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Delineation of molecular events that occur in a PKCα-KR-mediated murine model of CLL

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A thesis submitted for the Doctor of Philosophy
at the University of Glasgow

Faculty of Medicine

Submitted: June 2012

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Summary

Chronic lymphocytic leukaemia (CLL) is the most prevalent leukaemia of the Western world, and despite the recent evolution in clinical treatment of the disease, it remains incurable. Although current therapies such as allogeneic hematopoietic stem cell (HSC) transplantation have been successfully used to treat CLL, this is an option for only a minority, as most CLL patients are diagnosed over the age of sixty and cannot withstand the harsh transplantation procedures. Combination chemotherapy, such as fludarabine and cyclophosphamide, has been shown to significantly improve response rate and prolonged remission in CLL patients, however, no improvement in overall survival has been observed. Patients eventually relapse due to re-emergence of minimal residual disease (MRD). Therefore it is critical that further clinical therapies are investigated in order to eliminate MRD, and offer hope to patients that are unresponsive to current treatments.

CLL is marked by the presence of the accumulation of long-lived mature monoclonal B cells in peripheral lymphoid organs, bone marrow and peripheral blood with the specific phenotype of CD19$^\text{hi}$, CD5$^+$, CD23$^+$ and IgM$^\text{lo}$ that resist apoptosis. The in vivo accumulation of leukaemic lymphocytes is highly facilitated by interactions of CLL cells with other cells present in their microenvironment, including stromal cells and soluble factors such as IL4. Studies have established a variety of mechanisms potentially responsible for disease progression in CLL, including chromosomal abnormalities and intrinsic defects in the apoptotic machinery due to higher levels of the anti-apoptotic protein Bcl-2 family member proteins Bcl-2 and Mcl-1, thus making this disease extremely heterogeneous.

Although the apoptotic machinery is certainly dysregulated in CLL, it is not simply a disease of a clonal accumulation of B cells, rather, proliferation is occurring as well as apoptosis, accounting for up to 2% of the clone size per day. CLL B cell proliferation centres exist within lymph nodes (LN) and bone marrow (BM) where B cells receive signals from their B cell antigen receptor (BCR) to proliferate, generating a very aggressive form of the disease. In addition, evidence suggests that stimulation through the BCR plays a pivotal role in pathogenesis of CLL since CLL B cells have a phenotypic profile of B cells activated by antigen interaction and a genetic expression profile of antigen experienced B cells.

During the course of our studies assessing the impact of modulating protein kinase C (PKC) signaling in B cell development in vitro or in vivo, we developed a unique
model system to investigate the mechanisms underlying the induction of CLL. Introduction of full length, catalytically inactive PKCα (PKCα-KR) into HSCs derived from wild type mouse fetal liver (FL), and subsequent culture of the cells either in vitro or in vivo resulted in the generation of a population of B lymphocytes that are phenotypically similar to human CLL cells (CD19hi, CD5+, CD23+, IgMlo). PKCα-KR-expressing FL cells also expressed enhanced proliferative capacity over untransduced cells and were refractory to apoptosis. These results indicate that the subversion of PKCα signaling acts as an oncogenic trigger for developing B lymphocytes. The aim of this project was to identify similarities between our murine CLL (mCLL) model and human CLL and investigate putative translational therapeutic targets. The main findings of this study implicate PKCβII as an important survival and proliferation signal within mCLL. Cyclin D1 is also upregulated within mCLL, linked to an increase in the proliferative capacity of mCLL cells, and is regulated through transcriptional repressor 4EBP1, which appears inactive in both mCLL and human CLL. In addition, PKCα-KR transduced cells harbour the potential for lineage plasticity in a microenvironment-dependent manner, whereby PKCα-KR B cells lineage switch to T cells upon Notch ligation. The reprogramming occurs via a reduction in B cell specific genes and an upregulation of T cell specific genes, implicating the deregulation of PKCα activity/expression as a potential mechanism for lineage trans-differentiation during malignancies. Importantly, in human CLL, PKCα is downregulated at the transcript and protein levels implicating it a tumour suppressor, highlighting the translational capacity of our CLL mouse model.
Declaration

This work represents original work carried out by the author and has not been submitted in any form to any other University.

Milica Vukovic

June 2012
Acknowledgements

First and foremost I’d like to thank Dr. Alison Michie for her endless support over the years as a supervisor, a role-model and a friend. You are one of the most intelligent and caring people that I’ve encountered in my life. Thank you for always being so kind, honest and understanding. To Dr. Rinako Nakagawa, Dr. Emilio Cosimo and Dr. Alison McCaig, thank you for your help in the lab, it was much appreciated.

A significant mention goes to Professor Tessa Holyoake and the Paul O’Gorman Leukaemia Research Centre group for always being there to answer questions, find solutions and extend care and support to my career and to me personally. I thank you sincerely. To Dr. Kamil Kranc, thank you for your patience, support and helpful science chat.

To Dr. Susan Jamieson, an endless vehicle of inspiration, support and trust, I thank you wholeheartedly.

A very special thank you goes to Dr. Juan Manuel Iglesias for being the most patient and caring mentor that I’ve ever had, and for always having the time to provide expert advice. To Dr. Torsten Stein, my advisor, thank you for your support and encouragement. I’d like to thank Rod Ferrier, Clare Orange and Mark Catherwood for their immense help with immunohistochemistry.

I am very lucky to have Dr. Camille Huser, Dr. Daria Olijnyk, Dr. Verica Paunovic, Amy Sinclair and Maria Karvela who have been the most loyal and trustworthy colleagues and the most wonderful friends.

To my dearest, most wonderful fiancé Marcelo, thank you for helping me, believing in me and standing by me always; you are my love, my rock and my best friend.

Most importantly, I’d like to thank my parents Jovan and Slavica; the best parents in the world. You have always trusted me blindly, made me happy, loved and supported me unconditionally. To Jovs, my wonderful sister and best friend, you are so kind and caring and I couldn’t have done this without you. To Nina, my other wonderful sister and best friend, you are so warm-hearted and charismatic and I couldn’t have done this without you. I’d also like to thank Professor Luck for always being wise and inspirational. I hope to enjoy life as much as you do.

Last but not least, I’d like to thank the University of Glasgow, the Overseas Research Students Awards Scheme, the J.R. Robertson Endowment Scholarship and the Lady Tata Memorial Trust for supporting my research. Without this funding, my research would not have been possible.
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<td>avidin-biotin-complex</td>
</tr>
<tr>
<td>AID</td>
<td>activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>APRIL</td>
<td>a proliferation-inducing ligand</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Btk</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>c-myc</td>
<td>c-myelocytomatosis viral oncogene</td>
</tr>
<tr>
<td>CAP</td>
<td>cyclophosphamide/doxorubicin/prednisone</td>
</tr>
<tr>
<td>CB</td>
<td>cord blood</td>
</tr>
<tr>
<td>CDK6</td>
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<tr>
<td>CDR3</td>
<td>complementarity-determining region 3</td>
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<tr>
<td>CHOP</td>
<td>cyclophosphamide/doxorubicin/vincristine/prednisone</td>
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<tr>
<td>CLL</td>
<td>chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CR</td>
<td>complete remission</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>CREB</td>
<td>cyclin AMP-responsive element binding protein</td>
</tr>
<tr>
<td>CSR</td>
<td>class switch recombination</td>
</tr>
<tr>
<td>D</td>
<td>diversity</td>
</tr>
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<td>diacylglycerol</td>
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<td>DNA (cytosine-5')-methyltransferase 1</td>
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<td>early B cell factor</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>eGFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>eukaryotic initiation factor</td>
</tr>
<tr>
<td>EPAC</td>
<td>exchange protein activated by cyclic AMP</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated mitogen activated protein kinase</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
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<tr>
<td>FC</td>
<td>fludarabine and cyclophosphamide</td>
</tr>
<tr>
<td>FCR</td>
<td>fludarabine, cyclophosphamide and rituximab</td>
</tr>
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<td>FDCs</td>
<td>follicular dendritic cells</td>
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<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
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<td>foetal liver</td>
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<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>germinal centre</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled chemokine receptors</td>
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<tr>
<td>GSK3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HCL</td>
<td>hairy-cell leukaemia</td>
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<tr>
<td>HPCs</td>
<td>haematopoietic progenitor cells</td>
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<tr>
<td>HRP</td>
<td>horseradish-peroxidase</td>
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<tr>
<td>HSA</td>
<td>heat stable antigen</td>
</tr>
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<td>HSC</td>
<td>haematopoietic stem cell</td>
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<tr>
<td>i.p.</td>
<td>intra-peritoneally</td>
</tr>
<tr>
<td>IBLP</td>
<td>immunoblastic lymphoma plasmacytoid</td>
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<td>ICR</td>
<td>imprinting control region</td>
</tr>
<tr>
<td>Id</td>
<td>inhibitor of differentiation</td>
</tr>
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<td>Iono</td>
<td>ionomycin</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IgH</td>
<td>Ig heavy chain</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IκB kinases</td>
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<td>IKKα</td>
<td>IκB kinase-α</td>
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<td>IL7R</td>
<td>interleukin-7 receptor</td>
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<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>J</td>
<td>joining</td>
</tr>
<tr>
<td>L</td>
<td>leader sequence</td>
</tr>
<tr>
<td>LDL</td>
<td>lithium dodecyl sulphate</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>MALT</td>
<td>mycosa associated lymphoid tissue</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MBL</td>
<td>monoclonal B cell lymphocytosis</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>myeloid cell leukaemia sequence 1</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>mCLL</td>
<td>murine CLL</td>
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<tr>
<td>MDR</td>
<td>minimal deleted region</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>mLN</td>
<td>mesenteric lymph nodes</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propane sulfonic acid</td>
</tr>
<tr>
<td>MPPs</td>
<td>multipotent progenitor cells</td>
</tr>
<tr>
<td>MRD</td>
<td>minimal residual disease</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
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<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>NDC</td>
<td>no drug control</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<td>NGFIB</td>
<td>nerve growth factor IB</td>
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<td>NHL</td>
<td>non-Hodgkin’s lymphoma</td>
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<td>NLCs</td>
<td>nurse-like cells</td>
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<tr>
<td>Notch1</td>
<td>Notch homolog-1</td>
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<tr>
<td>NZB</td>
<td>New Zealand Black</td>
</tr>
<tr>
<td>OA</td>
<td>okadaic acid</td>
</tr>
<tr>
<td>Oct1</td>
<td>octamer transcription factor 1</td>
</tr>
<tr>
<td>Oct2</td>
<td>octamer transcription factor 2</td>
</tr>
<tr>
<td>OR</td>
<td>overall response</td>
</tr>
<tr>
<td>ORR</td>
<td>overall response rate</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PALS</td>
<td>periarteriolar lymphoid sheath</td>
</tr>
<tr>
<td>PAX5</td>
<td>paired-box-protein-5</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PDK1</td>
<td>3’-phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PFS</td>
<td>progression-free survival</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>pHSCs</td>
<td>pluripotent haematopoietic stem cells</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodine</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>pim-1</td>
<td>proto-oncogene serine/threonine-protein kinase</td>
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<td>PIP3</td>
<td>phosphoinositides</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein serine/threonine phosphatase A</td>
</tr>
<tr>
<td>pre-BCR</td>
<td>pre-B cell receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinyliden difluoride</td>
</tr>
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<td>RAG</td>
<td>recombination activation genes</td>
</tr>
<tr>
<td>Rag</td>
<td>ras-related GTPase</td>
</tr>
<tr>
<td>rapa</td>
<td>rapamycin</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>SCF</td>
<td>stem-cell factor</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficient</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromally secreted stromal cell-derived factor 1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SFFV</td>
<td>spleen focus forming virus</td>
</tr>
<tr>
<td>SHM</td>
<td>somatic hypermutation</td>
</tr>
<tr>
<td>SNBTS</td>
<td>Scottish National Blood Transfusion Service</td>
</tr>
<tr>
<td>Sox-4</td>
<td>Sex-determining region Y-box 4</td>
</tr>
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</table>
SV40  simian virus 40
SYK  spleen tyrosine kinase
T1  transitional 1
T2  transitional 2
TACI  transmembrane activator and calcium modulator and cyclophilin ligand-interactor
TCL1  T cell leukaemia 1
TCR  T cell receptor
TdT  terminal deoxynucleotidyl transferase
TMB  tetramethylbenzidine
TNF  tumour necrosis factor
TRAF2DN  TRAF2 mutant that lacks N' terminus RING and zinc finger domains
TRAFs  Tumour necrosis receptor associated factors
TSC2  tuberous sclerosis complex 2
VEGF  vascular endothelial growth factor
VH  variable heavy chain
ZAP70  70 kDa zeta associated protein
Chapter 1:
Introduction
1.1 Normal B cell development and function

