised cell processing facilities and can only be applied to individual patients (Kohn et al. 2011).

Over the last 20 years monoclonal antibodies such as rituximab, cetuximab and trastuzumab have merged as effective therapeutics for the treatment of various cancers (Dougan and Dranoff 2009). The relative success of these antibodies has led to a drive

towards designing novel antibody therapies including the development of bi-specific antibodies. Blinatumomab is a bi-specific single chain antibody construct classified as a bi-specific T-cell engager (BiTE). It is formed by the fusion of an anti-CD3 single chain variable fragment (scFV) to an anti-CD19 scFV via a short peptide linker. These bi-specific antibodies can retarget T-cells expressing CD3 towards tumour cells expressing CD19, resulting in activation of cytolytic response mechanisms in T-cells (Chames and Baty 2009). Blinatumomab has already shown clinical activity in a phase II trial in relapsed acute lymphoblastic leukaemia with 80% of patients achieving a complete molecular response (Topp et al. 2011) and in a phase I study in non-Hodgkin lymphoma (NHL) with high response rates seen in both patients with indolent and aggressive disease (Bargou et al. 2008). In addition ongoing phase II clinical trials are assessing the efficacy and safety of blinatumomab in relapsed/refractory diffuse large B-cell lymphoma (DLBCL) due for completion 2014.

The objectives of this chapter are to assess the potential use of blinatumomab as an immunotherapeutic agent in CLL. Several flow cytometry based assays will be used to determine whether blinatumomab can overcome the tolerance of CLL patients' T-cells *in vitro*. This will include measuring T-cell activation, proliferation and cytotoxic response to blinatumomab treatment in both treatment naïve patients and patients that have relapsed or are refractory to current chemoimmunotherapeutic regimens.

5.1 Blinatumomab activates T-cells from CLL patients

In order to assess whether blinatumomab could activate T-cells from CLL patients, CLL PBMCs were incubated in culture with or without the bi-specific antibody. Two concentration of blinatumomab (10 and 100ng/ml) were chosen for testing based on

pharmacological doses used in clinical trials in NHL and ALL. T-cells from these cultures were then analysed for their expression of activation markers at day 3 and day 7. T-cells from blinatumomab treated cultures showed an increase in the expression of the activation markers CD38 and HLA-DR ($CD4^+$: Figure 5.1A-B, $CD8^+$: 5.1D-E) by day 3, which further increased by day 7. Analysis of surface CD69 expression on T-cells showed it was also increased, peaking at day 3 on $CD4^+$ (Figure 5.1C) and $CD8^+$ T-cells (Figure5.1F).

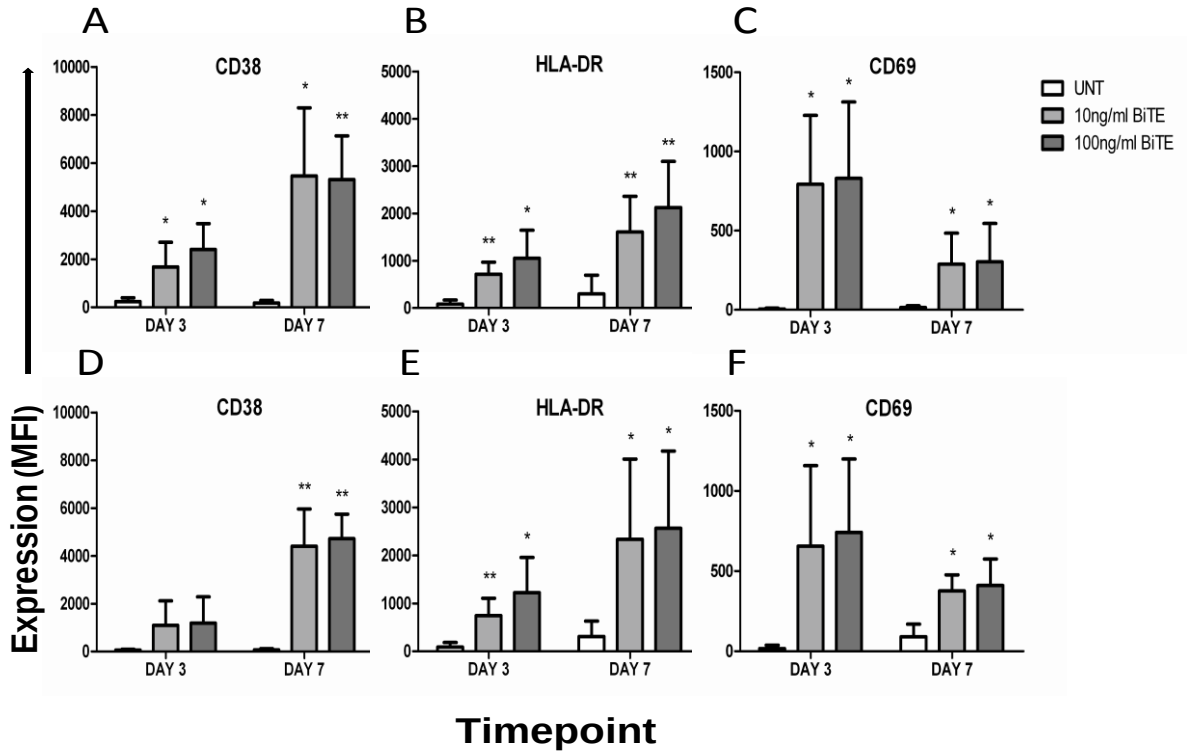


Figure 5.1. Expression of activation markers CD38, HLA-DR and CD69 on $CD4^+$ (A-C) and $CD8^+$ T-cells (D-F) after blinatumomab treatment (n=4). Activation markers were measured on T-cells from CLL PBMC cultures after 3 or 7 days by 8-colour flow cytometry (FACS Canto II). Statistical analysis was performed using a paired *t*-test comparing the level of activation markers in blinatumomab treated cultures (10 or 100ng/ml) with untreated controls. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$).

5.2 Blinatumomab induces T-cell dependent CLL cell activation.

Given that blinatumomab can activate T-cells from CLL patients, we went on to assess whether CLL cells were also being activated in response to blinatumomab treatment of CLL PBMCs. CLL cells were found to have an increased expression of the activation marker HLA-DR (Figure 5.2A and C) by day 3 in treated PBMC cultures. To determine whether this CLL activation was T-cell dependent, purified CLL cells were also incubated with blinatumomab and the expression of HLA-DR assessed. No significant increase in CLL cell HLA-DR expression was observed after incubation of purified CLL cells with blinatumomab (Figure 5.2B).

Overall these results demonstrate that blinatumomab can activate T-cells and CLL cells in PBMC cultures. The activation of CLL cells was subsequently shown to be T-cell dependent.

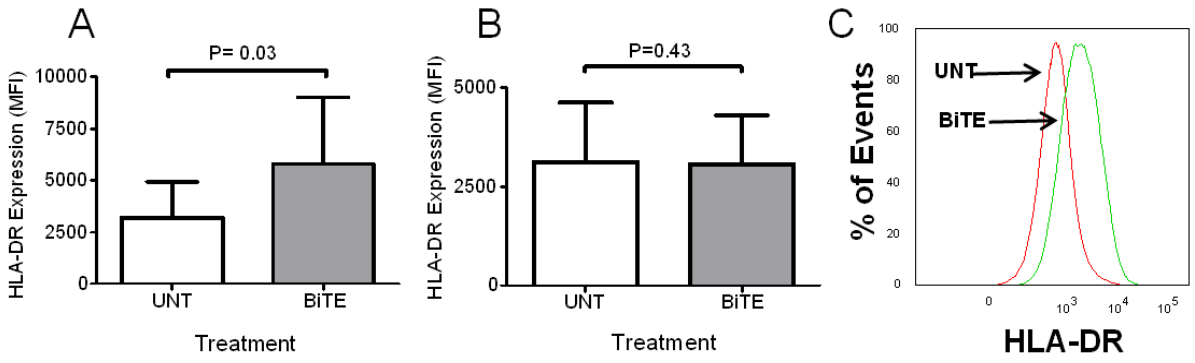


Figure 5.2. Activation of CLL cells in PBMC cultures treated with blinatumomab. Expression of HLA-DR on CLL cells taken from (A) PBMC or (B) purified B-cell cultures treated for 3 days with blinatumomab (10ng/ml) was measured using 8-colour flow cytometry (n=4). (C) Histogram showing HLA-DR expression on CLL cells taken from PBMC cultures treated with or without blinatumomab for 3 days. Unt= untreated, BiTE= Blinatumomab. Statistical analysis was performed using a paired *t*-test.

5.3 Blinatumomab mediates T-cell proliferation in primary CLL samples

To establish whether blinatumomab mediated T-activation also resulted in T-cell proliferation, T-cells from CLL PBMC cultures were analysed for expression of the Ki-67 protein after treatment with blinatumomab. Ki-67 is a nuclear protein present in cells in the active phases of cell cycle but absence in resting cells (Urruticoechea, Smith and Dowsett 2005). Intracellular Ki-67 expression in both $CD4^+$ and $CD8^+$ T-cells was significantly increased in cultures treated with blinatumomab after 3 and 7 days compared to untreated controls (Figure 5.3A-C). Interestingly blinatumomab treatment induced significantly more cycling $CD8^+$ T-cells than $CD4^+$ T-cells at day 3 ($P=0.04$). In order to determine if this T-cell cycling was maintained, the percentage of Ki-67 expressing T-cells was compared between day 3 and day 7. There was a significant increase in the percentage of Ki-67-expressing $CD4^+$ and $CD8^+$ T-cells from day 3 to day 7 after blinatumomab treatment ($CD4^+$: $P=0.0005$, $CD8^+$: $P=0.002$). As a positive control PBMC cultures were also activated with CD3/CD28 beads to determine the highest level of T-cell proliferation possible. Although T-cell proliferation induced by blinatumomab treatment was lower than that induced by CD3/CD28 beads at day 3, similar levels were achieved by day 7.

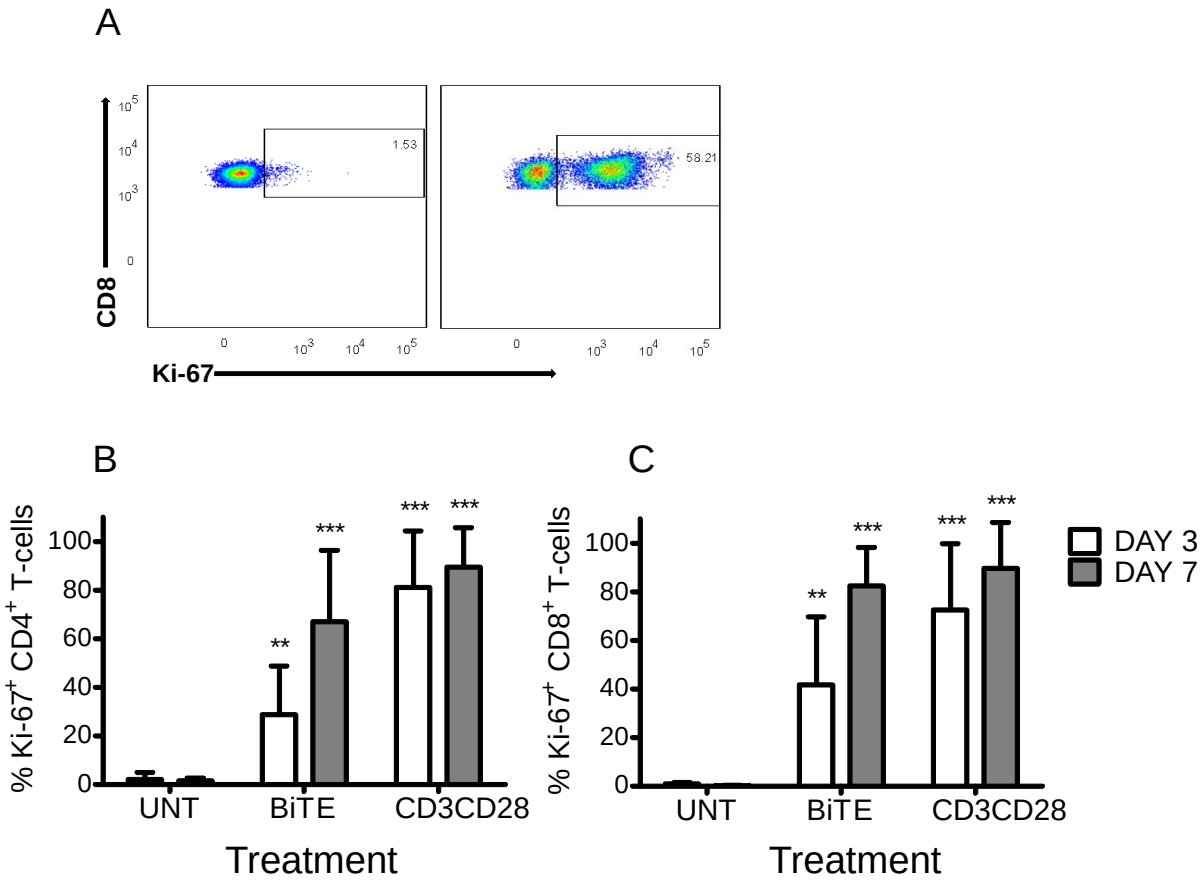


Figure 5.3. T-cell proliferation after incubation with blinatumomab. PBMCs extracted from CLL patients were cultured in the presence or absence of blinatumomab (n=7). Intracellular Ki-67 was used as a marker for proliferating $CD4^+$ and $CD8^+$ T-cells. The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). (A) FACS analysis showing the gating strategy used to separate Ki-67+/- T-cells (Left plot: untreated control, right plot: 10ng/ml blinatumomab treated). The percentage of Ki-67 expressing $CD4^+$ (B) and $CD8^+$ (C) T-cells was determined after incubation of PBMCs with blinatumomab (10ng/ml) or CD3/CD28 beads for 3 or 7 days in culture. *= (P<0.05), **= (P<0.01) and ***= (P<0.001). Statistical analysis was performed using a paired *t*-test.

5.3.1 Blinatumomab promotes the preferential expansion of T-cells displaying an effector memory phenotype

To identify the phenotype of T-cells cycling in response to blinatumomab treatment, the markers CCR7 and CD45RA were used to divide T-cells into Naïve, CM, EM and EMRA subsets (Figure 5.4). Analysis of the $CD4^+$ T-cell subset in untreated cultures showed they

were predominantly an effector memory phenotype followed by Naïve, CM and EMRA. After blinatumomab stimulation there was an initial increase in EMRA $CD4^+$ T-cells by day 3 (P=0.01) but by day 7 only $CD4^+$ T-cells with an EM phenotype were increased compared to untreated controls (P=0.02). In addition was a significant drop in naïve $CD4^+$ T-cells by day 7 (P=0.0098). Similar results were obtained from CD3/CD28 stimulated cultures with an increase in EM (P=0.004) and decrease in naïve $CD4^+$ T-cells observed at day 7 (P=0.01, Figure 5.4A).

Analysis of $CD8^+$ T-cells in untreated cultures showed that they are of a predominantly EM or and EMRA phenotype with few cells displaying either a Naïve or CM phenotype (Figure 5.4B). Blinatumomab induced the expansion of EM T-cells at Day 3 (P=0.03) and Day 7 (P=0.0002) whilst EMRA T-cells were reduced at the day 7 timepoint (P=0.0003). No significant changes in the percentage of naïve or CM $CD8^+$ T-cells were observed at both timepoints. CD3/CD28 stimulated cultures also showed an increase in EM $CD8^+$ T-cells at day 3 (P=0.0006) and day 7 (P<0.0001). The reduction in EMRA $CD8^+$ T-cells was however observed earlier at day 3 (P=0.0003) continuing until day 7 (P<0.0001). There was no change in naïve T-cells with only a slight increase in CM T-cells seen at day 3.

These results show that blinatumomab induces a preferential expansion of T-cells displaying an effector memory phenotype within the $CD4^+$ and $CD8^+$ T-cell compartments by Day 7. Concomitantly there was a reduction in the percentage of naïve $CD4^+$ T-cells and EMRA $CD8^+$ T-cells. A similar trend in $CD4^+$ and $CD8^+$ T-cell expansions was seen following CD3/CD28 stimulation by day 7.

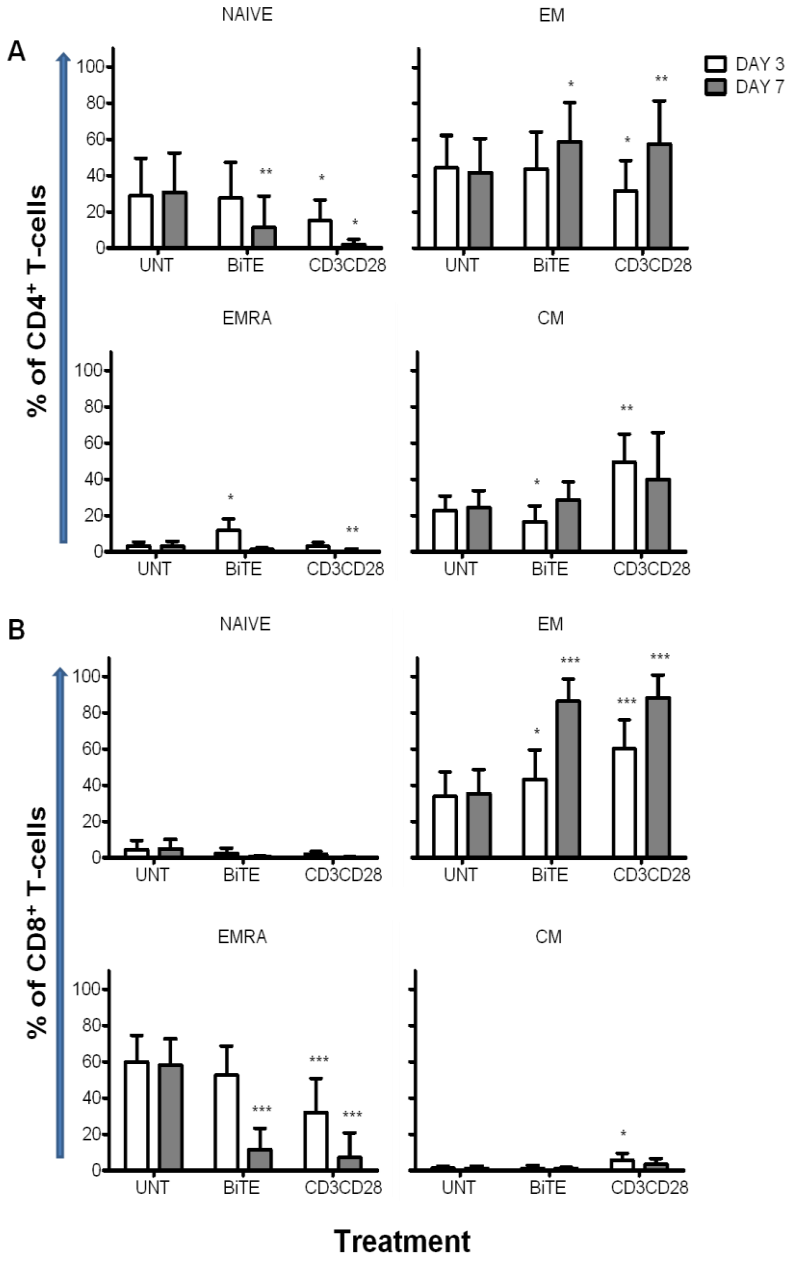


Figure 5.4 Blinatumomab preferentially induces the expansion of T-cells with an effector memory phenotype. T-cells from PBMCs treated with blinatumomab were stained with antibodies against CCR7 and CD45RA and analysed by 8-colour flow cytometry (n=7). T-cell subsets were defined as naïve ($CCR7^+CD45RA^+$), Central memory (CM) ($CCR7^+CD45RA^-$), Effector memory (EM) ($CCR7^-CD45RA^-$) and Highly differentiated effector memory (EMRA) ($CCR7^-CD45RA^+$). The percentage of naïve, EM, EMRA and CM T-cell subsets in (A) $CD4^+$ T-cells and (B) $CD8^+$ T-cells were determined at day 3 and day 7 post incubation with blinatumomab (10ng/ml) or CD3/CD28 beads. Each bar represents the combined data from 7 CLL patients. *= (P<0.05), **= (P<0.01) and ***= (P<0.001). Statistical analysis was performed using a paired *t*-test.

5.3.2 Cycling of T-cells subsets in response to blinatumomab treatment

In order to determine whether the increase in T-cells displaying an EM phenotype was due to the preferential proliferation of the EM subset, Ki-67 expression was measured in both $CD4^+$ and $CD8^+$ T-cell compartments after blinatumomab treatment. EMRA T-cells within the $CD4^+$ compartment and naïve and CM T-cells within the $CD8^+$ T-cell compartment were not included in the analysis due to their low frequency. Within the $CD4^+$ T-cell population T-cells with an EM phenotype had the highest percentage of cycling cells (Figure 5.5A) 7 days after blinatumomab treatment. When the percentage of cycling cells was compared between subsets, there was found to be a higher percentage of cycling EM (P=0.01) and CM (P=0.02) compared to naïve $CD4^+$ T-cells. There was also a higher percentage of cycling EM $CD4^+$ T-cells compared to CM but this did not reach significance (P = 0.19). Within the $CD8^+$ T-cell compartment the predominant T-cell phenotypes are EM and EMRA. By day 7 virtually all of the EM cells were cycling (91%) whereas only a minority of the EMRA cells were cycling (20%) within the $CD8^+$ T-cell compartment (Figure 5.5B; P=0.0001).

These results suggest that the expansion of EM T-cells seen within the $CD4^+$ and $CD8^+$ T-cell compartments after blinatumomab treatment is due to an increase in EM T-cell proliferation in comparison to other subsets.

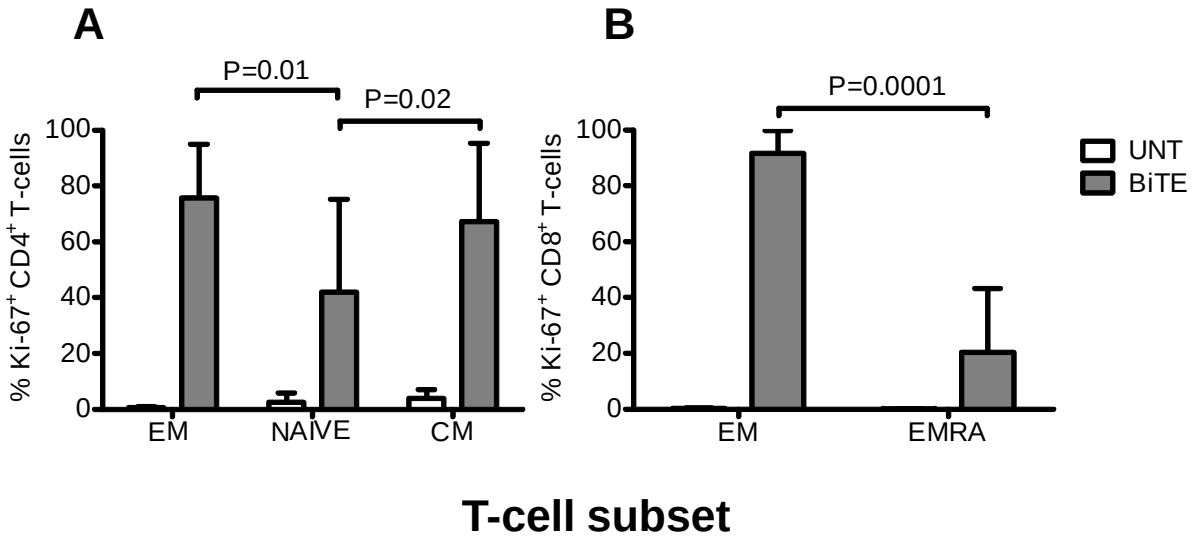


Figure 5.5 The frequency of cycling $CD4^+$ (EM, Naïve and CM) and $CD8^+$ (EM and EMRA) T-cells after blinatumomab treatment. PBMC from CLL patients were incubated for 7 days in the presence or absence or 10ng/ml blinatumomab (n=7). The percentage of cycling ($Ki\text{-}67^+$) cells were measured within T-cells subsets from $CD4^+$ (A) and $CD8^+$ (B) T-cell compartments by flow cytometry. T-cell subsets were defined as Naïve ($CCR7^+CD45RA^+$), Central memory (CM) ($CCR7^+CD45RA^-$), Effector memory (EM) ($CCR7^-CD45RA^-$) and Highly differentiated effector memory (EMRA) ($CCR7^-CD45RA^+$). Each bar represents the combined data from 7 CLL patients. Statistical analysis was performed using a paired *t*-test.

5.4 Blinatumomab promotes the release of pro-inflammatory cytokines from CLL patient PBMCs

Given that blinatumomab could induce T-cell activation, cytokines were measured to determine whether activation resulted in Th1 or Th2 type responses. To identify the types of cytokines released after blinatumomab treatment, supernatants from PBMC cultures were simultaneously analysed for the presence of 11 different cytokines using cytokine bead array technology (Figure 5.6). Three cytokines (IFN-γ, TNF-α and TNF-β) and 1 chemokine IL-8

were found to be significantly increased in the supernatants of cultures treated for 3 days with blinatumomab (Figure 5.7). IFN-γ was increased in 9/10 patients (P=0.03) and TNF-α in 10/10 patient samples (P=0.02). As a positive control CD3/CD28 beads were used to activate T-cells from these PBMC cultures and the supernatant analysed for various cytokines. A significant increase in IFN-γ, TNF-α, TNF-β and IL-8 levels were also observed in CD3/CD28 bead treated cultures similar to those cultures treated with blinatumomab (Figure 5.7). Analysis of IL-2, IL-5 and IL-10 showed that they were significantly increased in supernatants from CLL PBMC cultures treated with CD3/CD28 beads but not blinatumomab (Appendix 1). The remaining cytokines IL-1β, IL-12p70 and IL-4 were found at levels below the threshold of detection for this assay in untreated, CD3/CD28 bead and blinatumomab stimulated cultures.

These results show that blinatumomab treatment can promote the release of pro-inflammatory cytokines in CLL PBMC cultures.

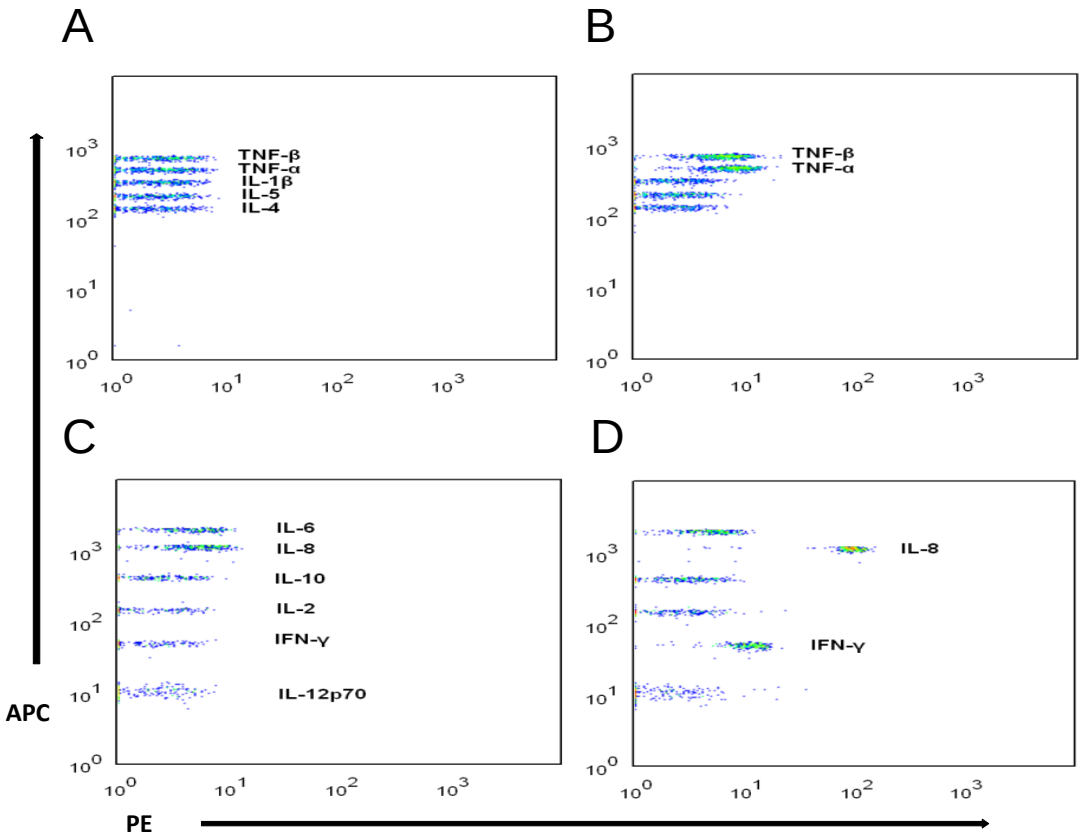


Figure 5.6. Gating strategy used to identify the levels of cytokines present in supernatant of PBMC cultures treated with blinatumomab. Cytokine levels were measured using 8-colour flow cytometry (FACS Canto II). (A+ C) FACS analysis showing the levels of cytokines present in untreated PBMC cultures. (B+ D) FACS analysis showing the levels of cytokines in PBMC cultures treated with blinatumomab. Cytokines from top to bottom: (A-B) TNF-β, TNF-α, IL-1β, IL-5 and IL-4. (C-D) IL-6, IL-8, IL-10, IL-2, IFN-γ and IL-12p70.

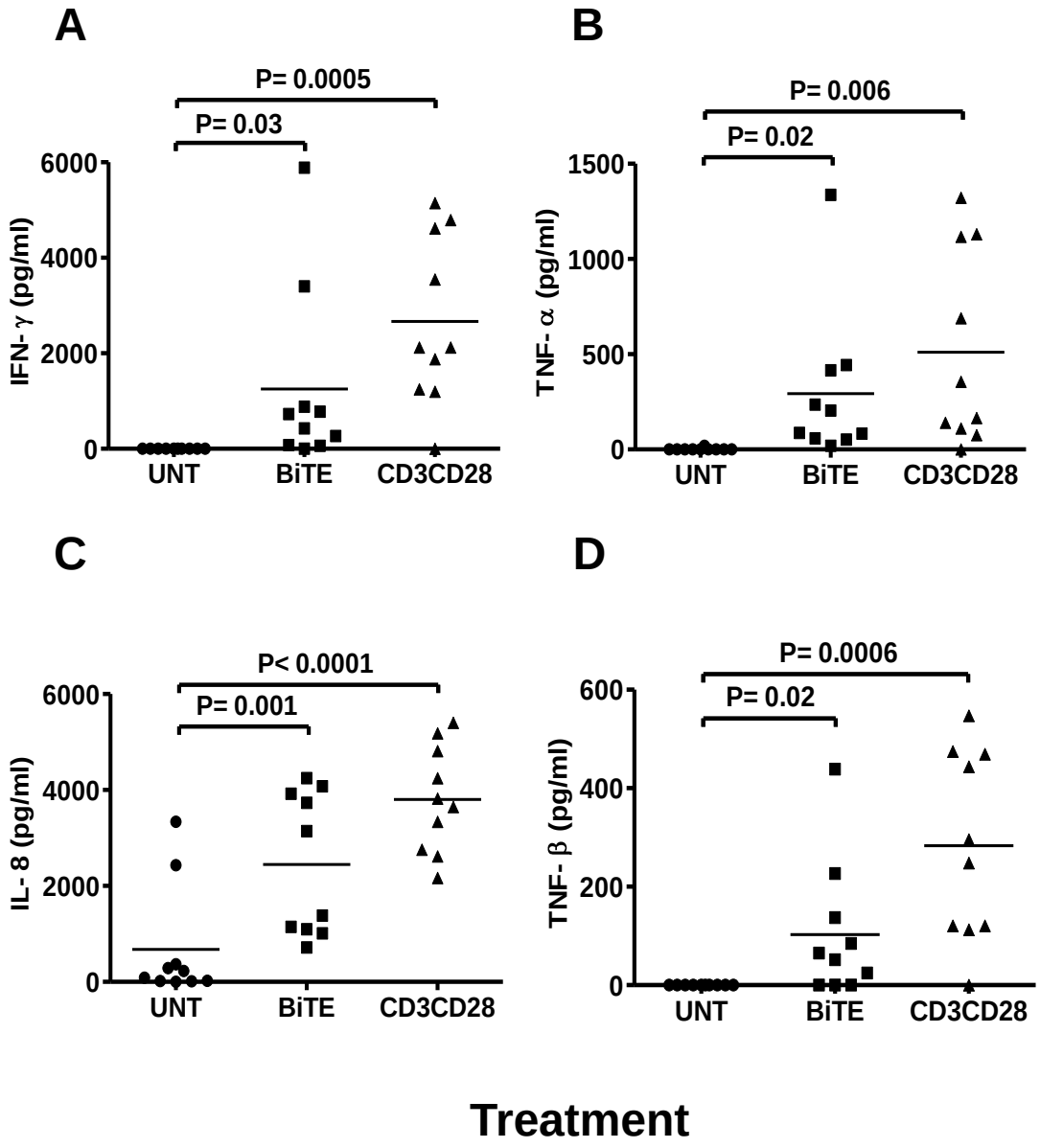


Figure 5.7. Pro-inflammatory cytokine release from CLL patient PBMCs after incubation with blinatumomab. Flow cytometric analysis of cytokines in supernatants taken from PBMC cultures treated with or without blinatumomab (n=10). Individual concentrations of (A) IFN-γ, (B) TNF-α, (C) TNF-β and (D) IL-8 in the supernatants of cultures treated with or without 10ng/ml blinatumomab or CD3/CD28 beads. The mean within each group is shown. Statistical analysis was performed using a paired *t*-test. Each dot represents 1 CLL patient and statistical analysis was based on 10 individual patient samples. BiTE=10ng/ml blinatumomab treated, CD3/CD28= CD3/CD28 bead treated cultures.