1.1.1 Summary of B cell development

B cell development is characterized by a series of steps marked by the upregulation or downregulation of specific genes that regulate differentiation, proliferation, migration, survival and apoptosis (Honjo & Neuberger 2004). At each stage of B cell development, gene rearrangements lead to either successful or unsuccessful immunoglobulin (Ig) protein chain products, allowing the cell to proceed to the next stage of development or to apoptose respectively (Murphy et al. 2008). It is crucial that a B cell expresses only one receptor specificity, and is therefore strictly monitored by inherent checkpoints to avoid dysfunctional development (Murphy et al. 2008). Therefore an immature B cell that binds self-surface antigen is removed through negative selection in the BM. A mature B cell bound to foreign antigen is activated and eventually gives rise to plasma and memory cells (Murphy et al. 2008).

1.1.2 B cell development

Pluripotent haematopoietic stem cells (pHSCs) develop into lymphoid progenitors that can generate B, T and NK cells and are phenotypically Lin⁻CD27⁺ckit⁺Sca-1⁺ within the mouse and Lin⁻CD34⁺CD38⁻ within the human (Honjo & Neuberger 2004; Nagasawa 2006) (Figure 1.1). Within the BM microenvironment lie stromal cells that provide cell-to-cell contact through cell adhesion molecules and their ligands and also secrete necessary cytokines and chemokines for the subsequent differentiation into B cells. Multipotent progenitor cells (MPPs) express cell-surface receptor tyrosine kinase called Flt-3 in human (Flt-3/Flk-2 in mice) that binds Flt-3 ligand on stromal cells. Through Flt-3 signaling, an MPP differentiates to a common lymphoid progenitor (CLP) that can give rise to both B and T cells (Honjo & Neuberger 2004). Flt-3 signaling together with activity of transcription factor PU.1 (also known as spleen focus forming virus (SFFV) proviral integration oncogene SPI1) induces expression of the interleukin-7 receptor (IL7R) in both human and mouse. IL7 secreted by the stromal cells is important for the development of B and T cells in mice (Honjo & Neuberger 2004). In human, although IL7R is expressed on HSCs from the BM and foetal liver (FL), stem cells within human cord blood (CB) that are CD34⁺CD38⁻CD7⁺ do not express IL7R, indicating that it may not be necessary in human B cell development (Hao et al. 2001; Milne & Christopher J Paige 2006; Hoebeke et al. 2007). In addition to IL7,
stem-cell factor (SCF) is another cytokine that stimulates growth of the haematopoietic lineage and B cell progenitors (Honjo & Neuberger 2004). SCF is a membrane bound cytokine that interacts with receptor tyrosine kinase ckit (CD117) on precursor cells. Stromally secreted stromal cell-derived factor 1 (SDF-1), also known as chemokine CXCL12 is also important in B cell development at this stage for retention of B cell precursors within the BM (Honjo & Neuberger 2004). CLP are marked by surface expression of CD45^Lin^ckit^lo^ Sca-1^lo^ in mouse and CD34^+^CD38^-^CD10^-^ in human (Honjo & Neuberger 2004; Nagasawa 2006) (Figure 1.1). At this point of development definitive B cell fate is specified by activity of E2A and early B cell factor (EBF) (see Section 1.1.4).

The CLP gives rise to the pre-pro-B cell, where Ig rearrangement begins with the heavy-chain locus driven by recombination activation genes (RAG)s (Figure 1.2). Phenotypically pre-pro-B cells are ckit^-B220^-Flt^-3^-CD43^-^BP^-1^- and heat stable antigen (HSA/CD24)^-^ in mouse and CD34^+^CD38^-^CD19^-^CD10^-^ in human (Figure 1.1) (Nagasawa 2006). Ig heavy chain (Ig_H) rearrangement begins with the coming together of diverse (D) to joining (J)_H regions at both alleles of the Ig_H locus (Figure 1.2). However these cells are not decisively B cells, as D_H-J_H gene rearrangements are also found in T, NK and myeloid cells (Honjo & Neuberger 2004). As the lymphoid progenitors continue differentiation toward the B cell fate, some cells begin to express Igα (CD79a). In addition transcription factors paired-box-protein-5 (PAX5) and aiolos are expressed within this population, and Id genes repressed (Honjo & Neuberger 2004).
Figure 1.1 B cell development: surface protein expression

At this point the cell becomes a pro-B cell that begins rearrangement of variable heavy chain (V_{\mu}) gene segment to DJ_{\mu} on one chromosome (Honjo & Neuberger 2004)(Figure1.2). If this rearrangement is unsuccessful, rearrangement on the other allele begins. Phenotypically pro-B cells are characterized as CD19^{+}ckit^{+}B220^{+}Flt-3^{+}CD43^{+}BP-1^{+}HSA^{+} in mouse and CD34^{+}CD38^{+}CD19^{+}CD10^{+} in human (Figure 1.1) (Nagasawa 2006; Igarashi et al. 2002).

Terminal deoxynucleotidyl transferase (TdT) is an enzyme that is expressed by pro-B cells. By adding non-templated nucleotides at the joints in between rearranged gene segments, TdT aids in the diversity of the BCR repertoire. Once V(D)J has rearranged, the rearranged heavy chain (IgV_{\mu}) must be functionally tested. The heavy chain is incorporated into a receptor along with two surrogate proteins that have similar structures to the light chains (which have not yet rearranged) (Ogawa et al. 2000; Gounari et al. 2002). Expression of these two surrogate proteins: \lambda 5 and VpreB are induced by E2A and EBF. Alongside these surrogate proteins, Ig\alpha (CD79\alpha) and Ig\beta (CD79b) are two other necessary proteins expressed by pro-B cells that are crucial components of the pre-B cell receptor
Formation of the pre-BCR marks the transition between a pro-B cell toward a large pre-B cell population which is CD19+ckit−B220+Flt-3−CD43−BP-1−HSA+ in mouse and CD34−CD19−CD10+ in human (Figure 1.1) (Nagasawa 2006; Murphy et al. 2008). Signaling via the pre-BCR involves key molecules such as B cell linker protein (BLNK) and Bruton’s tyrosine kinase (Btk), and ensures that only one of the two heavy chain alleles are expressed in a cell, a process termed ‘allelic exclusion’. Allelic exclusion involves a reduction in expression of RAG1 and RAG2 and reduction in access to heavy chain locus to the recombinase machinery. Pro-B cell to large pre-B cell transition is also marked by a proliferation event, whereby the population of cells that have successfully formed the pre-BCR expands before it becomes a population of small pre-B cells.

Expression of VpreB and λ5 is turned off at this point (Grawunder et al. 1995) through pre-BCR signaling via Igα/Igβ, spleen tyrosine kinase (SYK) and BLNK (Melchers 2005).

**Figure 1.2 V(D)J Recombination**

Modified from Murphy et al. 2008
Large proliferating pre-B cells eventually stop dividing and light chain rearrangement re-occurs. At this point TdT is activated in human cells (but not in mouse) (Honjo & Neuberger 2004; Payne & Crooks 2007).

Light chain rearrangement is initiated by the reactivation of RAG proteins. Each small pre-B cell can generate a unique rearranged light chain, thus again enlarging the overall pool of B cells with different antigen specificities. Since light chain loci lack D segments, rearrangement occurs by VJ joining (Figure 1.2). Once light chain rearrangement is successful on both alleles, a light chain pairs with a μ chain forming an intact IgM that is expressed on the cell surface and the cell enters the immature B cell stage. Allelic and isotypic exclusion occur after light chain rearrangement to produce B cells expressing only one type of light chain: κ or λ. Immature B cells are found in BM and spleen and express IgM but not IgD on their surface. They also continue to express RAG1, RAG2, and AA4.1, whereas CD21 and CD23 are not yet expressed (Honjo & Neuberger 2004). If immature B cells express autoantigen specific BCR, arrest in differentiation is initiated and apoptosis follows. On the other hand, low avidity to autoantigen can result in positive selection of immature B cells into the B1 cell compartment in the mouse, or periphery for human (Honjo & Neuberger 2004). Separate B1 and B2 compartments found in the mouse are yet to be identified in human (see section 1.1.2.1) (Payne & Crooks 2007). An immature B cell passes through a transitional stage (see section 1.1.2.2) where it can be positively selected for and allowed to mature, now expressing both IgM and IgD. A mature B cell bound to foreign antigen is activated and eventually gives rise to plasma and memory cells (Murphy et al. 2008).

1.1.2.1 B1 versus B2 cells

In mouse, B1 cells consist of B1a and B1b cells, which differ based on surface phenotype whereby B1a cells are IgMloIgDlo/CD5+ and B1b cells are IgMhiIgDlo−CD5lo−. B2 cells are IgMloIgDhiCD5− (Hayakawa & Hardy 2000). B1 cells are thought to be derived from FL, whereas B2 cells are of adult BM origin. B1 cells are found within spleen, pleural and peritoneal cavities and can express CD11b within the peritoneal cavity (Berland & Wortis 2002). Two models exist that explain the origin of a B1 and B2 cells. In the single lineage model, B1 and B2
cells are thought to derive from two distinct committed precursors, and are therefore distinct lineages. In the layered model, B1 cells are thought to develop as a result of antigen-driven differentiation and selection (Berland & Wortis 2002). B1 cells are thought to develop as a result of B cells encountering repetitive antigens during neonatal life, whereas B2 cells develop as a result of B cells being exposed to exogenous antigens during adult life. In this model, B2 cells can transition into B1 cells through BCR stimulation (Herzenberg 2000). However, Montecino-Rodriguez and colleagues show that B1 and B2 progenitors are distinct subsets based on CD138 and MHC class II molecule expression during early B cell development (Montecino-Rodriguez et al. 2006; Tung et al. 2006).