5.5 T-cells release cytotoxic granules and express high levels of IFN-γ and TNF-α after incubation with blinatumomab

To elucidate whether T-cells were the source of the pro-inflammatory cytokines found in the supernatants from PBMC cultures, T-cells were analysed for the intracellular expression of IFN-γ and TNF-α. There were significantly higher percentages of $CD4^+$ and $CD8^+$ T-cells expressing IFN-γ, TNF-α or both cytokines after treatment with blinatumomab (Figure 5.8A-B). The percentage of T-cells expressing IFN-γ was higher than that of TNF-α expressing T-cells ($CD4^+$: P=0.04, $CD8^+$: P=0.04).

The ability of blinatumomab to activate the cytolytic response mechanisms of T-cells was measured by staining for the intracellular expression of granzyme B in both $CD4^+$ and $CD8^+$ T-cell subsets. There was a significant increase in the percentage of $CD8^+$ (P=0.03) and $CD4^+$ (P=0.004) T-cells expressing granzyme B in PBMC cultures treated with blinatumomab compared to controls (Figure 5.8C and F). Further analysis revealed there was significantly more $CD8^+$ T-cells expressing granzyme B post blinatumomab treatment compared to $CD4^+$ T-cells (P=0.03). In order to assess whether or not T-cells were releasing granzyme B, surface expression of the degranulation marker CD107 was measured (Figure 5.8D and G).The percentage of $CD4^+$ (P=0.01) and $CD8^+$ (P=0.0004) T-cells expressing both granzyme B and surface CD107 increased after blinatumomab treatment. Statistical analysis showed that the percentage of $CD8^+$ T-cells expressing both granzyme B and CD107 were significantly higher than that seen in the $CD4^+$ T-cell population (P=0.04). PBMC cultures treated with CD3/CD28 beads were included as a positive control for the measurement of T-cell cytokines (IFN-γ and TNF-α) and markers of cytolytic activity (Granzyme B and CD107). CD3/CD28 beads induced similar levels of IFN-γ and TNF-α expression and granzyme B release from $CD4^+$ and $CD8^+$ T-cells.

These findings show that blinatumomab treatment of PBMC cultures increases IFN-γ and TNF-α expression and granzyme B release from $CD4^+$ and $CD8^+$ T-cells similar to PBMC cultures treated with CD3/CD28 beads.

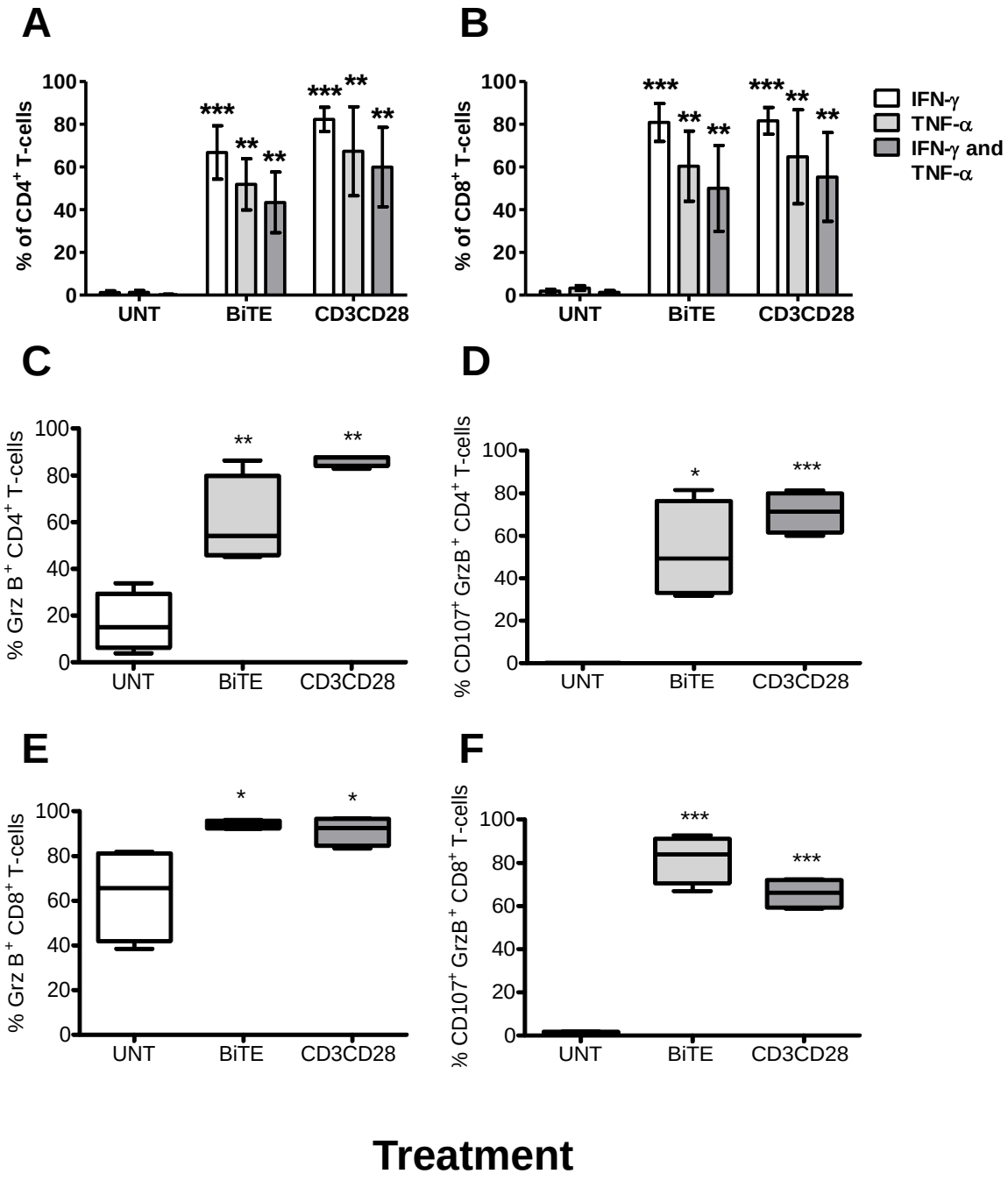


Figure 5.8 Blinatumomab treatment increases Th1 type cytokine expression and cytotoxic granule release from T-cells. Multicolour flow cytometric analysis of PBMCs from CLL patients treated with blinatumomab (n=4). (A-B) The percentage of $CD4^+$ and $CD8^+$ T-cells expressing IFN-γ, TNF-α or both IFN-γ and TNF-α. (C-F) The percentage of $CD4^+$ and $CD8^+$ T-cells expressing granzyme B or granzyme B and surface CD107 after treatment with blinatumomab. Statistical analysis was performed using a paired t-test and based on the relative levels of cytokines, CD107 and/or granzyme B expression in treated cultures compared to untreated controls. *= ($P<0.05$), **= ($P<0.01$) and ***= ($P<0.001$). BiTE=10ng/ml blinatumomab treated, CD3/CD28= CD3/CD28 bead treated cultures.

5.6 Blinatumomab induced activation of T-cells is CLL cell dependent

From an immunotherapeutic perspective it is important that blinatumomab does not induce T-cell activation and degranulation in the absence of CLL target cells, which could result in damage to healthy tissue. Purified T-cells from CLL patients were therefore incubated with blinatumomab in the presence or absence of CLL cell for 3 days. Intracellular Ki-67 expression was measured in both $CD4^+$ and $CD8^+$ T-cells, whilst granzyme B and surface CD107 were measured from $CD8^+$ T-cells (Figure 5.9A-C). There was no increase in the percentage of Ki-67 expressing $CD4^+$ or $CD8^+$ T-cells in T-cell cultures treated with blinatumomab compared to untreated controls. In contrast, T-cell cultures containing CLL cells and blinatumomab showed increased percentages of Ki-67 expressing T-cells in 3 out of 3 patients (Figure 5.10A-B). Similarly no increase in granzyme B^+ or granzyme B^+CD107^+ $CD8^+$ T-cells were observed in T-cell cultures treated with blinatumomab compared to untreated controls. T-cell cultures containing CLL cells however showed increased percentages of granzyme B^+ or granzyme B^+CD107^+ $CD8^+$ T-cells after blinatumomab treatment (Fig. 5.10C-D).

These findings show that T-cell activation, proliferation and $CD8^+$ T-cell cytotoxic granule release is strictly dependent on the presence of the tumour cells.

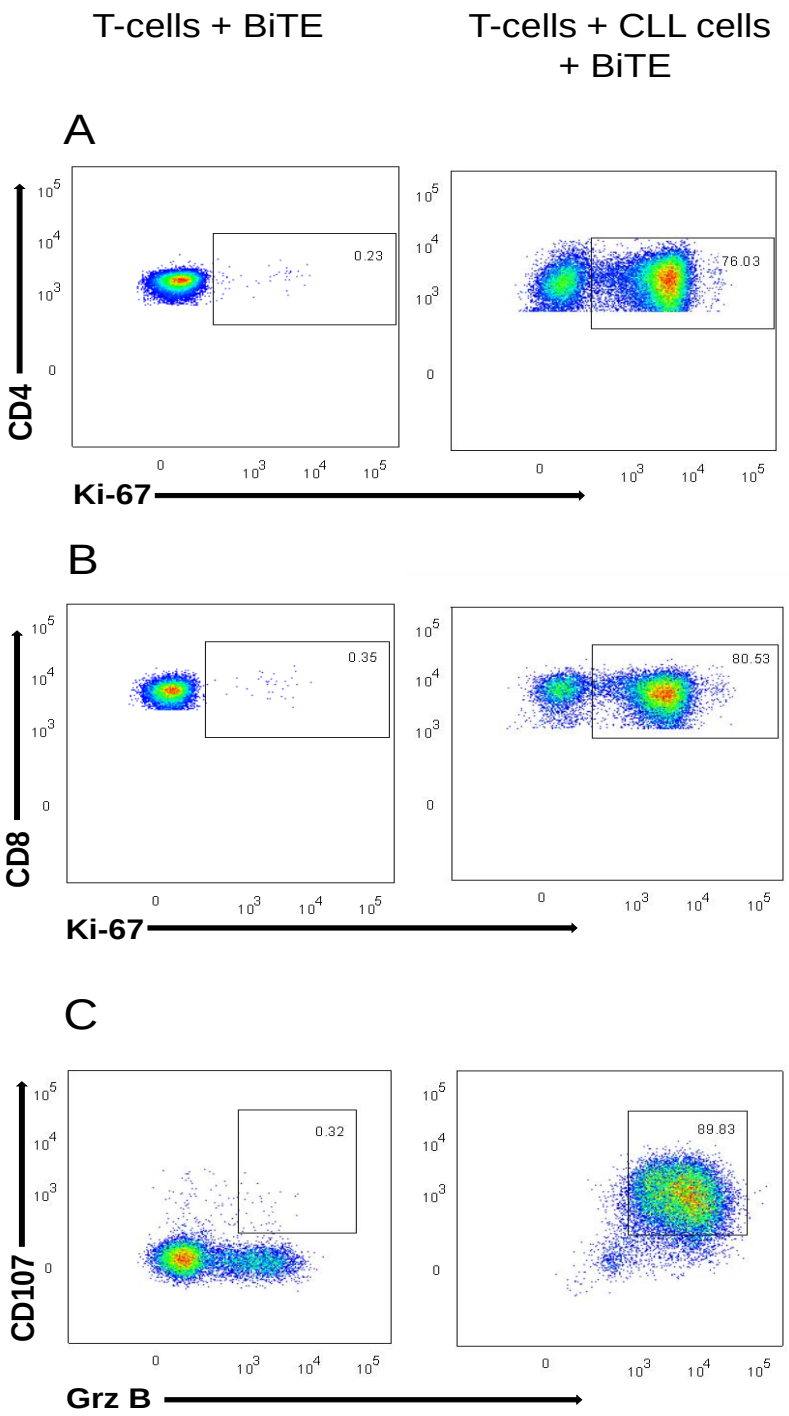


Figure 5.9. Gating strategy for measurement of Ki-67$^+$ and Granzyme B$^+$ CD107$^+$ CD4$^+$ and CD8$^+$ T-cells. T-cells were treated with blinatumomab (10ng/ml) in the presence or absence of CLL cells. Multicolour flow cytometric analysis showing the levels of intracellular Ki-67 expression in (A) CD4$^+$ and (B) CD8$^+$ T-cells after 3 days in culture with blinatumomab. Intracellular Granzyme B and surface CD107 expression were also measured in CD8$^+$ T-cells at Day 3 (C). Culture conditions: left panels T-cells and blinatumomab (10ng/ml), right panel T-cells, CLL cells and blinatumomab (10ng/ml).