1.1.2.2 Transitional B cells

Between BM immature B cells and peripheral mature B cells exist a subset of transitional B cells, termed transitional 1 (T1) and transitional 2 (T2) cells. The transitional stage is a key juncture where developing B cells gain access to the spleen, start responding to T-cell help and become insensitive to negative selection. Transitional B cells still resemble their BM counterparts (Chung et al. 2003). They are still susceptible to negative selection and are HSA^high AA4.1^+, express surface IgM at higher levels and CD22 and B220 at lower levels compared to mature B cells. Phenotypically, T1 B cells are AA4.1^+IgM^+IgD^−CD21^loCD23^− whereas T2 B cells IgM^hiIgD^hiCD21^+CD23^+. Allman et al describe a T3 subset which is similar to T2 but expresses slgM at lower levels than T2 cells (Allman et al. 2001).

When immature B cells exist in the BM, they emigrate to the spleen via terminal branches of central arterioles and enter the red pulp, penetrating the marginal zone sinuses and reaching the periarteriolar lymphoid sheath (PALS). T1 cells are limited to the outer PALS where the T-cell-B cell interphase lies, whilst T2 cells migrate toward B cell follicles, most likely due to their high surface expression of chemokine receptor CXCR5 compared to T1 cells (Chung et al. 2003). Whereas mature B cells induce proliferation upon BCR engagement, T1 cells do not proliferate and are vulnerable to apoptosis. It is unclear whether T2 cells proliferate and are resistant to apoptosis following BCR engagement (Loder et al. 1999; Allman et al. 2001; Su & Rawlings 2002; Petro et al. 2002; Chung et al. 2002; Chung et al. 2003). However, with T-cell help, T2 B cells do proliferate when stimulated and evade BCR-induced apoptosis, unlike T1 cells (Chung et al.
Both T1 and T2 cells, however, are capable of processing and presenting antigen like mature B cells. Therefore the transition from T1 to T2 is key in their subsequent development into mature B cells as T2 cells are more responsive to positive selection pressures in comparison to T1 cells.

### 1.1.2.3 Marginal zone B cells

Marginal zone (MZ) B cells are IgM^{hi} IgD^{low} CD21^{hi} CD23^{-} cells that can be found within the outermost portion of the white pulp within the spleen, in tonsil subepithelial areas, dome regions of Peyer patches and within the subcapsular portions of the LN (Chiorazzi & Ferrarini 2011). MZ B cells are capable of a T-cell independent response to bacterial polysaccharides and can carry either unmutated or mutated IgV rearrangements (Weill et al. 2009). In human, 70-80% of MZ B cells have mutated IgV_{H} rearrangements (Chiorazzi & Ferrarini 2011). MZ B cells can undergo IgV_{H} mutations and Ig isotype switching during responses in MZ areas. Activation-induced deaminase (AID) has been postulated by some to be responsible for inducing IgV mutations in MZ B cells, although some argue that AID cannot be detected in splenic MZ B cells (Chiorazzi & Ferrarini 2011; Willenbrock et al. 2005). A more accepted explanation is migration of B cells that have acquired mutations and undergone class switch recombination (CSR) within the germinal centre (GC) into the MZs. MZ B cells have been demonstrated to be antigen-experienced as confirmed by evidence of clonal expansion of IgM^{hi} IgD^{low} IgV unmutated B cells. Also, IgV mutated MZ B cells are capable of diversification and expansion within the MZ (Dono et al. 2000; William et al. 2002; Dono et al. 2007). Within MZ areas, IgG or IgA expressing B cells are found, termed switched memory B cells, that use mutated IgV_{H} genes and are most likely of post-GC origin (Chiorazzi & Ferrarini 2011).

### 1.1.3 The germinal centre reaction

If a foreign antigen invades the system, CD4^{+} helper T cells are activated by antigen-presenting cells in the periphery (Melchers 2005). These activated T cells travel into the peripheral lymphoid organs where the antigen activates resting mature B cells, forming GCs where centroblast and centrocyte B cells proliferate. After a few days, a GC becomes visible with histology and is marked by the presence of a light zone, rich in follicular dendritic cells (FDCs) and a dark zone filled with a compact pool of centroblasts (Figure 1.3). Within the GC, CD40 on B cells binds to CD40 ligand (CD154) on helper T cells and AID is expressed. AID
induces somatic hypermutation (SHM) in $V_H$ and $V_L$ regions of Ig, induces CSR via switch regions and induces replacement of V-region encoding segments (Melchers 2005).

**Figure 1.3 The germinal centre reaction**

The Igs on these B cells are now hypermutated and express $Ig_H$ classes other than IgM (such as IgG, IgE or IgA) (Klein & Dalla-Favera 2005). These cells can become long-living memory B cells that have high affinity to that particular antigen that will in turn get reactivated if the same/similar antigen invades the system (Klein & Dalla-Favera 2005). The hypermutated slg-expressing B cells can also turn into high-affinity antibody-secreting plasma cells. Both memory and plasma cells exit the GC and return to the BM (Melchers 2005). Alternatively if a B cell does not exhibit high affinity to an antigen, it is unlikely to survive, resulting in apoptosis.

SHM of mature B cells in response antigen stimulation by T cells not only leads to hypermutation of Ig $V_H$ and $V_L$ regions and Bcl-6 gene, but occasionally also other genes that are actively transcribed within the GC which can in turn lead to malignancies. For example, in diffuse large cell lymphoma, genes such as c-
myelocytomatosis viral oncogene (c-myc), proto-oncogene serine/threonine-protein kinase (pim-1) and PAX5 are also hypermutated (Melchers 2005).

1.1.4 Transcriptional Control

The decision of a lymphoid progenitor to become a B or T cell is controlled by the Ikaros gene which controls downstream target genes RAG, TdT, Ig\(\text{H}\) and Ig\(\text{L}\) chain genes and Ig\(\alpha\) (Honjo & Neuberger 2004). B cell commitment specifically, is initially mainly dependent on two transcription factors: the basic-helix-loop-helix protein E2A and EBF. E2A gene has two isoforms: E12 and E47 generated by alternate splicing. IL7 signaling promotes E2A expression that cooperates with PU.1 to induce EBF expression (Murphy et al. 2008). Together these transcription factors induce expression of B cell specific proteins and thus B cell fate. In fact, absence of E2A negatively affects B lineage differentiation. E2A\(^{-}\) mice do not rearrange Ig at the D\(_{H}\)-J\(_{H}\) nor V\(_{L}\)-J\(_{L}\) level (Bain et al. 1994; Zhuang et al. 1994; Lin & Grosschedl 1995; Sigvardsson et al. 1997; Kee & Murre 1998). Transcript levels of RAG1, RAG2, CD19, Ig\(\alpha\), Ig\(\beta\), VpreB, \(\lambda\)5 and PAX5 are reduced or abolished when E2A is downregulated. In addition, E2A isoform E47 induces expression of TdT and Ig\(\text{H}\) chain locus (Choi et al. 1996). Also, deficient EBF expression in mice results in a B cell developmental block similar to E2A\(^{-}\) mice (Lin & Grosschedl 1995).

Although E2A and EBF are important initially in B cell development, they are not sufficient to push B cell precursors to develop pre-BCRs and functional BCRs. Expression of transcription factor PAX5 is crucial to B cell development (Honjo & Neuberger 2004). It is expressed in all stages of B cell development apart from plasma cells (Urbánek et al. 1994; Busslinger & Urbánek 1995). In PAX5\(^{-}\) mice B cell development stops at the pro-B stage (Urbánek et al. 1994). These cells express VpreB and \(\lambda\)5 genes, Ig\(\alpha\) and Ig\(\beta\), RAG1 and RAG2, octamer transcription factors 1 and 2 (Oct1; Oct2), sex-determining region Y-box 4 (Sox-4), PU.1, Ikaros, E2A and EBF (Honjo & Neuberger 2004). PAX5\(^{-}\) cells do not initiate V\(_{H}\) to D\(_{H}\)-J\(_{H}\) rearrangement as normal pro-B cells and cannot therefore generate large pre-B cells that express the pre-BCR. Therefore, these cells cannot rearrange V\(_{L}\)-J\(_{L}\) and form immature or mature B cells.

PAX5 is not only necessary for B cell commitment, but also for the maintenance of B cell identity during subsequent differentiation (O'Brien et al. 2011). PAX5 activates the transcription of a number of downstream genes responsible for pre-
BCR signaling as well as other transcription factors that ensure B cell identity. More specifically, PAX5 regulates expression of pre-BCR components: V-DJ recombined IgH chain alongside surrogate light chains VpreB and λ5, and the Igα/β dimmer. Co-receptor CD19 which is responsible for positive amplification of pre-BCR signaling is also regulated by PAX5, alongside BLNK and phospholipase C gamma (PLCy)2 (Holmes et al. 2007). Signaling through the pre-BCR results in pre-B cell proliferation, activation of Igκ and allelic exclusion of the IgH locus. Expression of Aiolos which in turn represses λ5 resulting in termination of pre-BCR signaling, is also regulated by PAX5. In addition to activating B cell specific genes, PAX5 represses genes that are not B cell specific such as macrophage colony-stimulating factor (M-CSF) receptor encoding gene Csf1r important in macrophage development, Notch homolog-1 (Notch1) important in T cell development, and Flt3 which is important in early haematopoietic progenitors and early B cell development but downregulated during B cell commitment (Holmes et al. 2007). Therefore, PAX5 is a key regulator in the B cell gene expression program.

1.2 Chronic Lymphocytic Leukaemia
CLL is a disease characterised by the monoclonal expansion of mature B cells that highly express surface protein markers CD5 and CD23, and downregulate monoclonal slgM. Gene expression profiling data has highlighted the idea of phenotypic homogeneity within CLL, implicating transformation of a normal B cell. A B cell count of 5 x 10^9/L for 6 months or longer is used as a benchmark for CLL prognosis. Chromosomal abnormalities that are present in 80% of CLL cases are identified via fluorescence in situ hybridization (FISH), and are good predictors of progression (Desai & Pinilla-Ibarz 2012). Levels of β2-microglobulin and soluble CD23, lymphocyte doubling time, evidence of infiltration into the BM and lymphadenopathy, splenomegaly or hepatomegaly are also considered when assessing progression (Desai & Pinilla-Ibarz 2012). Mutational status of IgVH genes as well as surrogate markers ZAP70 and CD38 are also good determinants of disease stage (Rassenti et al. 2008).

Clinical staging is carried out using the Rai or Binet staging system (Desai & Pinilla-Ibarz 2012). Patients with the most advanced stage in either staging system have a 1-2 year median survival time whereas lowest stage CLL patients have a medium survival of more than 10 years (Rai et al. 1975; Binet et al. 1981).
Classically, CLL was considered a disease of accumulation rather than proliferation, because within the accumulation department (blood), most CLL cells are arrested in $G_0/G_1$ phase of cell cycle (Caligaris-Cappio & Hamblin 1999). However, current opinion highlights the importance of proliferative compartments within CLL, where cells are not resting yet actively proliferating and apoptosing (Messmer et al. 2005), eradicating the idea that CLL is a static disease, but rather dynamic. In fact, proliferation centres within the BM and LN of CLL patients display an aggregate of dynamic CLL cells that eventually make their way into the accumulation department (Granziero et al. 2001). Within proliferation centres are prolymphocytes and paraimmunoblasts surrounded by $CD4^+ CD40L^+ T$ cells. Of interest, stimulation of CD40 on CLL B cells through CD154 (CD40L) induces proliferation of otherwise resting CLL B cells (Buske et al. 1997; Kitada et al. 1999; Fluckiger et al. 1992) in vitro. This microenvironment mimicking system also allows for the upregulation of survivin, chemokine (C-C motif) ligand 17 (CCL17) and 22 (CCL22) which are characteristic of CLL cells within proliferation centres (Ghia et al. 2001; Granziero et al. 2001). Antigen stimulation together with T cell help, thus aids in clonal expansion of leukaemic cells which eventually accumulate within the blood through additional stromal help (Caligaris-Cappio & Ghia 2007). Proliferation compartments are a unique feature of CLL that other B cell malignancies do not share. Interestingly, however, in rheumatoid arthritis and multiple sclerosis, these proliferation centres do exist, substantiating the idea that a CLL cell is in fact antigen-experienced (Messmer et al. 2005), perhaps self-antigen-experienced due to the auto-immune characteristics of these two diseases.

### 1.2.1 IgV\textsubscript{H} mutational status

CLL cells have been demonstrated to be heterogeneous in terms of molecular and functional features such as clinical course (Klein et al. 2001; Caligaris-Cappio & Ghia 2007). While some CLL patients require immediate therapeutic intervention, others may never need therapy. The most prominent feature of CLL intraclonal heterogeneity arises from the two distinctive CLL subtypes based on IgV\textsubscript{H} gene mutational status. One group of CLL patients carries unmutated IgV\textsubscript{H} genes, while the other carries mutated IgV\textsubscript{H} genes. The two subsets have remarkable differential clinical outcomes whereby unmutated IgV\textsubscript{H} carrying patients display a worse overall prognosis than mutated IgV\textsubscript{H} carrying patients (Hamblin et al. 1999; Damle et al. 1999). CD38 expression and 70 kDa zeta associated protein
(ZAP70) positivity are another two distinguishing features within CLL patients, whereby patients that express CD38 and are ZAP70+ display a worse overall disease course (Damle et al. 1999; Crespo et al. 2003; Wiestner et al. 2003) (see sections 1.2.2 and 1.2.3). In fact, unmutated CLL cases have higher expression of ZAP70 and CD38 and can better activate key signal transduction pathways in response to BCR activation (see Section 1.2.5). Unmutated IgV H CLL cells also display greater proliferative capacity than mutated CLL cells (Klein et al. 2001; Rosenwald et al. 2001; Wiestner et al. 2003), supported by different telomere lengths (Damle et al. 2007; Messmer et al. 2005; Roos et al. 2008). In addition unmutated CLL cells are more likely to carry harmful genetic lesions such as 11q23 and 17p13 deletion (Kröber et al. 2002; Oscier et al. 1997; Stilgenbauer et al. 2007; Shanafelt et al. 2006).

At a functional level, intraclonal heterogeneity is also evident when CLL cells are stimulated through the BCR, CD40 or CD5 (Chen et al. 2002; Lanham et al. 2003; Moreau et al. 1997; Klein et al. 2001; Hamblin 2002; Hamblin et al. 1999; Damle et al. 1999; Crespo et al. 2003; Wiestner et al. 2003; Law et al. 1994; Zupo et al. 2000; Zupo et al. 1996; Lankester et al. 1995). There are also differences in expression of genes when considering different locations of a CLL cell within the body such as BM, LN and blood (Caligaris-Cappio & Ghia 2007). CLL cells residing within the proliferation centres, for example, have differential expression of survivin, CCL-17 and CCL-22 (Granziero et al. 2001). CD38 expression may also vary, whereby it is more present on CLL cells that have infiltrated the BM than those circulating within blood (Ghia et al. 2003).

1.2.2 Prognostic marker ZAP70

ZAP70 is a member of the ZAP70 protein tyrosine kinase family (of which SYK is another member) involved in T cell activation (Klein & Dalla-Favera 2005). When the T cell receptor (TCR) is engaged, ZAP70 interacts with immunoreceptor tyrosine-based activation motifs (ITAM) sequences of the TCR ζ-chains resulting in recruitment and activation of downstream signaling kinases (Au-Yeung et al. 2009). In normal B cells, it is exclusively expressed on mature tonsillar activated B cells and only transiently expressed in pro-B to pre-B cell transition (Schweighoffer et al. 2003). In CLL, its expression is used as a surrogate marker for the level of IgV gene mutation status, and thus as a predictor of clinical outcome (Crespo et al. 2003; Orchard et al. 2004). In concordance with this, stable expression of ZAP70
was shown to be correlated to 93% of unmutated CLL cases (Crespo et al. 2003; Wiestner et al. 2003). A study of 307 CLL patients revealed that ZAP70+ patient groups displayed a significantly shorter median time from diagnosis to treatment compared to ZAP70− CLL patients implicating ZAP70 to be a strong predictor of need for treatment in CLL (Rassenti et al. 2004).

1.2.3 Prognostic marker CD38

CD38 is a non lineage-restricted transmembrane glycoprotein that can function as an ectoenzyme, with the ability to catalyze cyclic ADP-ribose synthesis and dyrolysis that functions as a receptor. CD38 was originally also thought to be indicative of mutational status, whereby 30% of unmutated CLL cases investigated in one study expressed CD38 (Damle et al. 1999). More recently, no such association has been demonstrated (Poeta et al. 2001; Damle et al. 1999; D'Arena et al. 2001), however IgVH mutational analysis together with ZAP70 expression and CD38 expression can collectively prove to be of greater prognostic value than these factors individually (Morilla 2008; Giudice et al. 2005).

1.2.4 MicroRNA (miRNA)

Recent studies have highlighted the fact that CLL is a disease with substantial alterations of miRNA expression. miRNA gene expression profiles of normal versus CLL CD5+ cells revealed a significant proportion of differently expressed miRNAs including miR-183, miR-190, miR-24-1, miR-203, miR-16-1 and miR-15a all of which are down-regulated in CLL (Calin et al. 2004). Patients with 13q14 deletions have a better prognosis compared with patients with complex cytogenetic changes (Döhner et al. 2000). In addition 13q14.3 deletions are associated with the mutated subset of CLL which is also indicative of a good prognosis (Oscier et al. 2002). A comparative analysis of CLL samples either carrying or not carrying a 13q14 deletion revealed that miR-16-1, miR-24-2, miR-195, miR-203, miR-220, mirR221 were expressed at significantly lower levels and miR-7-1, miR-19a, miR-136, miR-154, miR-217 are expressed at significantly higher levels within the 13q14 deletion carrying patients (Calin et al. 2004). When mutated versus unmutated CLL subsets were analysed, five miRNAs were differentially expressed (miR-186, miR-132, miR-16-1, miR-102, miR-29c), highlighting the prognosis potential of miRNAs (Calin et al. 2004; Calin et al. 2005).
1.2.5 BCR Signaling in CLL

A stereotyped CDR3 is most commonly found within unmutated CLL cases, however 10% of mutated cases may carry an identical BCR. The notion of antigen exposure in CLL is underlined by the CLL cell’s signature of BCR-mediated stimulation (Damle et al. 2002). Signaling through the BCR is variable in CLL, where some cases, mostly unmutated have a significantly higher ability to signal through the BCR than their mutated counter parts (Lanham et al. 2003; Lankester et al. 1995). Intracellular regions of BCR components CD79a and CD79b contain ITAMs that interact with intracellular tyrosine kinases such as SYK and LYN (Zenz et al. 2010). These kinases in turn activate downstream pathways. The ability to transduce a BCR-mediated signal may depend on molecules such as ZAP70, HIS, CD38, or IgV\textsubscript{H} mutational status (Figure 1.4) (Zenz et al. 2010). For example, CLL cells that have unmutated IgV\textsubscript{H} genes and are ZAP70\textsuperscript{+} are stimulated through their BCR, resulting in recruitment of SYK and ZAP70 which phosphorylate and activate downstream targets BTK, phosphoinositide 3-kinase (PI3K), BLNK, PLC\textsubscript{γ} which in turn activate pro survival and anti-apoptotic signaling pathways such as protein-threonine protein kinase Akt, nuclear factor kappa B (NF-κB), extracellular regulated mitogen activated protein kinase (ERK) and mammalian target of rapamycin (mTOR) (Figure 1.4) (Zenz et al. 2010).
Figure 1.4 Signaling in CLL.

Constant BCR stimulation by low-affinity antigen could in fact lead to an overall worse prognosis associated with the unmutated CLL group through induction of survival and growth of the leukaemic clone (Caligaris-Cappio & Ghia 2007). On the other hand, in patients that have a better overall survival rate, a high-affinity self-antigen could also cause selection of a responsive clone, but would however lead to receptor desensitization and an anergic state (Melchers & Rolink 2006; Caligaris-Cappio & Ghia 2007).

Among the most common genetic alterations leading to a good prognosis is the deletion of tumour suppressor region 13q14. The 13q14 cluster contains miR-15a/16 which targets B cell CLL/lymphoma 2 (Bcl-2) anti-apoptotic protein, adding to the complexity of good and bad prognostic markers. Downregulation of DAPK1 through epigenetic silencing is another genetic alteration leading to a bad prognosis (Zenz et al. 2010). The interactions of CLL cells with their environment composing of soluble factors (such as vascular endothelial growth factor (VEGF)α, SDF1 and IL-4) and stromal cells and T cells (expressing CD40L) also contributes to pathogenesis through upregulation of anti-apoptotic proteins myeloid cell...
leukaemia sequence 1 (Mcl-1), Bcl-2 and survivin (Figure 1.4) (Zenz et al. 2010). Targeting BCR signaling has therefore been a major focus in CLL therapy with use of specific inhibitors, antisense therapy or BCR interference.