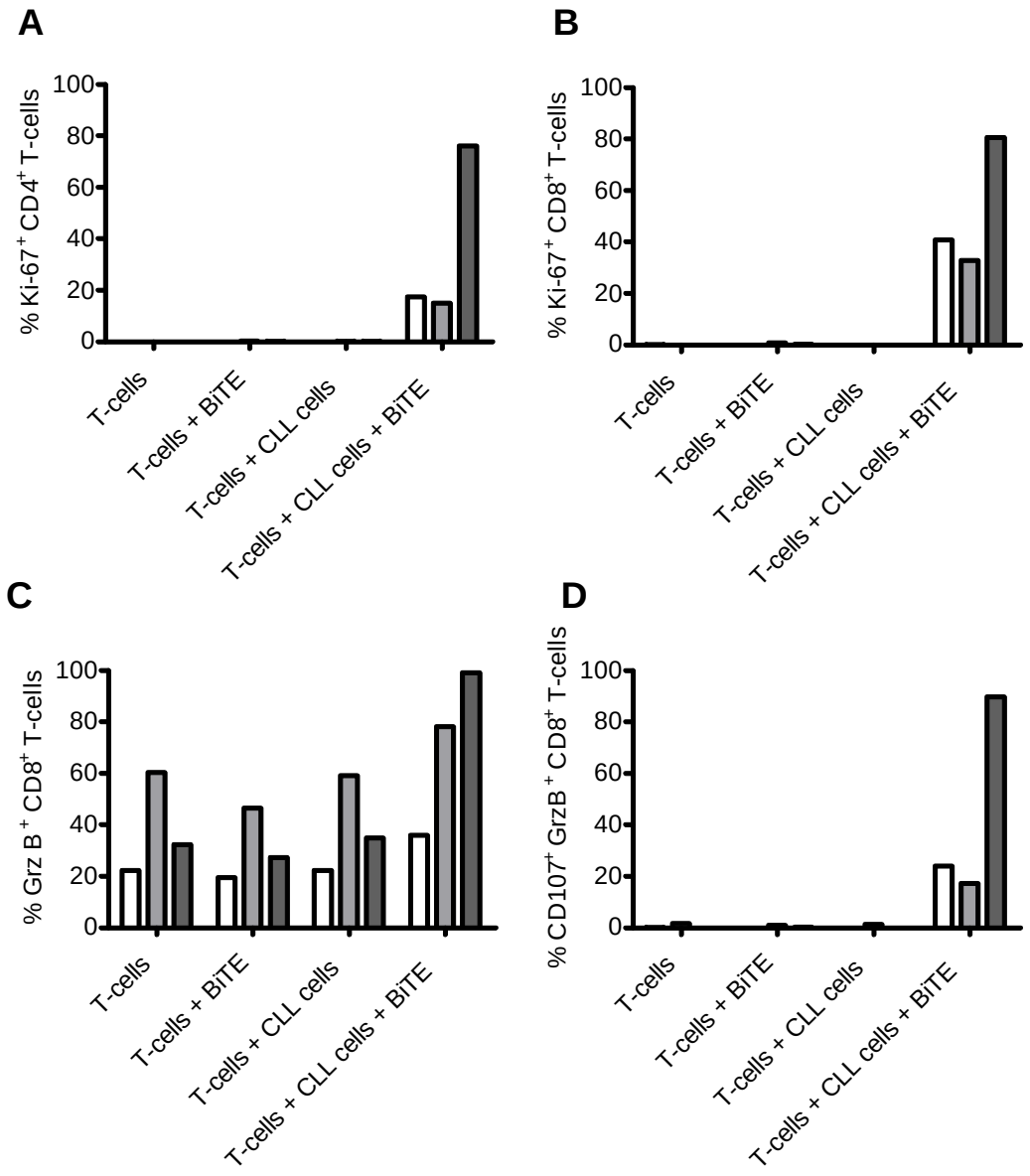


Figure 5.10. Blinatumomab induced activation of T-cells is CLL cell dependent. T-cells were treated with blinatumomab (10ng/ml) in the presence or absence of CLL cells. The percentage of (A) Ki-67$^+$ CD4$^+$ and (B) Ki-67$^+$ CD8$^+$ T-cells were measured after 3 days in culture by flow cytometry. Within the CD8$^+$ T-cell compartment the percentage of (C) Granzyme B$^+$ and (D) Granzyme B$^+$ CD107$^+$ cells were also measured. Each bar represents the results obtained from 1 patient (n=3).

5.7 Blinatumomab induces the expansion of T-cells and a concomitant reduction in CLL cells in PBMC cultures

To assess whether the total number of T-cells and CLL cells were changing in PBMC cultures treated with blinatumomab, the absolute number of these cell types were measured (Figure 5.11). Cultures treated with blinatumomab had a significantly higher absolute number of $CD4^+$ (P=0.01) and $CD8^+$ (P=0.01) T-cells after 7 days when compared to untreated controls (Figure 5.12A-B). Significant increases in $CD4^+$ and $CD8^+$ T-cells were also seen in cultures treated with an agonist anti-CD3 antibody or CD3/CD28 beads. Absolute numbers of CLL cells were significantly reduced in cultures treated with blinatumomab when compared to untreated controls (P=0.002). In contrast, cultures treated with the anti-CD3 antibody showed a significant increase in absolute numbers of CLL cells, whilst cultures treated with CD3/CD28 beads showed no significant change compared to untreated controls (Fig. 5.12C). Comparison of treatment naïve and previous treated patients within the blinatumomab, anti-CD3 antibody or CD3/CD28 bead treated groups showed no significant differences in absolute CLL cell counts.

In order to determine if the level of T-cell expansion correlated with the amount of CLL cell death in the cultures, the change in absolute numbers of $CD4^+$ and $CD8^+$ T-cells in each culture was plotted against the percentage change in CLL cells in treated cultures compared to controls (Figure 5.13). There was a significant correlation between the change in absolute numbers of $CD4^+$ and $CD8^+$ T-cells in blinatumomab treated cultures and the reduction in CLL cell numbers (CD4: P=0.05, CD8: P=0.04). In cultures treated with the anti-CD3 antibody or CD3/CD28 beads no such correlation was observed.

These observations show that blinatumomab and not anti-CD3 antibody or CD3/CD28 bead treatment of CLL PBMCs leads to a decrease in absolute numbers of viable CLL cells in

both treatment naïve and previous treated patients. Importantly the levels of T-cell proliferation in PBMC cultures treated with blinatumomab positively correlated with reduced levels of viable CLL cells.

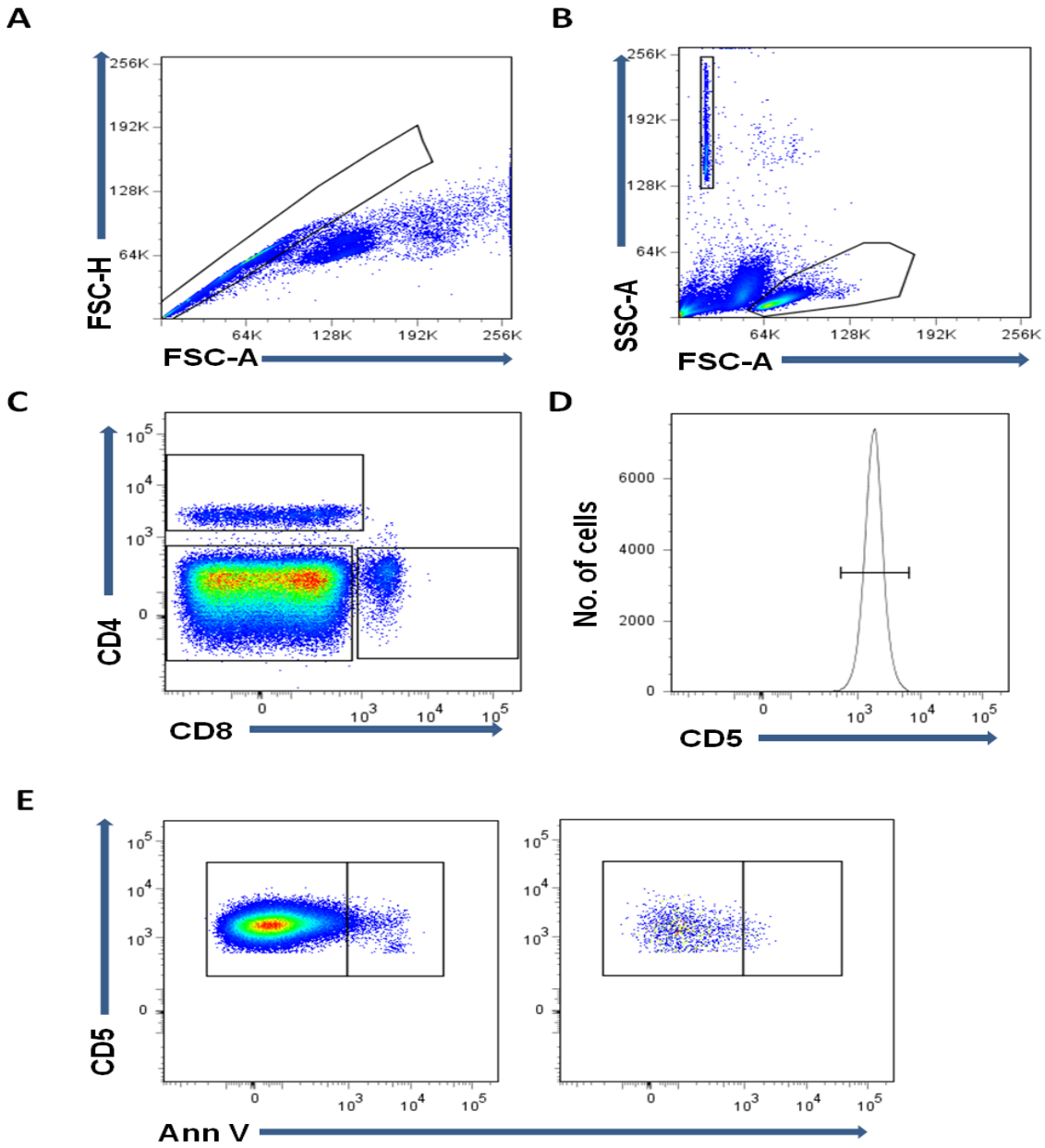


Figure 5.11. Gating strategy for the separation of T-cells ($CD4^+$ and $CD8^+$) and CLL cells for absolute count analysis. The number of positive cells was measured using 8-colour flow cytometry (FACS Canto II). (A) The lymphocyte population was gated by firstly excluding doublets using forward scatter height vs. area (B) followed by gating of lymphocytes and absolute count beads using forward and side scatter profiles. (C) T-cells were seperated based on their expression of CD4 and CD8 surface markers. (D) CLL cells were then gated based on their lack of surface CD4 and CD8 expression and positive expression of CD5. (E) Apoptotic cells were excluded based on the expression of Annexin V (left panel untreated, right panel 10ng/ml BiTE treated).

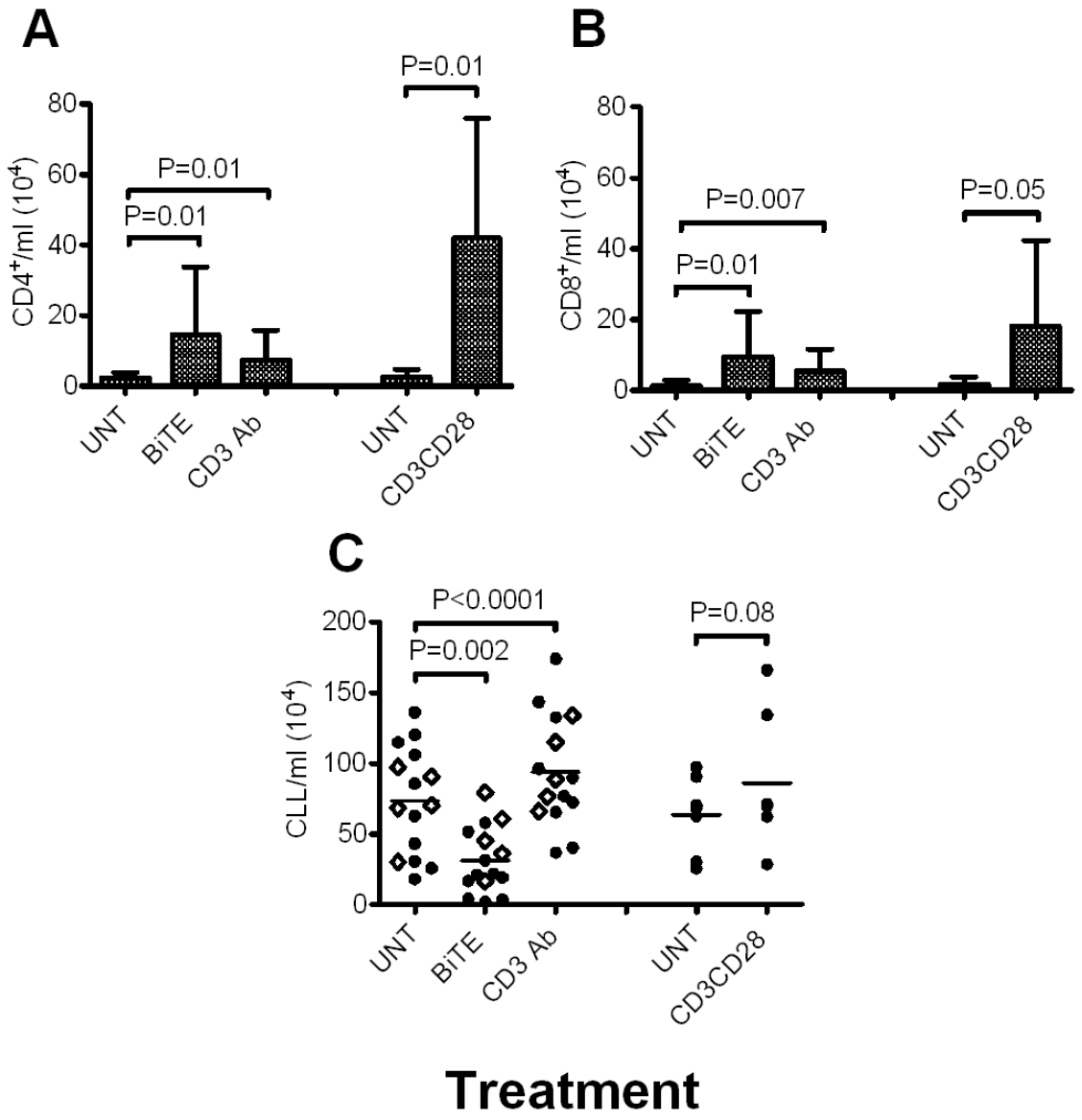


Figure 5.12. CLL PBMC cultures showed an increase in absolute numbers of T-cells and a decrease in absolute CLL cell numbers after treatment with blinatumomab. Absolute numbers of (A) $CD4^+$ T-cells, (B) $CD8^+$ T-cells and (C) CLL cells were calculated from CLL PBMC cultures after 7 days in the presence or absence of blinatumomab (10ng/ml). CD3/CD28 beads and an agonist anti-CD3 antibody were included as positive controls. Each bar represents the combined results with BiTE and CD3 Ab (n = 15) and CD3/CD38 (n = 7) respectively. The mean within each group is shown. Statistical analysis was performed using a paired t-test. For this analysis, patients were divided into treatment naïve (●) and previously treated patients (◊) respectively.

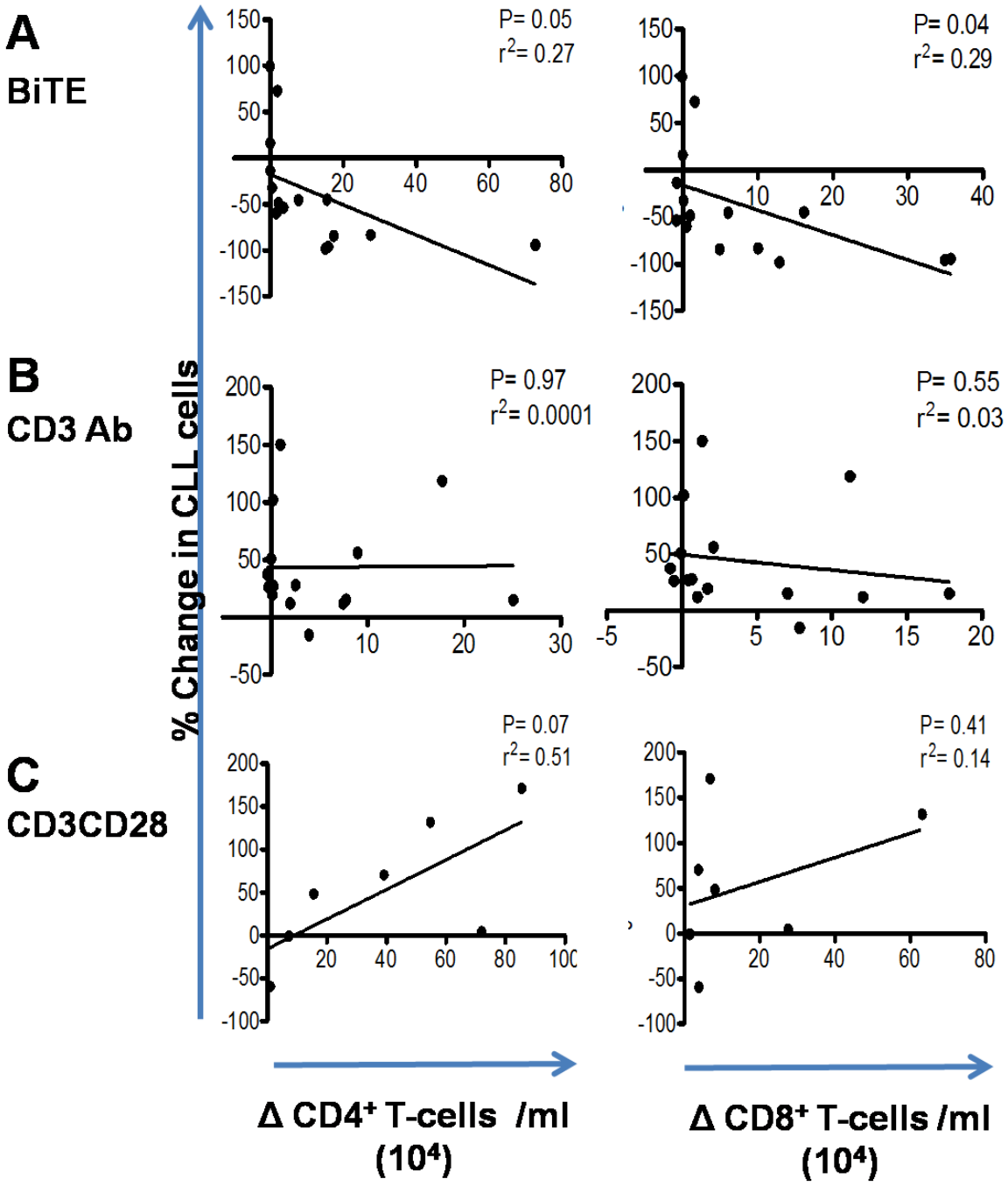


Figure 5.13. Linear regression plots for the percentage change in CLL cells vs. change in absolute numbers of T-cells in cultures treated with BiTE, an anti-CD3 antibody or CD3/CD28 beads. Linear regression analysis was used to analyse the effect T-cell expansions had on the percentage change in CLL cells in response to (A) BiTE (10ng/ml), (B) an agonistic CD3 antibody (27.7ng/ml) or (C) CD3/CD28 bead stimulation. Each dot represents data obtained from 1 CLL patient. BiTE and CD3 Ab treated (n = 15), CD3/CD28 treated (n = 7).