1.2.6 SHM and CSR

Ever since the discovery that CLL B cells express remarkably low levels of sIgM, the role of the BCR has been a feature of CLL research. As discussed above, in normal B cell development antigen stimulation leads to activation which in turn induces proliferation and differentiation, eventually generating an antigen-specific memory B cell or plasma cell. In CLL, the role of chronic antigen stimulation has been highlighted as an aid in malignant transformation (Mackay & Rose 2001). In other lymphomas, constant bacterial or viral stimulation has been shown to be a cofactor in disease development (Jelić & Filipović-Ljesković 1999; Ferreri et al. 2004). In addition BCR sequencing studies have underlined the role of antigen encounter in CLL (Stevenson & Caligaris-Cappio 2004a). SHM, normally a post antigen encountering event, occurs in at least half of CLL patients (Fais et al. 1998). This process generates long-lived, high-affinity antibody producing memory B cells from a naïve B cell precursor (Wabl et al. 1999). It is usually thought to occur within GCs in a T-cell dependent manner (Berek et al. 1991; MacLennan 1994a), however it can also occur in a T-cell independent manner and outside of GCs (William et al. 2002) (as discussed in section 1.2.10.2).

SHM is characterised by insertions/duplications or deletions within the sequence of the variable region of the BCR. Since it is a common phenomenon in normal, neoplastic or autoreactive B cells (Caligaris-Cappio & Ghia 2007), it is therefore difficult to associate SHM with CLL cells in particular, with the exception of CLL IgH-V3-21 expressing cells where a specific deletion was found in all stereotyped receptor cases (Tobin et al. 2003). In addition CLL cases show a bias toward certain IgVH genes and the complementarity-determining region 3 (CDR3) sequences. CDR3 sequences are unique for each B cell and its progeny and define BCR specificity. However, more than 20% of unrelated CLL cases in both mutated and unmutated CLL carry identical or closely homologous CDR3 sequences on IgH and IgL chains (Tobin et al. 2003; Widhopf et al. 2004; Tobin et al. 2004; Ghiotto et al. 2004; Messmer et al. 2004; Stamatopoulos et al. 2007; Ghia et al. 2005). This remarkable statistic implies that the CLL clone is selected for through recognition of a limited set of particular antigens (Caligaris-Cappio &
Antigen stimulation has also been implicated in the progression of CLL. For example, the CDR3 sequence a patient carries may correlate to overall clinical outcome. Patients with V3-21 carrying a short HCDR3 have an overall bad outcome regardless of mutational status (Ghia et al. 2005). In patients carrying the V\text{H}1-69 gene, two unmutated subsets display opposite clinical outcomes, whereby V\text{H}1-69/D2-2/J6 patients have a short lifespan compared to V\text{H}1-69/D3-10/J6 patients that live significantly longer (Stamatopoulos et al. 2007). In contrast, V\text{H}4-39 also predominates among the unmutated cases, whereas V\text{H}3-23, V\text{H}4-34 and V\text{H}3-07 predominates within the mutated CLL subgroup (Oppezzo & Dighiero 2005).

CSR and SHM allow a B cell to mature, however during the various stages of maturation, B cells become susceptible to abnormal development and generation of diverse forms of leukaemia (Fu et al. 1978). There are various mechanisms employed by CSR in isotype switching (Oppezzo & Dighiero 2005) such as deletional recombination between DNA-switch regions, duplication of sister chromatids and trans-splicing (Oppezzo & Dighiero 2005). These mechanisms can sometimes lead to the production of double isotype expression in the same cell. Similarly, in CLL, some sub-populations of B cells express clonal isotype switch, whereby different isotypes sharing same hypervariable domains are found within the same cell due to trans-splicing or duplication of sister chromatids mechanisms (Oppezzo & Dighiero 2005). V\text{H} rearrangement genes within these cells remain unmutated, indicating that CSR can occur without SHM (Siekevitz et al. 1987; Sideras et al. 1989). Normally, CSR is thought to follow SHM within the GC (Oppezzo & Dighiero 2005; Sideras et al. 1989; Siekevitz et al. 1987). Therefore the process of differentiation and diversification are not necessarily interconnected (Siekevitz et al. 1987; Sideras et al. 1989).

### 1.2.7 Role of antigen in CLL

V(D)J gene recombination of Ig loci, along with SHM and CSR after antigen encounter allow for the generation of many different BCR specificities against an array of exogenous pathogens. However, 20 percent of CLL patients share a restricted BCR repertoire with almost identical Ig sequences (Darzentas et al. 2009). Over-usage of Ig genes V\text{H}1-69, V\text{H}4-34, V\text{H}3-7 and V\text{H}3-21 in CLL and similar CDR3 regions in patients that were geographically distributed became apparent in CLL research (Fais et al. 1998; Potter et al. 2003; Widhopf et al. 2004;
Ghiotto et al. 2004; Tobin et al. 2004; Messmer et al. 2004). Ig light chain repertoire was also demonstrated to be frequently restricted and presented homogeneity within κ and λ CDR3 regions (Widhopf et al. 2004; Ghiotto et al. 2004; Tobin et al. 2004; Tobin et al. 2003). Because of the minimal likelihood of randomly selecting two B cells that harbour almost identical Ig rearrangements, CLL’s Ig repertoire restriction and CDR3 composition could probably not occur by chance. Therefore this implicates antigen selection of CLL cells, conferring a tumour cell’s growth advantage. In mucosa-associated lymphoid tissue (MALT) lymphoma and Burkitt’s lymphoma, infectious agents such as Helicobacter and Epstein-Barr virus (EBV) respectively have been implicated in development of disease (Wotherspoon et al. 1991; Vereide & Sugden 2009). In CLL, history of pneumococcal pneumonia has been linked with increased risk for CLL (Anderson et al. 2009; Landgren et al. 2007). Nevertheless, some studies indicate that a number of CLL monoclonal antibodies, similarly to natural antibodies, can recognize molecular structures existing in bacterial and apoptotic cells (Chu et al. 2008; Myhrinder et al. 2008; Catera et al. 2008), providing an alternative explanation to antigen selection of CLL clones. Therefore, CD5⁺ CLL cells were implicated to be derived from a subset of cells producing natural antibodies that have a function as scavengers of apoptotic debris as well as binding to bacterial cell structures. Collectively these data indicate that antigen/autoantigen, apoptotic cells and microbial pathogens can synergistically drive CLL pathogenesis by constantly stimulating the BCR.

1.2.8 Role of the Microenvironment

Ex-vivo culture of CLL B cells without stroma results in spontaneous apoptosis, thus highlighting the role of the microenvironment within CLL (Lagneaux et al. 1999; Chiorazzi & Ferrarini 2003). Within BM and secondary lymphoid tissues CLL cells interact with stromal cells and matrix, leading to increased survival, proliferation, CLL-cell homing, tissue retention and drug resistance of CLL cells (Burger 2011). Among the cells that make up the microenvironment are nurse-like cells (NLCs), mesenchymal stromal cells and T cells (Figure 1.5). CLL cells also express chemokine receptors and adhesion molecules that allow for contact with NLCs or BM stromal cells (BMSCs) (Burger 2011).
1.2.8.1 Role of NCL and BMSCs

NLCs protect CLL cells from spontaneous or drug-induced apoptosis, whereas BMSCs provide a nourishing niche where CLL cells are protected from cytotoxic agents (Burger et al. 2000; Tsukada et al. 2002). Co-culture of CLL cells with NLCs results in increased NFκB activation and upregulation of target genes CCL3 and CCL4 (Burger et al. 2009; Herishanu et al. 2011). NLCs secrete chemokine (C-X-C motif) ligand 12 (CXCL12 or SDF-1) and CXCL13, platelet/endothelial cell adhesion molecule 1 (CD31), plexin B1 (PLXNB1), B cell activating factor (BAFF), a proliferation-inducing ligand (APRIL) and vimentin (Figure 1.5) (Burger 2011).

NLCs also attract CLL cells through secretion of CXCL12 and CXCL13 whereas BMSCs secrete CXCL12 (Burger 2011). G-protein coupled chemokine receptors (GPCRs) CXCR4 and CXCR5 on CLL cells bind to CXCL12 and CXCL13 respectively. CXCR4 (CD184) is highly expressed on CLL cells and regulates cell chemotaxis, actin polymerization, and migration over vascular endothelium and under BMSCs (Burger 2011). BCR signaling downregulates CXCR4 and upregulates chemotaxis toward CXCL12 and CXCL13 (Quiroga et al. 2009; Vlad et al. 2009). Compared to ZAP70− cells which are correlated to a better prognosis, ZAP70+ cells which are correlated to higher responsiveness to BCR stimulation and worse prognosis, survive better in response to CXCL12 (Richardson et al. 2006; Chen et al. 2002). In addition, ZAP70+ and CD38+ CLL cells display higher levels of chemotaxis toward CXCL12 (Richardson et al. 2006; Deaglio et al. 2007).
CXCR5 (CD185) binds to chemokine CXCL13, regulating lymphocyte homing and situating within the lymph follicles (Burger 2011). Deleting CXCR5 in mice results in an absence of inguinal LNs and a defect in development of primary follicles and GCs within the spleen and Payer patches (S. N. Mueller & R. N. Germain 2009). Like CXCR4, CXCR5 is highly expressed on CLL cells. When CXCR5 is stimulated with CXCL13, an activation of PI3Ks, and ERK signaling occurs leading to actin polymerization, chemotaxis and endocytosis (Bürkle et al. 2007). Other receptors including CXCR3 and CCR7 are also expressed on CLL cells and are important in lymphoid tissue homing.

NLCs highly express CD31 which is the ligand for CD38 expressed by normal B and T cells and poor prognosis CLL cells (Deaglio et al. 2005). In vitro co-culture of CLL cells with murine fibroblasts expressing CD31 resulted in proliferation of CLL cells (Deaglio et al. 2005). Increased proliferation was shown to correlate with upregulation of survival receptor CD100 and down regulation of CD72 which is an inhibitory receptor on CLL cells (Deaglio et al. 2005). Integrins such as VLA-4 (CD49d) work in concert with chemokine receptors to establish cell-cell adhesions (Deaglio et al. 2005).

1.2.8.2 Role of T cells

T cells have also been implicated as necessary components of the CLL microenvironment. CLL is marked by a dysregulation of T lymphocyte function that may correlate to clinical features in CLL such as hypogammaglobulinaemia and autoimmune haemolytic anaemia (Mellstedt & Choudhury 2006). CD4⁺ and CD8⁺ T cells are elevated during CLL progression and display oligoclonality (Mellstedt & Choudhury 2006; Serrano et al. 1997). Within proliferation centres where CLL cells reside, CD38⁺ CLL cells co-localize with activated CD4⁺ T cells, suggesting that these T cells may play a role in the CLL clone’s expansion (Patten et al. 2008). Within a CLL-transfer model where CLL cells were injected into immunodeficient mice, activated CD4⁺ T cells were demonstrated to support CLL-cell proliferation (Bagnara et al. 2011).

CLL cells are also activated through interactions of CD40 with CD40L present on T cells, a member of the tumour necrosis factor (TNF) family, along with BAFF, and APRIL (Burger 2011). Activation through CD40L, BAFF and APRIL leads to immune recognition, increased survival and increased outgrowth of CLL cells. Binding of BAFF can occur via BAFF receptor (BAFF-R) or interaction with
receptors B cell maturation antigen (BCMA) or transmembrane activator and calcium modulator and cyclophilin ligand-interactor (TACI) on a CLL cell (Mackay et al. 2003). APRIL also interacts with BCMA and TACI (Mackay et al. 2003). BAFF overexpression leads to increased B cell proliferation and autoimmunity in mouse models (Shanafelt et al. 2010), whereas overexpression of APRIL leads to a B-1 lymphoproliferative disorder similar to human CLL (see section 1.2.12). BAFF and APRIL have been shown to protect CLL cells from drug-induced and spontaneous apoptosis in vitro (Nishio et al. 2005), by activating the canonical NFκB pathway (Endo et al. 2007). In addition, analysis of serum levels of APRIL in CLL patients revealed elevated expression compared to normal B cells, and negatively correlated to survival (Nishio et al. 2005). In addition, activation of the BCR on CLL cells leads to cytokine secretion which may attract other cells to the microenvironment (Ghia et al. 2002; Burger et al. 2009).

1.2.9 Genetic homogeneity

CLL cells exhibit exceptionally high genetic homogeneity indicative of their clonal outgrowth (Klein et al. 2001; Caligaris-Cappio & Ghia 2007). In fact, unsupervised hierarchical gene expression cluster studies of CLL cases displayed a common gene expression profile, independent of mutational status of CLL or expression of surrogate marker CD38 (Klein et al. 2001; Rosenwald et al. 2001; Dürig et al. 2003). Therefore, although genotypic and clinical differences exist between the two subgroups of CLL, CLL represents a homogenous disease (Klein & Dalla-Favera 2005). It is therefore unlikely that the hypothesis stating that different origins of CLL are based on different subgroups of CLL is correct. Genetic homogeneity indicates that all CLL B cells derive from a common precursor (Klein & Dalla-Favera 2005). Microarray analyses of CLL compared to other lymphoma subtypes demonstrated that the CLL signature is very distinct from that of other lymphomas (Rosenwald et al. 2001; Wang et al. 2004; Jelinek et al. 2003). CLL specific genes that were identified include ZAP70 and exchange protein activated by cyclic AMP (EPAC) (Klein et al. 2001; Tiwari et al. 2004). Another observation within the CLL signature was the comparatively low expression of cycle-associated genes as compared to normal and transformed B cells analyzed (Klein et al. 2001). However, the source of the cells analysed does not represent cells from proliferation centres within lymphoid organs and thus may have led to a generalization of all CLL cells, regardless of location within the body (Chiorazzi & Ferrarini 2003).
When a supervised gene expression analysis was conducted on the data set, a small set of genes were discovered that were different between the two mutational subgroups of CLL (Klein et al. 2001; Rosenwald et al. 2001). Another study investigated gene expression differences between CD38^+ and CD38^- CLL cases, and found very few differences (Dürig et al. 2003). Within this study, an unsupervised clustering did identify some gene profile differences between two subgroups: one with a more favourable outcome and one with a less favourable outcome (Dürig et al. 2003). Another group identified genetic differences according to patient survival and clinical staging (Stratowa et al. 2001). Similarly, Jelinek et al demonstrated the presence of a set of genes that are distinguishable between low risk and high risk patients based on Rai stage (Jelinek et al. 2003). However, Klein and Dalla-Favera point out complications with the above studies (Stratowa et al. 2001; Dürig et al. 2003) arising from lack of CLL B cell purification prior to analysis and lack of independent panel validation (Klein & Dalla-Favera 2005).

### 1.2.10 Origin of a CLL cell

To date, there is no consensus as to the normal cell counterpart to CLL, however evidence suggests that it is an antigen-experienced B cell (Chiorazzi & Ferrarini 2011). It is also unclear whether a single or multiple normal precursors evolved into a CLL cell.

As mentioned above (section 1.2.1), CLL can be divided into two patient subgroups with distinct clinical courses based on mutational status of IgV_H genes. This finding gave rise to the hypothesis that unmutated and mutated CLL clones come from distinct cellular origins. CLL IgV_H repertoire is non-random and is different in unmutated versus mutated CLL subsets. IgV_H repertoire between normal and CLL cells is also different. The higher frequency of poly/auto-reactive BCRs, particularly within unmutated CLL cases and retention of BCR signaling in unmutated CLL suggest that specificity of antigen-binding and capacity of the BCR to signal both influence clonal expansion, resulting in increased tumour survival and growth (Chiorazzi & Ferrarini 2011). However, microarray gene expression studies suggest that although the CLL cell gene profile differs from the normal B cell, very few genetic differences exist between unmutated and mutated CLL cells implying a single cell of origin (section 1.2.9).
1.2.10.1 CD5 expression

One of the notable features of a CLL cell is high expression of CD5. CD5 is normally considered a T-cell molecule that inhibits signaling in T cells (Brossard et al. 2003). Mouse B1 cells have been implicated to be the mouse equivalent of the human CLL cell of origin because of their high expression of CD5 (Caligaris-Cappio et al. 1982). B1 cells within the mouse are responsible for producing multireactive antibodies and lack the ability to form GCs and undergo isotype class switching (Montecino-Rodriguez & Dorshkind 2006). Unlike murine CD5\(^+\) B cells, human CD5\(^+\) B cells do not respond to T-independent antigens and do not produce autoreactive antibodies (Chiorazzi & Ferrarini 2003; Hervé et al. 2005). In adult mice, these B1 cells remain constant in numbers and can clonally expand (Stall et al. 1988).

Mouse B1 CD5\(^+\) cells express unmutated IgV genes (Kocks & Rajewsky 1989), similar to healthy human CD5\(^+\) B cells as demonstrated by single cell PCR of CD5\(^+\) B cells from healthy individuals (HBrezinschek et al. 1997; Fischer et al. 1997; Geiger et al. 2000). CLL cells, however can express both unmutated and mutated IgV\(_H\) genes, therefore a CD5\(^+\) B cell cannot alone be considered a precursor to all CLL. Therefore, Fischer et al made an assumption that IgV mutated CLL cells derive from CD5\(^+\) B cells that only occasionally proliferate and mutate their Ig genes in the GC reaction (Fischer et al. 1997). These assumptions were based on the canonical B cell development pathway where unmutated IgV B cells are naïve B cells and somatically mutated B cells are memory B cells (Klein & Dalla-Favera 2005).

Some argue that expression of CD5 is a consequence of activation requirements (Wortis et al. 1995). Although high expression of CD5 on CLL cells is a constant occurrence, CD5 can serve as an activation marker within CD5\(^-\) B cells and CD5\(^+\) B cells can downregulate CD5 upon activation (Morikawa et al. 1993; Caligaris-Cappio et al. 1989). In addition, some healthy older individuals (above 65) carry a small population of CLL-like CD5\(^+\)CD19\(^+\) cells within circulating blood (Ghia et al. 2004; Rawstron et al. 2002).

Studies ruled out CD5\(^+\) B cells as the normal counterpart to the CD5\(^+\) CLL B cell through gene expression profile analysis that demonstrated that CD5\(^+\) B cells derived from cord blood displayed a very different profile than CLL B cells (Klein et al. 2001; Rosenwald et al. 2001). However, CD5\(^+\) B cells from cord blood may be
different from adult CD5⁺ B cells (Klein & Dalla-Favera 2005). Therefore CD5 cannot alone be used as a distinguishing marker of B-CLL. However, high expression of CD5 coupled with low expression of CD20 is characteristic of CLL (Rawstron et al. 2001).

1.2.10.2 Marginal Zone B cell/Memory B cell

Another speculative suggestion for the origin of a CLL cell is the MZ B cell (Chiorazzi & Ferrarini 2003; Klein & Dalla-Favera 2005). In most GC malignancies, chromosomal translocation occurs within the GC, and since CLL B cells lack chromosomal translocations, it is therefore possible that they are derived from post-GC MZ B cells in which Ig class switching and SHM are no longer active (Figure 1.6) (Klein & Dalla-Favera 2005). Of note, a small subset of CLL cells can class switch. Gene expression analysis of CLL cells compared to different subsets of normal B cells indicated high similarity of CLL cells with CD27⁺ memory B cells found in the tonsil (Klein et al. 2001). Indeed, IgM-expressing CD27⁺ B cells also respond to T-independent antigens, eluding to their MZ B cell identity (Dono et al. 2001; Werner-Favre et al. 2001). In mouse and human cells, SHM was demonstrated to occur outside of the GC and in a T-independent manner (Weller et al. 2004), and therefore the origin of a CLL cell may not lie within the GC (Figure 1.6). In addition, other studies (Weller et al. 2004; Kruetzmann et al. 2003) indicate the possibility of somatically mutated IgM-expressing B cells generated in an antigen-independent fashion, and elude to the idea that SHM can give rise to a diversified pre-immune repertoire (Klein & Dalla-Favera 2005). These T-independent IgM⁺ IgV mutated cells are generated in the spleen through an unknown pathway (Kruetzmann et al. 2003; Weller et al. 2004).

However CD27 can be found on B cells other than post-GC memory B cells reacting to T-dependent antigens (Klein & Dalla-Favera 2005). CD27⁺ B cells can carry unmutated IgV genes, however CD27⁺ B cells are IgM⁺IgD⁺ and thus naïve in Ig status. Therefore CD27⁺ cells can be: classical GC-generated memory B cells; antigen experienced B cells reacting to T-independent antigens; or somatically mutated B cells generated in a T-cell dependent or independent manner. Since all of these cells reside within the MZ of peripheral lymphoid organs, they can be referred to as MZ B cells (Klein & Dalla-Favera 2005).
Figure 1.6 Speculative origins of a CLL cell

The above arguments support the hypothesis that MZ B cells derived from T cell dependent/independent stages of development are the normal counterpart to the human CLL B cell. Indeed, CD27 is often expressed on tumour cells of human B cell malignancies (Oers et al. 1993). Since CLL B cells display a restricted IgV gene repertoire (in both mutated and unmutated subsets) and antigen selection occurs within mutated CLL subsets, it can be concluded that antigen stimulation plays an important role within CLL pathogenesis (section 1.2.7). Interestingly, hairy-cell leukaemia (HCL) cells also resemble CD27+ B cells in terms of gene expression profile (Basso et al. 2004). In addition, like CLL cells, HCL cells lack chromosomal translocation (Haglund et al. 1994; Sambani et al. 2001). This leads to the hypothesis that CLL and HCL cells arise from a similar precursor, a CD27+ MZ or a memory B cell (Klein & Dalla-Favera 2005).