5.8 Blinatumomab induces CLL cell death by apoptosis that is enhanced in the presence of CD40L

As CLL cells readily undergo apoptosis in liquid culture, CLL cells were incubated in co-culture with a CD40L transfected fibroblast cell line to promote cell survival. In keeping with previous findings, CLL cells were found to be protected from apoptosis in the presence of both non-transfected mouse fibroblasts (NTLs) and mouse fibroblasts transfected with CD40L (TL(CD40L)) when compared to liquid culture (LC) alone (Figure 5.14A). Despite the increased survival of CLL cells conferred by co-culture, blinatumomab treatment increased the percentage of apoptotic CLL cells in liquid culture (P=0.04) and in co-culture with NTLs (P=0.002) or TL(CD40L) (P=0.02, Figure 5.14B). Furthermore, the percentage of apoptotic CLL cells induced by blinatumomab in the TL(CD40L) cultures was similar to that observed in LC. Interestingly increased percentages of apoptotic CLL cells were observed in blinatumomab treated TL(CD40L) cultures compared to TL cultures (BiTE: 10ng/ml P=0.06, 100ng/ml P=0.05) suggesting that CLL cell activation following CD40L stimulation may enhance the effects of blinatumomab (Figure 5.14C). When purified CLL cells were incubated in LC in the presence of blinatumomab (10ng/ml) no increase in the percentage of Annexin V^+ CLL cells was observed when compared to untreated controls (Figure 5.14D, P=0.63).

These findings show that blinatumomab treatment of CLL PBMC cultures leads to an increase in the percentage of apoptotic CLL cells *in vitro.* Importantly these increases in apoptotic CLL cells detected after blinatumomab treatment are maintained even in the presence pro survival and activation signals.

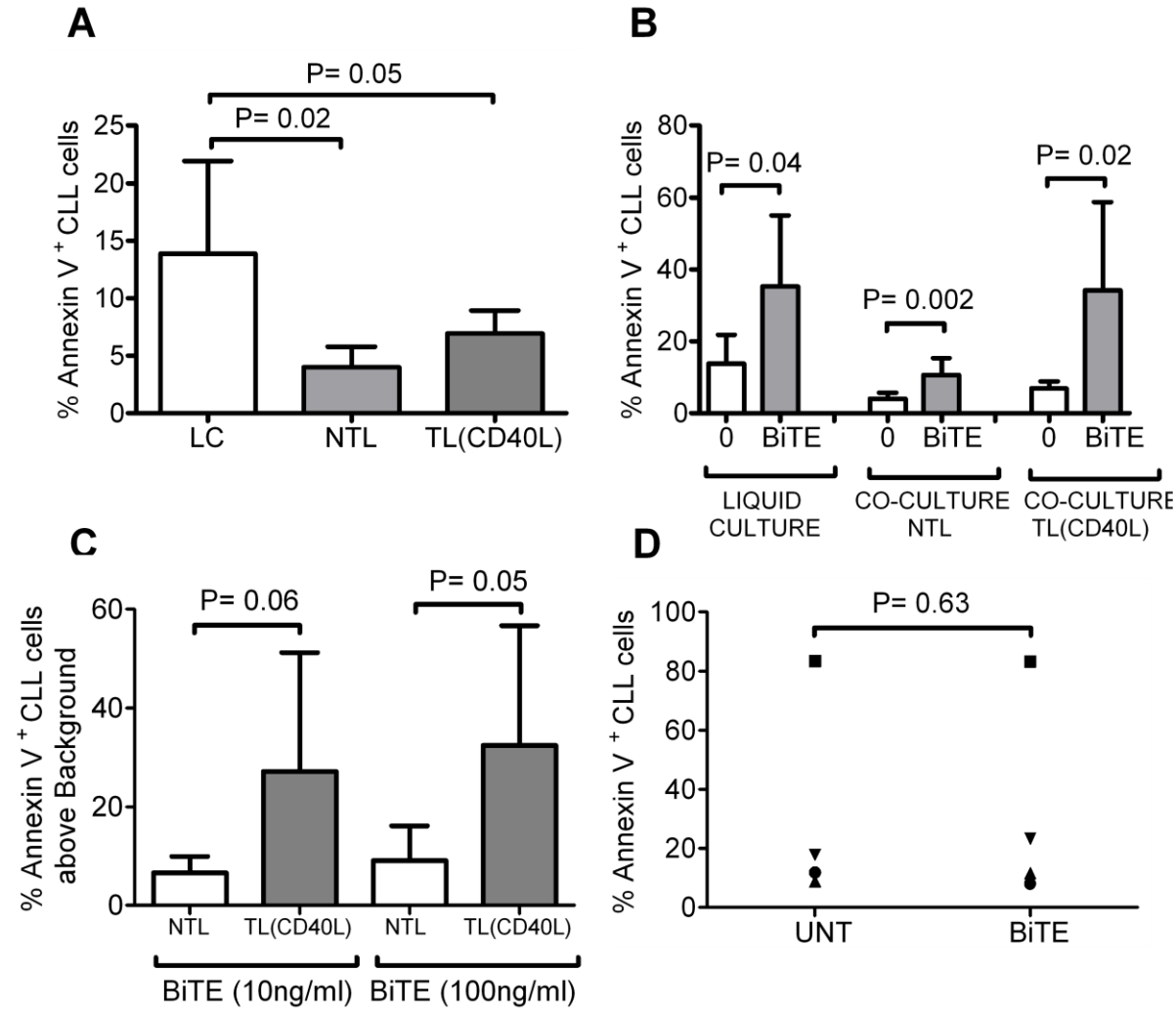


Figure 5.14. Blinatumomab induces CLL cell death in PBMC cultures, which is maintained even in the presence of pro-survival signals. PBMCs from CLL patients were incubated for 7 days in liquid culture (LC) or co-cultured on fibroblast cells transfected with TL(CD40L) (n=7). (A) The percentage of Annexin V^+ CLL B-cells after 7 days of incubation in LC or on NTL or TL(CD40L) (B) The percentage of Annexin V^+ CLL cells measured in cultures treated with or without blinatumomab (10ng/ml). Each graph shows the combined data from 7 CLL patients. (C) The percentage of Annexin V^+ CLL cells above background controls in PBMCs treated with blinatumomab (10ng/ml and 100ng/ml) and co-cultured on NTL or TL(CD40L). (D) The percentage of Annexin V^+ CLL cells measured in purified CLL cell cultures incubated for 7 days in the presence or absence of blinatumomab (10ng/ml). Each dot represents 1 CLL patient (n=4). The mean within each group is shown. Statistical analysis was performed using a paired *t*-test.

5.9 Blinatumomab induces clustering of CLL cells around T-cells

The ability of blinatumomab to bind both CD3 and CD19 is thought to promote the bridging of T-cells and tumour cells. In order to determine if this bridging effect would occur in CLL, PBMCs from CLL patients were cultured in the presence of blinatumomab and visualised by phase contrast microscopy. Incubation of CLL PBMCs with blinatumomab caused the formation of cell clusters after 12 hours in culture. These clusters were not observed in cultures treated with an anti-CD3 Ab or CD3/CD28 beads (Figure 5.15). Interestingly, the size and number of clusters observed after treatment with 100ng/ml BiTE compared to 10ng/ml BiTE is indicative of a dose-dependent relationship. In order to identify the cellular composition of these clusters, purified CFSE-labelled T-cells (CFSE Red) and CLL cells (CFSE Green) were incubated in culture with blinatumomab (Figure 5.16). Fluorescence confocal imaging of these clusters showed a single T-cell (Red) surrounded by multiple CLL cells (Green, Figure 5.16B). A computer generated 3-D reconstruction of these clusters revealed one T-cell closely interacting in a centralised position with 11 CLL cells (Figure 5.16C).

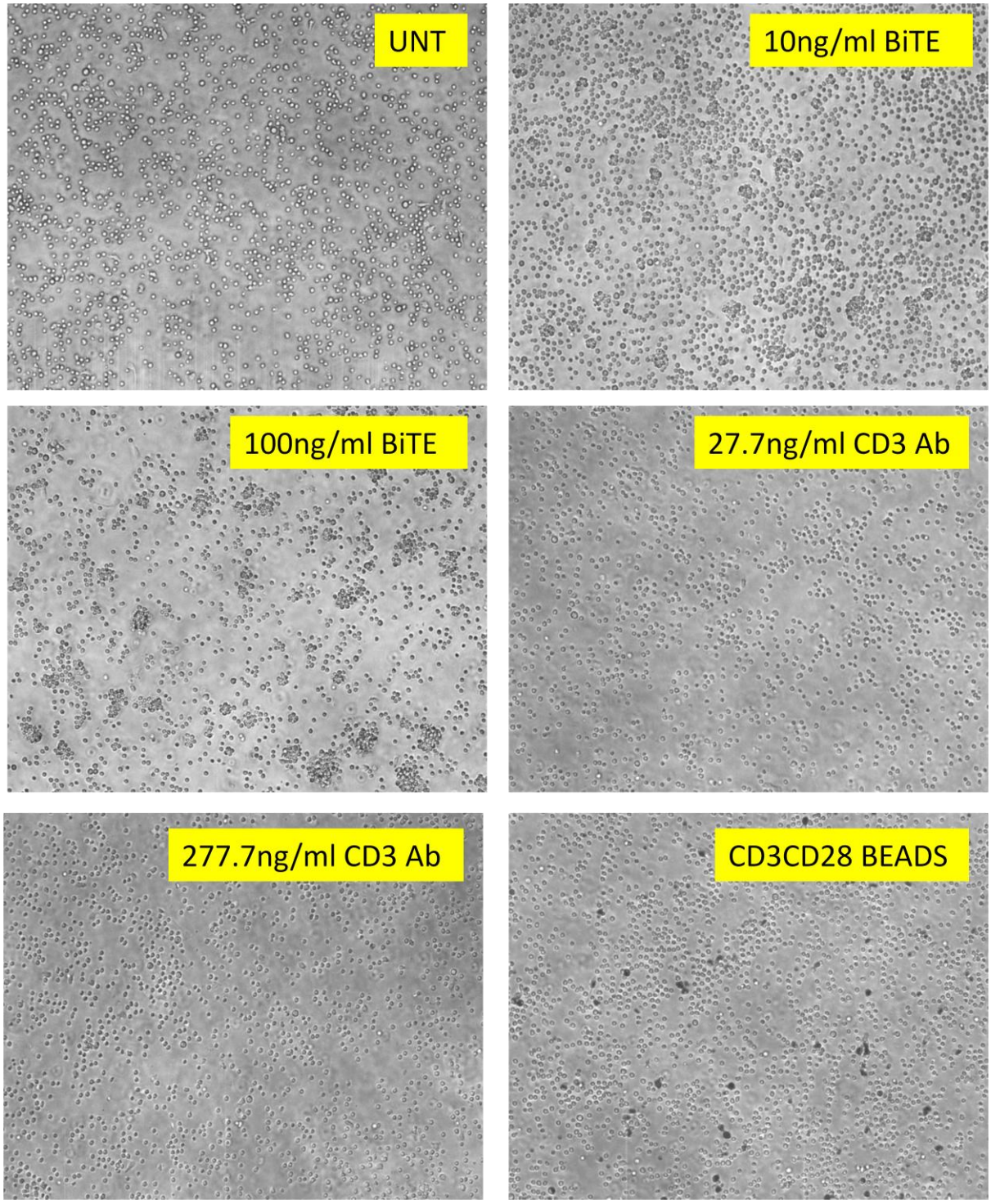


Figure 5.15 CLL patient PBMCs treated with blinatumomab, an anti-CD3 antibody or CD3/CD28 beads. Phase contrast images taken using light microscopy after 12hours in culture. Each image is labelled with the concentration and treatment added to each culture. UNT = untreated, BiTE = blinatumomab (10 or 100ng/ml), CD3 = anti-CD3 Ab (27.7 or 277.7ng/ml) and CD3/CD28 beads. Data represents one of 3 patients tested.

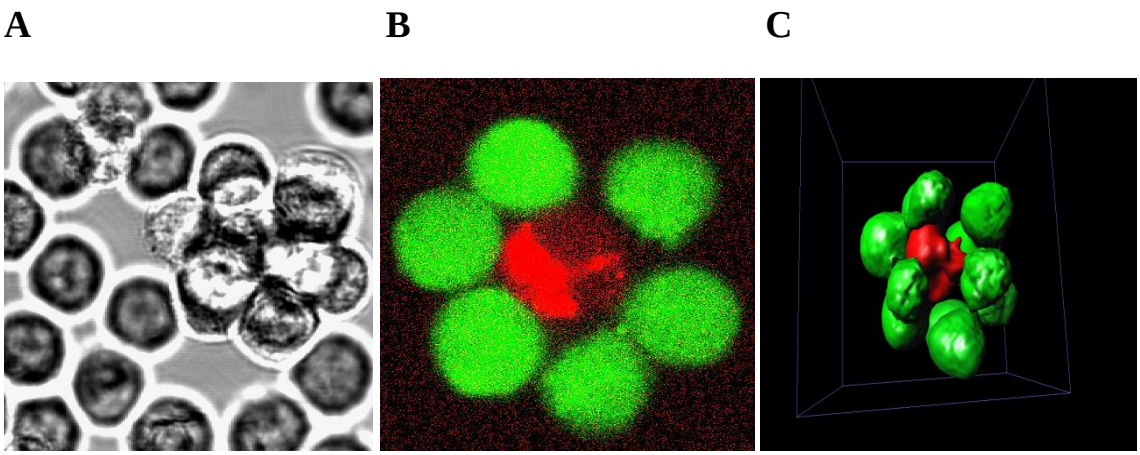


Figure 5.16 Visualisation of CLL and T-cell clustering in the presence of blinatumomab. T-cells and CLL cells were stained with CFSE (red and green) and visualised after incubation for 12 hours in the presence of blinatumomab (100ng/ml). (A) Phase contrast image showing a cell cluster formed in the presence of blinatumomab. (B) Visualisation of CLL cells (Green) and T-cell (Red) in clusters by confocal microscopy. (C) 3-D reconstruction of cluster using imaris imaging software; T-cells (Red) and CLL cells (Green). Results represent images obtained from 1 of 3 CLL patients.

5.10 Mechanism of blinatumomab directed T-cell killing of CLL cells

Taking into account all the information obtained from the experiments detailed above, a mechanistic timeline of blinatumomab-directed T-cell killing of tumour cells was developed (Figure 5.17). Firstly, blinatumomab engages both CD19 on CLL cells and CD3 on T-cells, bringing both cell types into close proximity allowing the formation of T-cell-CLL cell conjugates. The delivery of activation signals to T-cells induces their proliferation and differentiation into a predominately effector memory phenotype capable of secreting Th1 type cytokines. This process can take up to 3 days after which T-cells are capable of degranulating as evidenced by increased surface expression of CD107, leading to tumour cell-directed killing.

Like blinatumomab, CD3/CD28 beads can also induce the proliferation of T-cells with a predominately effector memory phenotype that are capable of secreting Th1 type cytokines.

CD3/CD28 beads however lack the ability to recruit CLL cells to the T-cell surface resulting in the failure of secreted granzyme B to enter the CLL cells and induce apoptosis. CLL cells therefore continue to survive in these cultures aided by the release of T-cell derived cytokines including IFN-γ, TNF-α and IL-8 which have been shown to promote CLL cell survival (Buschle et al. 1993, di Celle et al. 1994, Bojarska-Junak et al. 2002).

Overall these result indicate that blinatumomab mediated killing of CLL cells is strictly dependent on the bridging effect of the antibody between CLL cells and T-cells. Activation of T-cells in the absence of T:CLL cell bridging may result in the failure of T-cell cytotoxic mechanisms to successful target CLL cells.

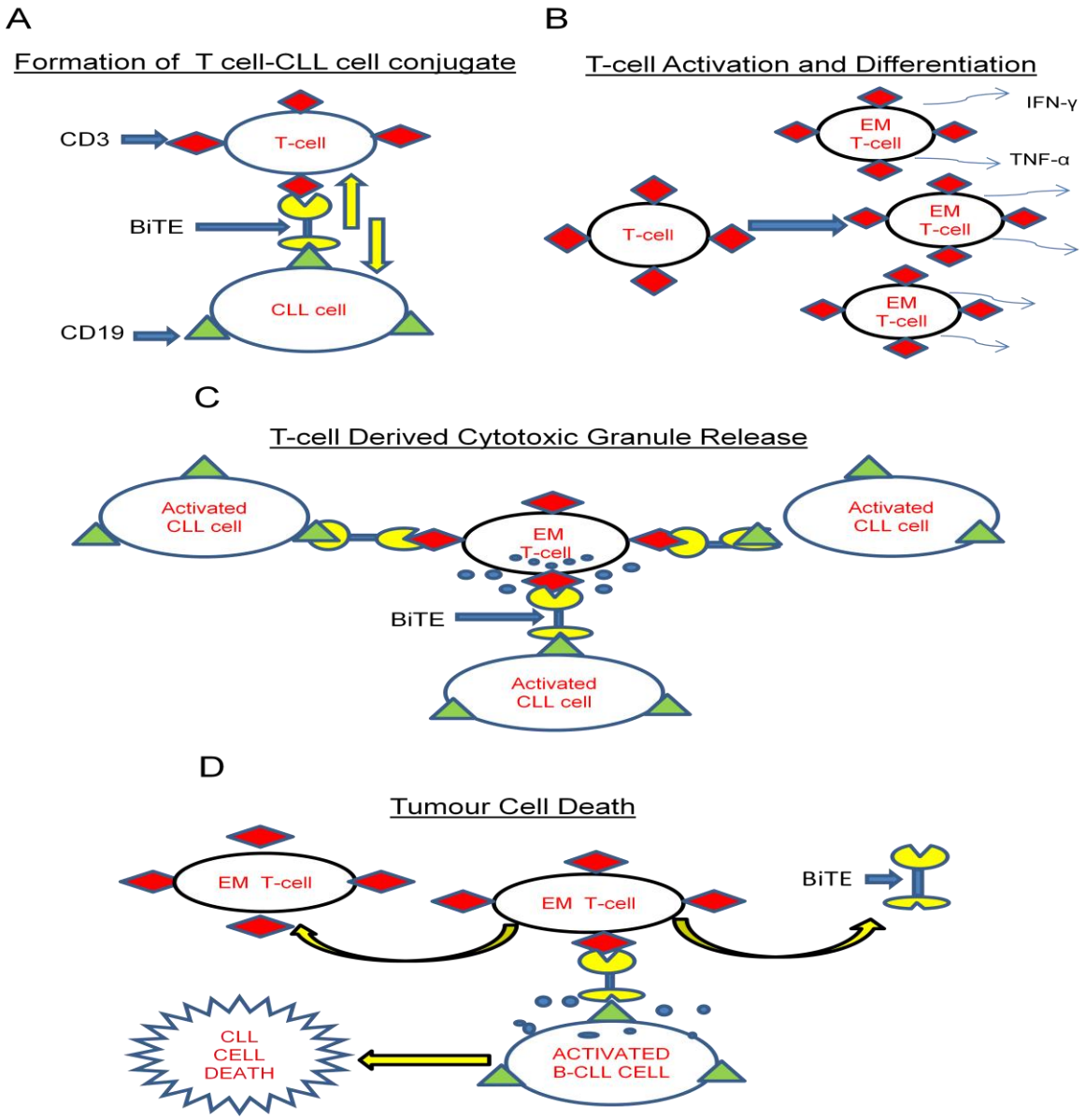


Figure 5.17. Proposed mechanism of blinatumomab directed T-cell killing of CLL cells. (A) Blinatumomab engages both CD19 on CLL cells and CD3 on T-cells bringing both cells into close proximity. Activation signals are then delivered to both cell types. (B) Activated T-cells proliferate and release pro-inflammatory cytokines including IFN-γ and TNF-α whilst also differentiating into an effector memory phenotype. (C) Blinatumomab brings effector memory T-cells and multiple CLL cells into close proximity, allowing targeted release of cytotoxic granules from both $CD4^+$ and $CD8^+$ T-cells towards the CLL cell surface. (D) Cytolytic granules enter the CLL cell inducing death by apoptosis. Blinatumomab disengages and is free to bind other T-cells and CLL cells.

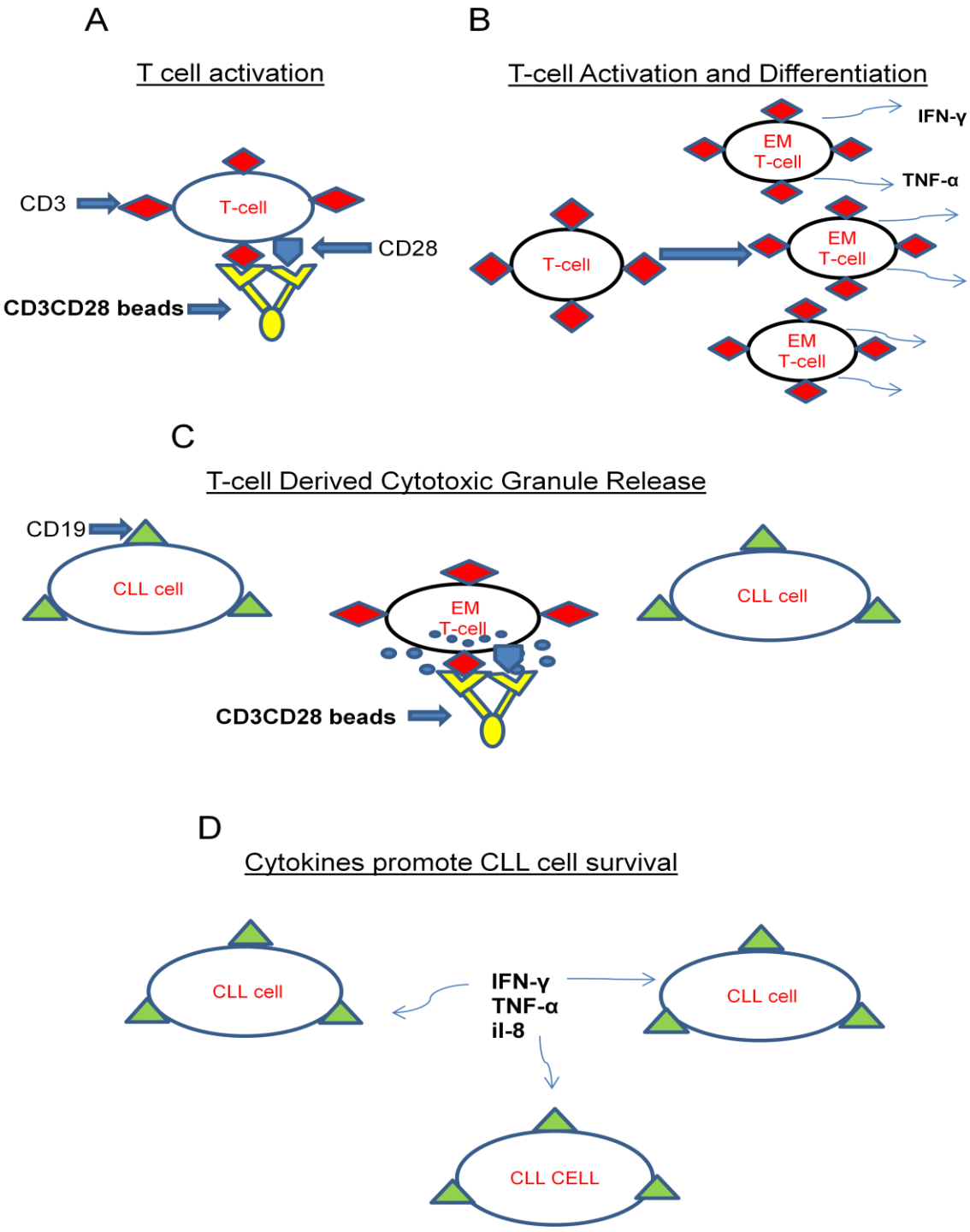


Figure 5.18. Proposed mechanism of CD3/CD8 bead promotion of CLL cell survival. (A) CD3/CD28 beads bind to CD3 and CD28 on the surface of T-cells inducing activation. (B) Activated T-cells proliferate and release pro-inflammatory cytokines including IFN-γ and TNF-α whilst also differentiating into an effector memory phenotype. (C) CD3/CD28 beads bind to effector memory T-cells inducing the release of cytotoxic granules which fail to enter and kill CLL cells due to distance between effector and target cells (D) The pro-inflammatory milieu of cytokines released by PBMCs upon incubation with CD3/CD28 beads aids the activation and survival of CLL cells.

5.11 Discussion

This chapter provides detailed *in vitro* evidence showing that blinatumomab, a bi-specific antibody targeting CD3 and CD19, can activate and induce the proliferation of T-cells from CLL patients *in situ* without having to provide additional CD28 stimulation or IL-2. Analysis of T-cell subsets showed that blinatumomab treatment induced the preferential expansion of T-cells with an effector memory phenotype; a phenomenon previously reported in NHL patients treated with blinatumomab (Bargou et al. 2008). EM T-cells are known to have a low-activation threshold and are capable of vigorous proliferation in response to stimulation (Sallusto, Geginat and Lanzavecchia 2004), which may explain their preferential expansion in blinatumomab-treated cultures. In addition, EMRA T-cells have been shown to have a low proliferation potential in comparison to TCM and TEM which supports the loss of EMRA T-cells after blinatumomab stimulation, particularly in the $CD8^+$ T-cell compartment (Sallusto et al. 2004). EM T-cells have also been reported to carry large amounts of perforin and are known to display rapid effector function upon stimulation (Sallusto et al. 2004). The generation of increased numbers of T-cells displaying an EM phenotype is likely to be important for blinatumomab-mediated cytotoxic T-cell responses against CLL cells. In colorectal cancer high densities of tumour infiltrating EM T-cells has been associated with increased overall survival (Pagès et al. 2005). Furthermore activation of tumour-associated $CD8^+$ EM T-cells have also been shown to promote tumour destruction and the production of tumour specific $CD8^+$ T-effector cells in mouse models (Pagès et al. 2005, Kilinc et al. 2009). In this regard, it would be interesting to investigate whether the elevated EM T-cell population is maintained in CLL cell cultures even after cessation of blinatumomab treatment.

$CD4^+$ and $CD8^+$ T-cells activated in response to blinatumomab treatment were shown to have increased expression of granzyme B and the surface degranulation marker CD107. These observations indicate that blinatumomab is indeed capable of inducing cytotoxic responses from $CD4^+$ and $CD8^+$ T-cells in CLL patients. Traditionally $CD4^+$ T-cells are thought to provide cytokine and co-stimulatory support to cytotoxic $CD8^+$ T-cells, with cytotoxic $CD4^+$ T-cell responses being relatively rare in healthy subjects (Man et al. 1990). However, an expanded population of cytotoxic$^+$ $CD4^+$ T-cells have been described during CMV infection and also in CLL patients (Walton et al. 2010, Casazza et al. 2006). It would be of interest to further characterise both the $CD4^+$ and $CD8^+$ T-cells post blinatumomab treatment, potentially giving further insight into how blinatumomab overcomes T-cell tolerance in the disease. This may include a detailed analysis of exhaustion and senescence markers, the frequency of regulatory T-cells and the expression levels of immunosuppressive molecules.

In this chapter, blinatumomab treatment induced CLL cell death in PBMC cultures, with the level of cell death shown to positively correlate with the degree of T-cell expansion. Interestingly, an agonistic CD3 antibody and CD3/CD28 beads were also capable of inducing proliferation of T-cells but unlike blinatumomab they did not cause a decrease in CLL cell survival. Indeed incubation with the agonistic CD3 antibody led to a significant enhancement of CLL survival in culture. This result is not unprecedented given that Patten *et al* showed that stimulation of T-cells with CD3/CD28 beads could increase the viability of CLL cells *in vitro* (Patten et al. 2005). In keeping with these findings we showed that incubation of CLL PBMC with CD3/CD28 led to a release of pro-inflammatory mediators including IFN-γ, TNF-α and IL-8 which are known to promote the survival of CLL cells *in vitro* (Buschle et al. 1993, di Celle et al. 1994, Bojarska-Junak et al. 2002). Although similar levels of these

cytokines were seen in cultures treated with blinatumomab, increased CLL cell death was observed in these cultures suggesting that it is not T-cell activation and cytokine release *per se* that promotes CLL cell killing.

The disparagy in CLL cell death detected after blinatumomab and CD3/CD28 or CD3 treatments indicates that the bridging of CLL cells and T-cells mediated by blinatumomab may be crucial for promoting the redirection of cytotoxic granules onto the CLL cell surface. Light microscope images from this study showed that blinatumomab could induce the clustering of CLL PBMCs *in vitro*. These clusters were subsequently shown to be composed of a centralised T-cell surrounded by CLL target cells. As T-cells express multiple CD3 molecules on their surface it is possible that blinatumomab facilitates the binding of several CLL cells to one T-cell allowing cytotoxic molecules to be delivered to multiple targets. Importantly, these clusters were not formed in the presence of CD3 or CD3/CD28 beads suggesting this bi-specificity is crucial for this T-cell/tumour cell conjugate formation and the subsequent cytotoxicity of the CLL cells. This concept is further supported by the fact that CD3/CD28 beads can induce granzyme B release from T-cells, but unlike blinatumomab this does not result in CLL cell death. Taken together these observations have important implications for adoptive T-cell therapy being trialled in CLL as this data suggests that transfer of CD3/CD28 activated T-cells will not be effective in treating CLL patients, without the bridging between CLL cells and T-cells (Wierda and O'Brien 2001, Ramsay and Gribben 2008).

The efficacy of blinatumomab in PBMC cultures is all the more remarkable given the fact that CLL cells typically outnumbered T-cells by up to 50:1, creating very low effector to target ratios. Blinatumomab may therefore represent a potential therapy for patients with high tumour burdens. Furthermore therapy with blinatumomab may not require prior

lymphodepleting regimes; which can restrict patients' suitability for treatment. The results of this study also suggests blinatumomab could have efficacy in patients where treatment options are extremely limited, including those patients that have relapsed from first-line therapy and those patients with poor prognostic markers including p53 mutations (Appendix 2).