1.2.10.3 Antigen-experienced B cell

The complexity of understanding the origin of a CLL cell arises from the lack of unique molecular abnormality in CLL, lack of balanced chromosomal translocation and CLL’s long natural history (Caligaris-Cappio & Ghia 2007). The low levels of
sIg may imply that a CLL cell has been exposed to antigens (Chiorazzi & Ferrarini 2003). However, this anergy-related feature of CLL cells does not mean that BCR signaling is impaired as demonstrated by the ability to stimulate some CLL cells in vitro through sIgM (Lanham et al. 2003; Chen et al. 2002). CLL B cells express activation markers, express transcripts of many cytokines and secrete some as seen in activated B cells (Caligaris-Cappio & Ghia 2007). Therefore, it is plausible to consider that a CLL cell’s normal equivalent is an antigen-experienced B cell, especially within the somatically mutated IgV gene carrying CLL cases (see section 1.2.7) (Rajewsky 1996; MacLennan 1994) (some studies highlight that somatically mutated IgM-expressing cells can be generated in an antigen-independent manner). In fact, telomere expression and length studies demonstrate that some CLL cells, particularly of the unmutated subset, display a history of cell division prior to leukaemic transformation, implying a post-antigen encountering event (Caligaris-Cappio & Ghia 2007). Some believe that mutated IgV carrying B cells come from antigen experienced B cells whereas unmutated IgV carrying CLL B cells come from antigen-inexperienced, naïve B cells (Oscier et al. 1997; Fais et al. 1998; Hamblin et al. 1999; Küppers et al. 1999). However, as discussed above (section 1.2.10.2) others have demonstrated through microarray studies comparing mutated versus unmutated CLL subsets that both subsets display an antigen-experienced memory B cell phenotype (Klein et al. 2001). Collectively interpretations of studies done to date have not resulted in a clear answer as to the origin of a CLL cell, but have certainly highlighted the complexity of understanding the initiating factors leading to this very heterogeneous disease.

1.2.11 CLL prognosis and Current Therapies

As CLL is a heterogeneous disease and presents with a highly variable clinical course, some patients need immediate treatment whilst others do not need treatment at all (Yuille et al. 2000). Traditionally, alkylating agents such as chlorambucil or cyclophosphamide have been used to treat CLL. Monoclonal antibody therapies in combination with chemotherapy have proven to be much more effective and increase progression-free survival (PFS) and overall survival (OS) (Desai & Pinilla-Ibarz 2012).
1.2.11.1 Single Agent Chemotherapy/Monotherapy

Chlorambucil, an alkylating agent has been the standard in CLL treatment for over four decades however response is variable between 30-70% within previously untreated patients (Desai & Pinilla-Ibarz 2012). Treatment of previously untreated CLL patients with single agent fludarabine, a purine analog, has resulted in superior overall response (OR) and complete remission (CR) rates when compared to alkylating agents alone (Rai et al. 2000; Leporrier et al. 2001; Eichhorst et al. 2009). It was also shown to be better than combination regimens cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP) or cyclophosphamide/doxorubicin/prednisone (CAP) in inducing CR rates but overall survival did not improve (Leporrier et al. 2001). It is important to note that patients with p53 (17p) dysfunction are often refractory to purine analogues such as fludarabine, thus making treatment very difficult (Döhner et al. 1995).

Bendamustine is another alkylating agent that is similar structurally to both alkylating agents and purine analogs. It restores p53 function by activating DNA-damage stress response, and downregulates mitotic checkpoint regulation genes (Desai & Pinilla-Ibarz 2012). Phase III clinical trial of previously untreated CLL patients with bendamustine versus chlorambucil demonstrated improved OR/CR rates over chlorambucil but no improvement of OS rate (Desai & Pinilla-Ibarz 2012).

1.2.11.1.1 Monoclonal Antibodies

Targeting CD20 and CD52 with monoclonal antibodies rituximab and alemtuzumab has been popular in CLL therapy. Rituximab, which targets CD20, has been shown to be more effective in previously untreated patients as CD20 levels subside with treatment (Desai & Pinilla-Ibarz 2012). In addition, CD20 is present in plasma of CLL patients which interferes with treatment (Huhn et al. 2001; Byrd et al. 2001; O’Brien et al. 2001). Therefore single agent monotherapy with rituximab is not as effective as rituximab and chemotherapy combined.

Alemtuzumab targets CD52 and has been demonstrated to induce cell death of CLL cells in vitro (Mone et al. 2006). When compared to chlorambucil alone OR/CR rates increased drastically (83/24% for alemtuzumab versus 56/2% for chlorambucil) (Hillmen et al. 2007). However, more than half of alemtuzumab treated patients developed cytomegalovirus (CMV) reactivation compared to 2% within the chlorambucil arm (Hillmen et al. 2007). Newer monoclonal antibodies
such as ofatumumab and GA-101 are currently being tested for improved effectiveness over rituximab or alemtuzumab (Desai & Pinilla-Ibarz 2012)

1.2.11.2 Combination Therapies

Combination therapy of purine analogs and alkylating such as FC resulted in improvements in OR, CR and PFS over fludarabine monotherapy (Eichhorst et al. 2006; Flinn et al. 2007; Catovsky et al. 2007). However, patients subjected to FC therapy often developed neutropenias (Desai & Pinilla-Ibarz 2012). Combination therapy of fludarabine and rituximab in one study demonstrated evidence of synergy where OR/CR rates of combination therapy increased over fludarabine alone (84/38% for combination therapy versus 63/20% for fludarabine monotherapy) (Byrd et al. 2005).

FCR was shown to increase CR and OR rates, and increase MRD negative-status over FC treatment alone but not in patients harbouring a 17p/11q deletion. Other combination therapies are being investigated including FCR/Alemtuzumab, Cladribine/Rituximab, and Bendamustine/Rituximab among others, in order to generate a therapy that is more effective and less toxic than other combination therapies (Desai & Pinilla-Ibarz 2012).

1.2.11.3 Small molecule inhibitor therapies

Newer treatments targeting the BCR within CLL and the microenvironment have been a key focus in current therapy development. SYK inhibitors (such as Fostamatinib R788), BTK inhibitors (such as PLC32765), PI3δ inhibitors (such as CAL101), and CXCR4 antagonists (such as Plerixafor) have all shown to be effective in targeting CLL cells (Burger 2011). In summary, CLL therapy is evolving to increase OS, PFS, OR and decrease MDR, however no current therapy is 100% effective. Although CLL still remains incurable, new knowledge in CLL biology is leading to novel therapies that may prove beneficial.

1.2.11.3.1 Inhibiting PKCβ

Recent studies have highlighted a role for PKCβII in CLL. BCR signaling is important to the survival of CLL cells and a key mediator is PKCβ that regulates deactivation of BTK and activation of IkB through phosphorylation events after engagement of the BCR (Abrams et al. 2007a). PKCβII was shown to be overexpressed and enzymatically active in primary cells from CLL patients (Abrams et al. 2007; Abrams et al. 2010). Additionally, its activity correlates to
BCR engagement response in CLL, amplifying survival signals (Zent et al. 2010). Treatment of CLL cells with enzastaurin, a PKCβ inhibitor resulted in CLL cell-specific apoptosis (Abrams et al. 2007; Holler et al. 2009). Activation of PKCβII was shown to be stimulated with VEGF, contributing to the enhanced survival and malignant phenotype of CLL cells (Abrams et al. 2010). In a murine model of CLL, the Eμ-TCL1 transgenic mouse model (see section 1.2.12), PKCβ was shown to be necessary for the development of CLL, as PKCβ-deficient TCL-1 transgenic mice did not develop CLL (Holler et al. 2009). Therefore, inhibiting PKCβ may prove to be beneficial in treatment of CLL.

1.2.11.3.2 Inhibiting mTOR Signaling

mTOR proteins belong to a group of evolutionarily conserved serine/threonine protein kinases that regulate the balance between protein synthesis and degradation in response to intracellular energy and nutrients such as amino acids (Memmott & Dennis 2009; Raught et al. 2001) directing cell growth and proliferation. These processes contribute to tumour formation, and many cancers are characterized by abnormal activation of the mTOR signaling pathway (Memmott & Dennis 2009). In CLL, mTOR has also been demonstrated to be active (Aleskog et al. 2008).

In vitro treatment of CLL cells with rapamycin showed anticancer activity (Aleskog et al. 2008) by inducing apoptosis (Hayun et al. 2009), and attenuating proliferation by induction of G1 arrest in cycling CLL B cells through downregulation of cyclins and survivin (Decker et al. 2003). A Phase II pilot trial of advanced CLL patients with rapamycin analogue RAD001 (everolimus) was shown to be promising, however also demonstrated high toxicity (Thomas Decker et al. 2008). Another study of 22 CLL patients demonstrated clinical responses and CLL cell mobilization in response to RAD001 treatment (Zent et al. 2010). In the Eμ-TCL1 model, treatment of mice with rapamycin significantly prolonged life span. Collectively targeting the mTOR pathway in combination with other agents may prove to be beneficial in CLL.

1.2.12 Mouse Models of CLL

1.2.12.1 Eμ-TCL1 mouse model

TCL1 is an oncogene that was initially associated with T cell leukaemias through inversions/translocations at locus 14q32.1. TCL1 rearranges with the TCR α/β at
locus 14q11 in a reciprocal manner. When activated, TCL1 is associated with mature T cell leukaemias, preleukaemic conditions, and AIDS-related immunoblastic lymphoma plasmacytoid (IBLP) (Narducci et al. 1995; Thick et al. 1996; Pekarsky et al. 2007; Teitell et al. 1999). Activation of TCL1 is possibly due to inversions inv(14)(q11;q32) or translocation of t(14;14)(q11;q32) (Brito-Babapulle & Catovsky 1991; Virgilio et al. 1994; Narducci et al. 2000). In T cells, TCL1 is only expressed during the early stages of development at the double negative (CD4−CD8−) stage. In B cells, TCL1 is expressed in early pre-B cells, and IgM expressing cells and to some extent in GC B cells, but not in plasma cells (Pekarsky et al. 2007; Narducci et al. 2000).

**Figure 1.7 TCL mouse model of CLL**

TCL1 is involved in the Akt oncogenic pathway (Laine et al. 2000; Pekarsky et al. 2007) which is important in proliferation, survival and death (Chan et al. 1999). In particular Akt has been demonstrated to be important in the survival and proliferation of T and B cells (Chan et al. 1999). As indicated in the Figure 1.7, PI3K is activated through growth and survival factors such as insulin, platelet-
derived growth factor (PDGF) and epidermal growth factor (EGF) in the plasma membrane (Pekarsky et al. 2007).

The two domains of Akt, the pleckstrin homology (PH) and kinase domain have distinct roles (Figure 1.7). The PH domain binds phosphoinositides (PIP$_3$) and is also responsible for protein-protein interactions and membrane recruitment whereas the kinase domain regulates Akt’s enzymatic activity. TCL1 and Akt have been demonstrated to physically interact through the PH domain of Akt. When Akt binds to TCL1, there is an increase in kinase activity of Akt thereby implicating TCL1 as Akt’s co-activator (Laine et al. 2000; Ahmed et al. 1993; Pekarsky et al. 2000). In addition TCL1 mediates Akt’s translocation into the nucleus (Pekarsky et al. 2000).