Given the potential for activated T-cells to damage healthy tissue it was important to show that blinatumomab-mediated T-cell activation and cytotoxic granule release was only induced upon binding of T-cells to CLL cells. This study showed that blinatumomab mediated T-cell activation, proliferation and cytotoxic granule release from T-cells was strictly dependent on the presence of CLL cell targets. A potential side effect of blinatumomab could be the elimination of healthy B-cells in CLL patients leading to reduced antibody responses. Hypogammaglobulinemia is however a feature of many CLL patients and can be managed with donor antibody infusions. Blinatumomab therefore represents a highly targeted therapy which may lack the numerous off target toxicities associated with chemotherapeutic agents.

There is a growing appreciation that conventional chemotherapeutic agents have significantly less potency against CLL cells found in microenvironmental niches including the lymph nodes and bone marrow (Munk Pedersen and Reed 2004). Drug resistance conferred by these microenvironmental niches may be responsible for the residual disease seen in patients after conventional therapy, which ultimately aids the repopulation of CLL cells in the periphery and the eventual relapse of patients. Microenvironmental signals including interactions between CLL cells and CD40L-expressing T-cells, stromal and nurse-like cells are thought to increase the apoptotic-threshold of CLL cells (Plander et al. 2009, Burger et al. 2000, Buggins et al. 2010, Hamilton et al. 2012). Furthermore CD40-CD40L

interactions have been shown to augment the antigen presenting capacity of CLL cells due to increased surface expression of adhesion molecules (CD54) and co-stimulatory molecules such as CD80, CD86 and CD70 leading to CLL cell activation and protection from fludarabine-mediated killing (Kitada et al. 1999, Ranheim and Kipps 1993, Romano et al. 1998). Importantly this study showed that CLL cells receiving activation signals through CD40-CD40L interactions in a co-culture model system were still susceptible to blinatumomab-mediated killing. Indeed it would appear that CD40L-mediated activation of CLL cells may augment blinatumomab-induced T-cell killing of CLL cells, possibly due to the upregulation of co-stimulatory molecules such as CD80 and CD86 on the CLL cell surface enhancing T-cell activation and targeting of CLL cells (Ranheim and Kipps 1993).

Given these *in vitro* findings, it seems likely that blinatumomab promotes the formation of T-cell-CLL cell conjugates through the bridging of CD3 and CD19. T-cells are both activated and proliferate into predominately an EM phenotype, which are capable of producing Th-1 type cytokines. These T-cells have cytolytic potential as they produce and release granzyme B as evident by the expressing of surface CD107 leading to tumour cell-directed killing. Importantly this killing mechanism is undiminished even in the presence of pro-survival co-culture conditions.

In conclusion the bi-specific antibody platform represents a novel immunotherapeutic strategy in CLL. Blinatumomab has the ability to overcome T-cell tolerance of CLL cells *in situ* which does not require the *ex vivo* separation of T-cells or gene transfer technology which can be technically challenging. Furthermore, it induces antigen-independent autologous T-cell activation *in situ* resulting in serial T-cell-mediated CLL cell killing. This chapter also demonstrates the efficacy of blinatumomab in variety of patients, including those who are treatment naïve and those that have relapsed or are refractory to conventional

therapy. Finally the data generated from this study strongly supports the clinical development of blinatumomab as a therapeutic agent in CLL.

CHAPTER 6

Final Discussion

6.1 Discussion

Despite recent advances in the treatment of CLL, it still remains an incurable B-cell malignancy with a median overall survival time of approximately 10 years regardless of the patient's age at diagnosis. For a proportion of CLL patients, treatment will never be required and they may die from causes unrelated to their disease. However for the majority of patients; particularly younger patients and those presenting with aggressive disease, their CLL will require treatment and they will invariably die from disease related complications (Dearden 2008).

Chemotherapy has traditionally been the backbone to any therapeutic approach in CLL. It does not however, induce long-term disease free survival, and patients are at high risk of relapsing and developing chemotherapy-resistant disease. Moreover there are relative few effective treatments for those patients that do relapse or are refractory to chemotherapy including patients with p53 mutations. Novel therapeutic strategies aimed at improving the treatment and survival of these types of patients is therefore warranted.

CLL remains an attractive model for immunotherapeutic approaches aimed at harnessing the body's natural immune system to target tumour cells. CLL patients have a relatively long natural disease course in comparison to other cancers, potentially allowing the development and progression of immune responses to tumour cells. Indeed evidence of improved clinical responses after the addition of immunotherapy to current chemotherapeutic regimes supports the use of such approaches. In addition the potential curative GVL effect observed after allo-HSCT, demonstrates that T-cells can recognise and target CLL cells.

There are however several obstacles to the successful implementation of immunotherapy in CLL including the profound immunosuppression and immune dysfunction associated with the disease. Such immune defects including the ability of CLL cells to induce T-cell tolerance compromises tumour vaccine and adoptive T-cell therapy, resulting in poor clinical responses (Ramsay and Gribben 2008).

In this study immunophenotypic analysis of T-cells from CLL patients was performed in order to better characterise their dysfunction status within the disease. Conceivably better identification and understanding of T-cell defects in CLL, may lead to the development of novel targeted therapies aimed at restoring T-cell function. Indeed such therapies may improve T-cell responses to vaccination and infection as well as potentially restoring tumour immunosurveillance in CLL patients. In-depth analysis of CLL patient in this study revealed a skewing of T-cells towards a highly differentiated effector memory phenotype with an increase in markers associated with limited ability to proliferate and increased propensity to undergo apoptosis (Brenchley et al. 2003, Serrano et al. 1997, Weekes et al. 1999, Van den Hove et al. 1998). These results provided evidence that T-cells are not simply bystander cells in this B-cell malignancy but are subject to chronic stimulation leading to exhaustion.

As part of the immunophenotyping study a novel prognostic marker in CLL patients based on patients CD4:CD8 T-cell ratio was identified (Nunes et al. 2012). CLL patients found to have an inversion in their CD4:CD8 T-cell ratios were shown to have a shorter time to first treatment and thus inferior prognosis when compared to normal ratio patients. T-cells from the CLL^{IR} patient group had a preferential expansion of antigen experienced $CD8^+$ T-cells with a $CD28^-CD27^-CD57^+$ and PD-1^+ phenotype when compared to the CLL^{NR} group. These observations further support a role for T-cells in the pathogenesis of CLL with chronic $CD8^+$ T-cell stimulation seemingly a feature of poor prognostic patients.

Given that most patients in our cohort were stage A, it would have been interesting to assess whether the ratio of CD8:CD4 T-cells increased with disease progression by studying patients with stage A, B and C disease. In addition, it would be of interest to assess what effect treatment with chemotherapy had on the distribution and phenotype of T-cells in CLL patients including the CD4:CD8 ratios. This aside, the identification of inverted CD4:CD8 ratios as a novel prognostic marker in CLL patients represents a significant finding, particularly in an age where therapy is becoming increasingly personalised. Currently the decision to initiate treatment of CLL patients is based on clinical symptoms indicative of disease progression (Gribben 2010) with prognostic markers somewhat sidelined due to several reasons. Firstly the multitude of prognostic markers and the lack of clinical trials comparing their individual and combined prognostic values have made it difficult to evaluate their true potential in CLL treatment. In addition large clinical trials looking at the clinical benefit of different therapeutic regimens on patients separated based on various prognostic markers are still lacking (Van Bockstaele et al. 2009). It is therefore hard to fully evaluate the prognostic power of CD4:CD8 ratios in CLL. Having said that CD4:CD8 ratios remains the only prognostic marker to date that is not based on the tumour cell and this study further showed it correlates with LDT, which has recently been shown to be the most important determinant of TTFT in stage A patients (Pepper et al. 2012). Completion of clinical trials evaluating prognostic markers will hopefully allow clinicians to utilise prognostic markers in CLL to better predict the prognosis of patients at diagnosis and direct when and what types of treatments are given to patients. Such prognostic markers will likely include immunological (CD4:CD8 ratio), tumour (LDT, CD38 positivity, IgVh mutational status) and genetic (cytogenetic aberrations e.g. deletion 17p or 13q) markers.

One interesting finding from chapter 3 was the identification of PD-1 as a marker that is upregulated on a subset of $CD8^+$ T-cells within the CLL^{IR} group. Interaction of PD-1 on T-cells with its ligand PDL-1 has previously been shown to inhibit TCR signalling (Sheppard et al. 2004) and may play an important role in maintaining T-cell tolerance in solid and haematological malignancies (Zhang et al. 2008, Berger et al. 2008). Conceivably blockade of such interactions in CLL may help overcome T-cell tolerance in the disease and may restore normal T-cell function including tumour surveillance. This study showed that PD-1 is over expressed on T-cells in CLL patients and the ligand for PD-1(PDL-1) on CLL cells can be upregulated in response to T-cell derived cytokines including IL-4 and following CD40L stimulation *in vitro*.

Unfortunately one drawback to this study was the expression and regulation of PD-1/PDL-1 could only be analysed on cells obtained from the peripheral blood of CLL patients and not from the lymph nodes. The microenvironment of the peripheral blood varies considerably from the lymph node where CLL cells are subject to pro-survival and activation signals, with proliferating tumour cells co-localising with activated $CD4^+$ T-cells in pseudofollicles (Patten et al. 2008). It would be interesting to obtain CLL lymph node samples, in order to test whether immunosuppressive molecules are over expressed in secondary lymphoid organs as well as the peripheral blood and to investigate their potential function at these sites.

Antibodies capable of blocking the signalling of immunosuppressive molecules have recently come under intense investigation as therapeutic molecules in solid and haematological malignancies (Berger et al. 2008, Brahmer et al. 2010). Blockade of immunosuppressive molecules may overcome tumour cell suppression of T-cells in CLL potentially restoring T-cell function. In this study antibody blockade of PD-L1 and CD200

signalling was found not to enhance CLL patient T-cell responses *in* vitro. However given the growing number of immune evasion mechanisms known to be utilised by CLL cells to suppress T-cell responses including reduced expression of molecules involved in antigen presentation, promotion or Treg production and secretion of immunosuppressive cytokines (Riches et al. 2010), it is possible that blockade of a single immunosuppressive molecule is insufficient to overcome T-cell tolerance in CLL patients (Kater, van Oers and Kipps 2007). Indeed it is possible that certain immune evasion strategies in CLL may play more important roles in maintaining T-cell tolerance than others. Future investigations are therefore required to evaluate the importance of individual immune evasion mechanisms in CLL so therapeutic agents can be designed targeting those mechanisms crucial for suppressing T-cell responses.

Due to the failure of blocking antibodies targeting known immunosuppressive molecules to enhance T-cell responses from CLL patients, this study investigated a more direct approach of promoting T-cell activation. Blinatumomab a bi-specific antibody targeting CD19 and CD3 has previously been shown to have clinical activity in ALL and NHL (Topp et al. 2011, Bargou et al. 2008) but its potential use in CLL was unknown. Despite evidence of T-cell exhaustion and suppression in CLL patients (Serrano et al. 1997, Wadhwa and Morrison 2006, Pallasch et al. 2009), this study showed that it is possible to induce significant activation of T-cells from CLL patients with blinatumomab *in vitro*. Moreover T-cell activation was shown to be accompanied by T-cell proliferation, production of pro-inflammatory cytokines and the release of granzyme B from both $CD4^+$ and $CD8^+$ T-cells.

From the experimental data collected this study was able to outline a mechanism of blinatumomab-mediated killing of CLL cells. Blinatumomab likely promotes the formation of T-cell/CLL cell conjugates facilitating the activation and proliferation of T-cells into a

predominately effector memory phenotype. Confocal microscope images obtained from this study provided evidence of these conjugates with tight clustering of CLL cells around a centralised T-cell after blinatumomab treatment; a phenomena not seen after anti-CD3 or CD3/CD28 stimulation. The bridging effect mediated by the bi-specific antibody appears to help direct T-cell cytolytic mechanisms in the form of granzyme B release towards the CLL cell surface inducing tumour cell death by apoptosis. This hypothesis was supported by evidence of increased CLL cell death in PBMC cultures treated with blinatumomab, which was notably absent in cultures treated with anti-CD3 or CD3/CD28beads. In addition, a study by Offner *et al* showed that bi-specific antibodies can mediate T-cell adhesion to target cells inducing T-cell signalling and the formation of regular lytic synapses (Offner et al. 2006). These findings support the idea that the bridging effect of blinatumomab is crucial for T-cell/tumour cell conjugate formation and the subsequent cytotoxicity of the CLL cells. It also provides evidence that activation of T-cells in the absence of T cell-CLL cell bridging can promote CLL cell survival *in vitro*, which may help explain the poor clinical responses of patients receiving autologous T-cells activated with CD3/CD28 stimulation (Hami et al. 2004, Ramsay et al. 2008).

It is likely that future therapeutic agents will be aimed at providing efficacy in all types of CLL, including those patients with chemotherapy refractory disease, the frail and elderly and those with pre-existing co-morbidities. This study provides evidence that blinatumomab may have efficacy not only in treatment naïve patients but also in those patients who are refractory or have relapsed from chemotherapy. In addition, blinatumomab appears to work even at high target to effector cell ratios. This suggests that prior lympho-depleting regimens designed to reduce tumour burden, but which limit the applicability of treatments in the frail, elderly and patients with co-morbidities, may not be necessary with blinatumomab therapy.

Lastly, evidence obtained from this study suggests that blinatumomab-mediated T-cell activation, proliferation and effector function is strictly dependent on the presence of the tumour cell, which may minimise off target toxicities.

Resistance mechanisms, the potential for clonal evolution and the emergence of tumour variants also remain significant obstacles hampering successful implementation of therapeutic agents in CLL. As blinatumomab targets CD19, an important co-receptor involved in BCR signalling, it seems unlikely that CD19 negative CLL clones would develop post treatment. In addition, drug resistance conferred by microenvironmental niches may play a crucial role in maintaining residual CLL cell populations after chemotherapy, allowing the repopulation of CLL cells in the periphery and contributing to the relapse of CLL patients (Foster et al. 2008). Evidence from this study suggests blinatumomab may maintain efficacy in pro-survival microenvironmental niches including lymph nodes and bone marrow. These observations are supported by results from a clinical trial in NHL patients showing that blinatumomab treatment cleared tumour cells from the blood, lymph nodes, spleen and bone marrow (Bargou et al. 2008). Overall these *in vitro* results suggest blinatumomab therapy could be highly effective in a wide variety of CLL patients with limited treatment-related toxicities and reduced chance of relapse.

The logical step forward would be to utilise data generated from this *in vitro* study to set-up a phase 1 clinical trial with blinatumomab here in Cardiff. Analysis of T-cells and B-cells from the blood of CLL patients, pre and post blinatumomab treatment would allow us to determine whether these *in vitro* results are translated *in vivo*. It would also be interesting to look at the effects of combining blinatumomab with different therapeutic agents. This study did investigate the potential synergistic properties of blinatumomab therapy with blocking antibodies against immunosuppressive molecules PD-1 and CD200 but was unable to show

any enhancement to T-cell responses *in vitro*. It would however be interesting to look at CLL cell survival as an indicator of synergy between these two therapeutic strategies, especially given previous *in vivo* data which suggested single agent blocking antibodies against PD-1 can provide clinical benefit in patients with haematological malignancies (Berger et al. 2008). In addition, it would be of great interest to investigative the potential synergistic properties of blinatumomab with lenalidomide or monoclonal antibody therapy (d'Argouges et al. 2009). Lenalidomide has previously been shown to induce CLL cell activation and upregulation of the co-stimulatory molecule CD80 (Aue et al. 2009). Given that CLL cell activation is observed prior to blinatumomab mediated T-cell killing of CLL cells in this study it would be interesting to assess both therapeutic agents for synergistic properties in CLL. It is however important to note that this study also demonstrates potent T-cell activation with blinatumomab as a single agent. Therefore caution should be used when trying to combine this type of therapy with other immunostimulatory agents to prevent over activation of the immune system *in vivo*.

Bi-specific antibodies aimed at directing T-cell responses towards tumour cells displaying specific antigens represents an interesting and widely applicable therapeutic strategy for treating cancers. Bi-specific antibodies have advantages over other types of immunotherapy designed to enhance T-cell responses in CLL including tumour vaccines and adoptive transfer of CD3/CD28 activated T-cells or TCR modified T-cells. Firstly, bi-specific antibodies can recognize and bind antigens presented on the tumour cell surface, without the need for MHC presentation, meaning they can be used to treat patients irrespective of HLA-genotype (Chames and Baty 2009). In addition the ability of bi-specific antibodies to recognize native surface antigens may limit the effects of tumour immune escape mechanisms including defective antigen processing and reduced MHC expression. Such

mechanisms are thought to hamper the generation of effective anti-tumour immune responses with vaccines or adoptive T-cell therapy. Secondly bi-specific antibodies can recognise non-protein antigens including carbohydrates and glycolipids allowing for an expanded range of tumour antigens that can be potentially targeted. Finally, bi-specific antibodies unlike adoptively T-cell therapy do not require the *ex vivo* expansion or genetic manipulation of T-cells with lentivirus/retrovirus technologies required for chimeric T-cells that can be technicallychallenging. ACT/CAR therapies also require T-cell preparations for each patient usually at onsite GMP labs whereas bi-specific antibodies can be produced offsite and can be administered to any patient.

B-cell malignancies generally represent a promising group of diseases for bi-specific antibody therapeutics due to their expression of CD19. CD19 is expressed on virtually all B-cell lineage leukaemias and lymphomas making it an excellent tumour-associated antigen. In addition, it is not present on haematopoietic stem cells or non B-cell lineages therefore limiting the chance of off target toxicities. Expanding the bi-specific antibody platform into solid cancers however may represent a more difficult challenge. Although many tumour antigens have been defined for solid cancers with respect to T-cells, few antibody targets that are applicable to a wide range of tumours have been defined. The few defined antigens that can be targeted by antibodies are often not consistently expressed between patients, may only be expressed on a proportion of tumour cells and may be present to some degree on healthy tissue (Igney and Krammer 2002). Despite this, a number of bi-specific antibodies have been developed targeting antigens in solid cancers such as EpCAM and HER2/neu (Baeuerle and Reinhardt 2009). Future identification of novel cancer antigens by serological testing e.g. SEREX, will no doubt aid the development of bi-specific antibodies with new target specificities that can be clinical applied in solid cancers.

In conclusion, the results of this study provided clear evidence of T-cell dysfunction in CLL patients, including a skewing of T-cells towards a memory phenotype with increased expression of exhaustion/senescence markers and immunosuppressive molecules. Moreover, it demonstrated the difficulty of trying to enhance T-cell responses from CLL patients using blocking antibodies against immunosuppressive molecules to PD-1 or CD200 *in vitro*. Despite these observations, this research did demonstrate the ability of a bi-specific antibody called blinatumomab to overcome T-cell tolerance in CLL allowing retargeting of T-cell cytotoxic killing mechanisms towards autologous tumours cells *in vitro*. Such therapy appeared to have efficacy not only in treatment naïve patients but also in patients that have relapsed or are refractory to current chemotherapy. Finally this research strongly advocates progression of blinatumomab into clinical trials as a novel therapeutic agent in CLL.

6.2 The future of immunotherapeutic agents in CLL

Immunotherapeutic agents aimed at harnessing the body's own immune system to target cancer cells remain a particularly appealing strategy for the treatment of CLL. The successful application of such approaches designed to enhance T-cell responses to CLL cells has however been limited due in part to the suppressive effects mediated by the tumour microenvironment (Kater et al. 2007). Future advances in our understanding of the tumour microenvironment, including identifying the key mechanisms that are utilised by tumour cells to suppress T-cells, will likely aid the development of novel therapeutics capable of blocking such pathways. In addition, immunotherapeutic agents are now being developed, which include bi-specific antibodies that can promote T-cell directed killing of CLL cells through mechanisms that minimise the suppressive effects of tumour cells.

It is likely that novel therapeutic agents, which block the suppressive mechanisms of the tumour microenvironment will form a key part of any immunotherapeutic strategy aimed at enhance anti-tumour immune responses *in vivo*. In addition, immunotherapy may be used alongside other cytotoxic agents, enabling killing of CLL cells through multiple mechanisms in order to avoid clonal evolution, the emergence of tumour escape variants and tumour relapse. Currently the 'gold standard' of treatment for CLL patients involves chemotherapy given alongside the monoclonal anti-CD20 antibody rituximab (FCR) (Robak et al. 2010). In the future, toxic chemotherapy will most likely be replaced by more targeted therapies with an emphasis on investigating *in vitro/in vivo* their modes of action and potential synergistic properties with immunotherapy. Indeed immunotherapy is already being considered alongside antagonists of BCR-associated kinases (Bruton's tyrosine kinase (BTK) and PI3Kδ) which have shown promising results in early phase I/II clinical trials in CLL (Woyach et al. 2012). In addition, new and current prognostic factors will become increasing important in the management of CLL, helping to identify those patients at risk of rapid disease progression, likely to be refractory to treatment and those that have worse overall survival. These 'high risk' patients may benefit from early treatment prior to the development of symptoms (Figure 6.1).

In regard to curing CLL, it is difficult to envision a single-agent being used to treat all types of CLL patients without the emergence of drug resistant disease. As such it is likely immunotherapy including blinatumomab would form part of a multi-strategy approach. Initial therapy may be designed to deplete the lymphoid compartments and peripheral blood of CLL cells including chemotherapy and BTK inhibitors. Once CLL cell numbers have reached a critical threshold, immunotherapy could be employed to eliminate residual CLL cells whilst minimising the potential emergence of tumour escape variants.

Future advances in the understanding of CLL, together with extensive clinical trials involving immunotherapeutic agents, will no doubt lead to improvements in clinical care and ultimately enhance the quality of life of CLL patients.

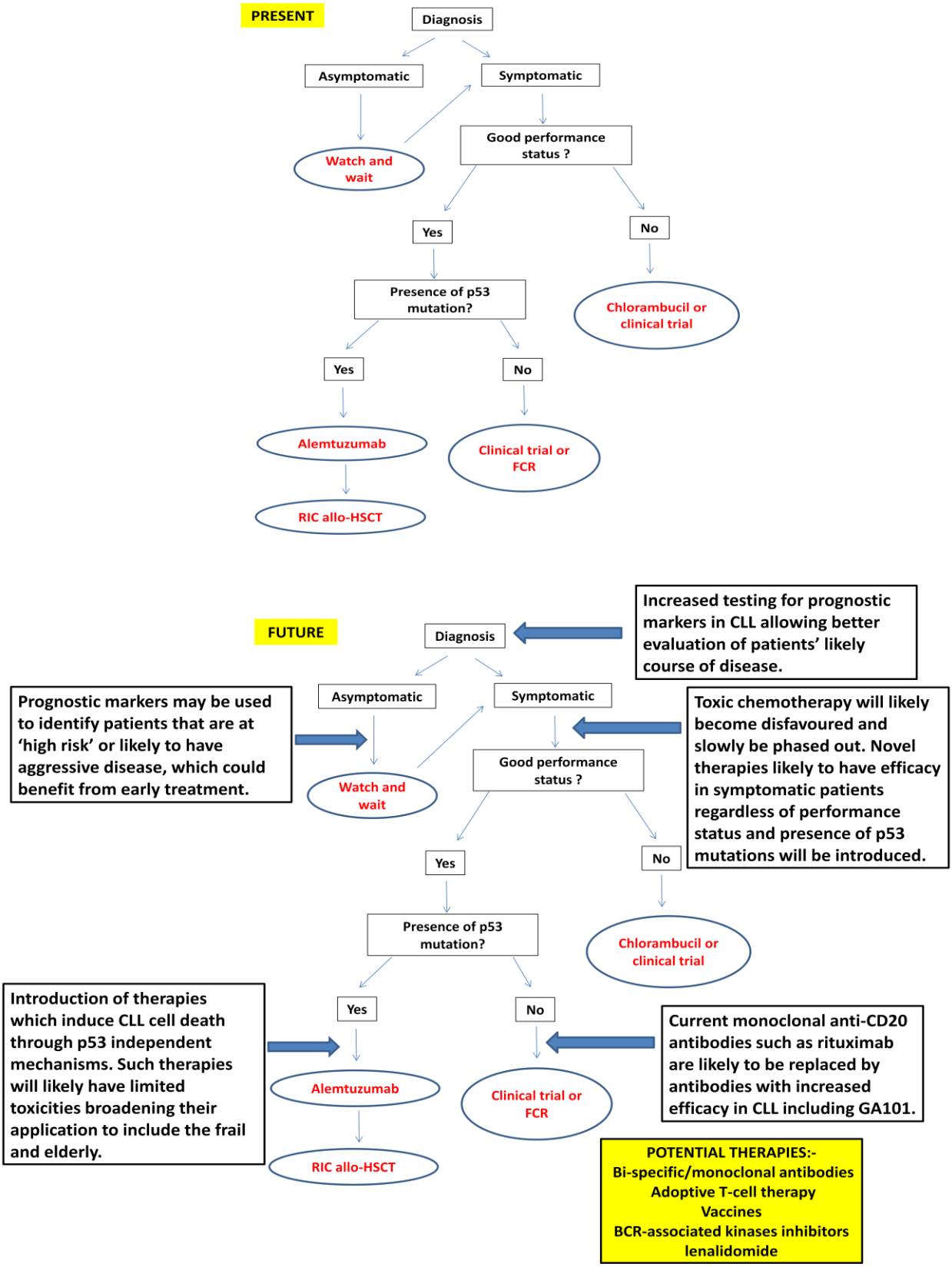


Figure 6.1 Present and future directions of therapy in CLL. Adapted from (Gribben 2010).

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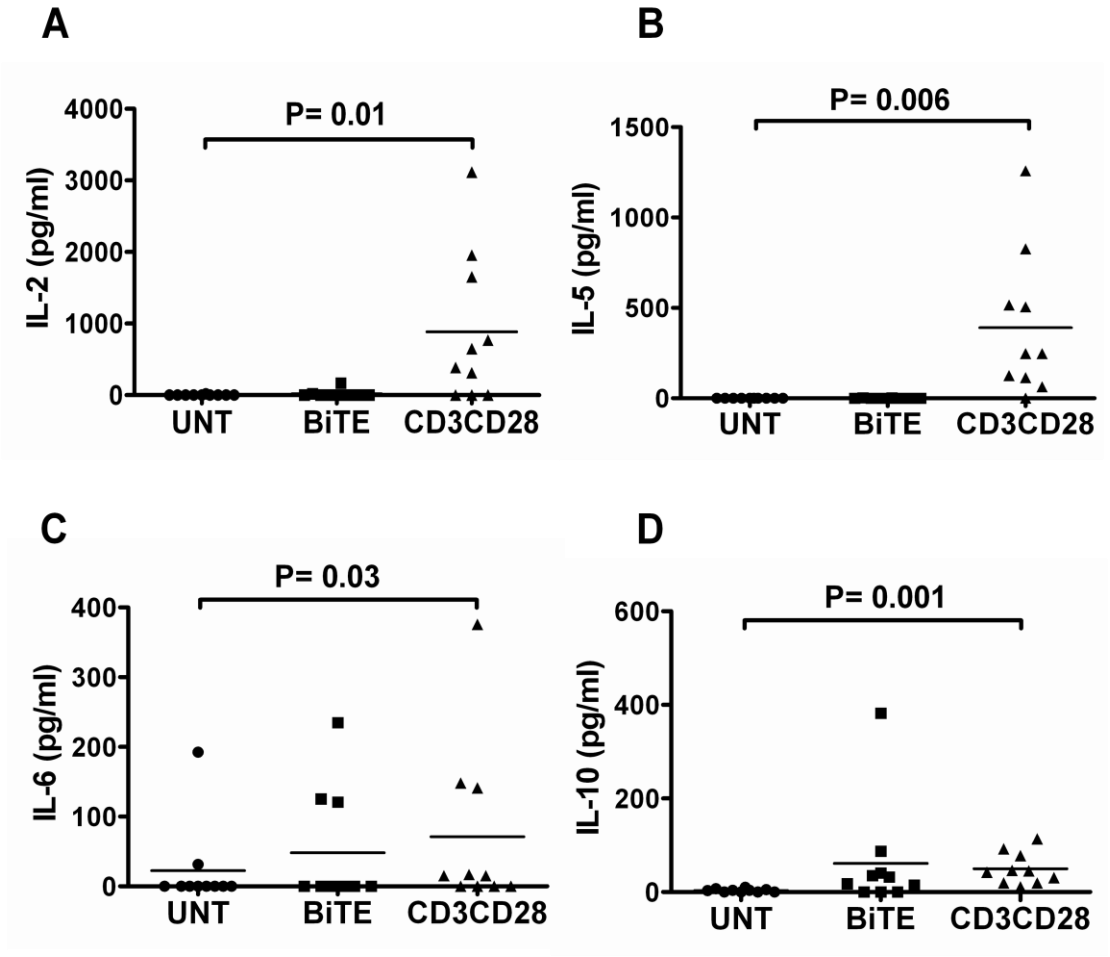
APPENDIX

Appendix 1 Concentrations of IL-2, IL-5, IL-6 and IL-10 released from CLL patient PBMCs after incubation with blinatumomab or CD3/CD28 beads.



Appendix 2 Characteristic profiles of CLL patients used in the blinatumomab study

ID	AGE	Binet Stage	LDT	% CD38	Genetics	IGHV Status	IGHV Usage	% ZAP-70	Treatment
001	73	A	<12	35.0	N	100.00%	Vh 3-11	31.6	U
002	60	B	ND	ND	13q-	ND	ND	ND	U
003	67	A	>12	8.7	ND	ND	ND	3.4	U
004	72	A	>12	0.4	N	ND	ND	5.5	U
005	69	A	>12	2.3	13q-	89.00%	Vh 3-7	2	U
006	58	A	<12	5.0	N	90.00%	Vh 4-34	34	T
007	83	A	>12	0.9	ND	92.00%	Vh 3-23	10.3	U
008	72	A	>12	99.0	ND	91.00%	Vh 4-61	66	U
009	65	A	>12	6.9	N	89.00%	Vh 3-53	1.4	U
010	73	A	<12	10.1	ND	94.00%	Vh 4-39	2.4	T
011	64	A	<12	10.5	ND	ND	ND	3.1	U
012	53	A	<12	19.4	13q-	ND	ND	0	U
013	70	A	>12	3.3	ND	84.00%	Vh 3-11	0.2	U
014	79	A	>12	6.7	ND	ND	ND	1.5	U
015	61	A	>12	0.4	N	90.00%	ND	0.6	T
016	64	A	>12	97.0	13q-	99.00%	Vh 3-30	43	U
017	79	A	>12	7.0	13q-/17p-	ND	ND	4	T
018	78	A	>12	ND	ND	ND	ND	ND	U
019	82	A	<12	2.7	ND	87.00%	Vh 3-74	20	U
020	58	A	>12	0.8	13q-	ND		19	U
021	58	A	>12	81.0	N	98.00%	Vh 3-48	85	U
022	73	A	<12	50.2	N	96.00%	Vh 3-48	0.3	T
023	61	A	ND	7.0	ND	ND	ND	18	U
024	65	B	ND	10	ND	ND	ND	ND	U
025	53	A	ND	8.2	13q-	ND	ND	0.4	U
026	72	A	>12	ND	ND	ND	ND	ND	U
027	54	A	>12	3.0	13q-	92.00%	Vh 3-23	10.3	U
028	72	A	ND	ND	ND	ND	ND	ND	U
029	78	A	>12	ND	6q-	ND	ND	ND	T
030	64	A	ND	16.0	Trisomy 12	100.00%	Vh 3-23	81	T
031	66	A	>12	2.3	ND	ND	ND	ND	T
032	57	ND	ND	ND	ND	ND	ND	ND	U
033	42	ND	>12	ND	N	ND	ND	ND	U
034	73	A	>12	5.5	Trisomy 12	91.00%	Vh 4-34	0.3	T
035	40	B	ND	6.0	11q-	96.00%	Vh 4-34	3.6	T

LDT: lymphocyte doubling time; ND: not determined

$13q^-$ and $17p^-$: any FISH or karyotypic abnormality of 13q or 17p; N: normal cytogenetics

IGHV status: <98% sequence homology with the closest germline sequence (mutated); >98% sequence homology with the closest germline sequence (unmutated)