When Akt binds to PIP$_3$, it moves to the plasma membrane where it becomes activated through phosphorylation at residue Thr308 by 3’-phosphoinositide-dependent kinase 1 (PDK1) and at residue Ser473 by mTOR. The now active Akt phosphorylates and thus regulates a variety of pro- and anti-apoptotic factors such as IκB kinase-α (IKKα) leading to NFκB activation, mTOR, the nerve growth factor IB (NGFIB) also known as Nur77, glycogen synthase kinase-3 (GSK3), Bcl-2 pro-apoptotic family member Bad, and cyclin AMP-responsive element binding protein (CREB) among others (Figure 1.7) (Chan et al. 1999; Cross et al. 1995; Ozes et al. 1999; Mok et al. 1999; Pekarsky et al. 2007).

When human TCL1 is expressed in mice under control of immature and mature B cell specific V$_H$ promoter enhancer Ig$_H$-Eµ (Bichi et al. 2002), it results in a pathogenesis similar to human CLL. Peripheral blood lymphocytes of Eµ-TCL1 mice at 1-9 months displayed a characteristic CLL phenotype marked by the expression of CD5, and were also B220$^+$/IgM$^+$. Sorted peripheral blood malignant B cells displayed cell cycle arrest at G$_0$/G$_1$ phase, as seen in human CLL (Bichi et al. 2002). By 6 months, an expansion of these cells was seen in all mice, but no signs of disease. Eventually all Eµ-TCL1 transgenic (10-20 months) exhibited an overall increased white blood cell count, enlarged LN, spleen, liver with evidence of malignant cell infiltrations that were TCL1 positive (Bichi et al. 2002). Analysis of Ig gene rearrangement demonstrated the presence of pre-leukaemic and leukemic clones, similar to human CLL (Bichi et al. 2002). Some mice exhibited lymphoadenopathy as seen in some cases of CLL (Bichi et al. 2002). Secondary non-haematological malignancies were also seen in Eµ-TCL1 mice similar to poor prognosis human CLL cases (Kyasa et al. 2004).
When TCL1 is expressed in mice within both B and T cells, a similar phenotype as the Eμ-TCL1 transgenic model is seen (Hoyer et al. 2002). This TCL1 model shows evidence of accumulation of malignant B cells with increased proliferative capacity in vitro and higher survival capabilities in vitro compared to normal counterparts (Hoyer et al. 2002).

Importantly, TCL1 protein is present in 90% of human CLL cases (of 213 patients studied) as assessed by flow cytometry, immunohistochemistry (IHC) and western blot (Herling et al. 2006). In addition, TCL1 expression was shown to positively correlate to ZAP70 expression and was more frequent within the unmutated CLL subset (Herling et al. 2006). As in human CLL, there is a bias toward V\textsubscript{H}11, V\textsubscript{H}12 and V\textsubscript{H}4 gene usage in Eμ-TCL1 mice (Yan et al. 2006). In human CLL, the unmutated subset exhibits long CDR3 containing tyrosine and serine rich sections that favour polyreactivity (Pekarsky et al. 2007). V\textsubscript{H}1-69 and V\textsubscript{H}4-39 rearrangements in particular, are always associated with poor outcome (Ghiotto et al. 2004). Similarly, within the TCL1 mouse, CDR3s also contain charged amino acids that are often not coded by germline D and J\textsubscript{H} segments (Pekarsky et al. 2007). Collectively, the TCL1 mouse model is representative of the unmutated, more aggressive form of CLL.

BCR signaling has also been demonstrated to play an important role within the TCL1 mouse model. In fact, TCL1 mice that also had a PKC\textbeta knockout failed to develop CLL even though they did develop an expanded CD5\textsuperscript{+} B cell population (Holler et al. 2009). The TCL1 transgenic mouse is the best known CLL mouse model to date and has been used as a tool to test pre-clinical drugs. Transformed lymphocytes from the Eμ-TCL1 mouse express Bcl-2, Mcl-1, DNA (cytosine-5)-methyltransferase 1 (DNMT1), PDK1 and are sensitive to agents currently used in therapy such as fludarabine and flavopiridol (Johnson et al. 2006).

1.2.12.2 Simian virus 40 (SV40) large T antigen model

SV40 is an oncogene that is able to transform a variety of cell types (Suda et al. 1987; Ahuja et al. 2005) and has been associated with a number of cancers (Gazdar et al. 2002; Vilchez et al. 2003) including non-Hodgkin’s lymphoma (NHL). It functions by inactivating p53 and Rb proteins thereby inducing genomic instability (White & Khalili 2004). Introduction of SV40T gene under the control of Ig\textsubscript{H} enhances results in increased proliferation of haemopoietic cells, similar to occurrences in myelodysplastic syndromes (Inoue et al. 1994). Introduction of the
SV40T gene that lacked a promoter in the reverse orientation between IgH D and JH segments, led to the generation of a disease similar to human CLL (Brugge et al. 2009).

The aim was to generate sporadic SV40 T antigen expression through homologous recombination in embryonic stem (ES) cells. Briefly, SV40 large and small T antigen-coding unit was introduced in between DQ52 and JH1 in the IgH locus (Brugge et al. 2009). An extra copy of the Eµ enhancer was introduced in one construct. Homologous recombination in ES cells was selected for and chimeric mice generated that carried the targeted IgH allele. Neomycin generated excision occurred through the cre-loxP system. The mice generated above were crossed with mice expressing Cre recombinase under control of CMV immediate early enhancer-chicken beta-actin hybrid promoter. Mice expressing a single targeted allele were bred with C57BL/6 mice generating heterozygous offspring IgH.TEµ or IgH.T. Analysis of 8-week old IgH.T, IgH.TEµ or wild type mice revealed no difference in BM and spleen cellularity and no difference in proportions of B cells (different stages of B cell development and also different types of B cells such as follicular, MZ or spleen cells) (Brugge et al. 2009).

In addition there was no bias of use of either targeted or wildtype IgH allele usage. SV40 T gene and protein expression in spleen was higher in the IgH.TEµ than IgH.T as expected due to Eµ promoter (Brugge et al. 2009). No SV40T transcription/translation was evident within the wildtype mice.

Interestingly, a subpopulation within the IgH.T and IgH.TEµ cohorts showed accumulation of monoclonal B cells. These B cells phenotypically resemble human CLL by expression of CD19, CD5. Leukaemia formation in these mice was evident within the blood, spleen and BM within 10 months. IgH.TEµ mice had higher incidence than IgH.T mice due to the extra copy of Eµ within the D-JH region which may be due to increased antisense transcription. In addition, crossing of IgH.TEµ mice with p53-deficient background increased incidence of CLL. Whereas p53−/− littermates developed T cell tumours, both IgH.T and IgH.TEµ mice biased toward B cell leukaemia. However, age of mice at evidence of malignancy increased with loss of 53, and tumour formation was not increased as a result of p53 loss.

In some cases, it was demonstrated that usage of IgVH genes within the IgH.TEµ cohorts that developed leukaemia resembled unmutated human CLL whereas
characterisation of \( V_H \) regions revealed predominantly germline sequences. In other cases, high levels of hypermutation was seen with use of \( V_HJ558 \). Within the unmutated set, there was evidence of non random \( V_H \) family usage (mostly \( V_H11 \)) and serine/tyrosine rich CDR3 regions. Interestingly two mice expressed \( V_H11 \) domains identical to those from a TCL1 CLL clone.

Also, the unmutated group expressed AID at high levels, similar to human CLL whereas low AID expression was seen in the rest of the cases. AID\(^{hi}\) and AID\(^{low}\) cases had similar transcription factor expression profiles supporting the idea that both subsets originate from a common post-GC cell.

The authors suggest that the predominant use of \( V_H11 \) in the Ig\(_H\).TE\(_\mu\) leukaemic cells demonstrated that the cell of origin was not a CD5\(^+\) B-1 cell that had randomly transformed because only 10\% of this B cell population use \( V_H11 \). The authors imply use of auto antigen-driven selection within the clonally expanded CD5\(^+\) B cells in aging mice that leads to disease. The fact that a number of CLL Ig\(_H\).TE\(_\mu\) cases demonstrated almost identical CDR3 regions was evidence of a restricted BCR repertoire.

Collectively, the authors show a mouse model that is able to generate both mutated and unmutated forms of CLL with similarities to the TCL1 mouse model and human CLL in terms of characterization of the BCR (Brugge et al. 2009).

### 1.2.12.3 miR15a/16-1 mouse model

As previously discussed, deletions in chromosomal region 13q14 are common in CLL implicating that this region contains a tumour suppressor gene (Klein et al. 2010). Minimal deleted region (MDR) of 13q14 encodes the deleted leukaemia (DLEU2) gene, and the micro RNA cluster miR-15a/16-1 which are considered to be tumour suppressors, targeting anti-apoptotic proteins like Bcl-2, and have been shown to be downregulated in CLL (Calin et al. 2004). DLEU2’s function is not well characterized because its sequence is not similar to any known non-coding RNA. miR15a/16-1 cluster has been characterized as a negative regulator of proliferation and apoptosis (Calin et al. 2005). Deletion of the MDR of 13q14 leads to proliferation in both human and mouse B cells and can result in clonal lymphoproliferative disorders (Bandi et al. 2009; Calin et al. 2008; Klein et al. 2010).
Klein et al developed a transgenic mouse that harboured conditional alleles that mimicked MDR deletion of miR-15a/16-1 or specifically deleted this region without any effect on DLEU2 (Klein et al. 2010). Homozygous deletion of MDR region or miR-15a/16-1 did not have an effect on lymphocyte development in the early months, however at 12 months both cohorts displayed an expansion of CD5+B220<sup>low</sup> cells within the peritoneal cavity. At 15-18 months, a clonal CD5+B220<sup>low</sup> population was evident within peripheral blood. Within a subpopulation of these mice infiltration of CD5<sup>+</sup> B cells into lymphoid organs was evident, and histopathologically resembled human CLL. These mice harboured enlarged splenic white pulp, smudge cells and aggregates of a clonal population of small lymphocytes in the BM similar to human CLL. Collectively 42% of MDR<sup>−/−</sup> and 26% of miR-15a/16-1<sup>−/−</sup> cohorts at 15-18 months developed B cell lymphoproliferative disorders of clonal origin (Klein et al. 2010).

MiR-15a/16-1<sup>−/−</sup> mice developed a milder disease than MDR<sup>−/−</sup> mice and had no apparent increase in survival than wild-type littermates, unlike MDR<sup>−/−</sup> mice that died earlier (Klein et al. 2010). When miR-15a/16-1 and MDR was deleted particularly within B cells, development of clonal lymphoproliferations occurred in a B cell autonomous manner in both MDR<sup>−/−</sup> and mir-15a/16-1 cohorts.

When IgV genes were sequenced within tumours of homozygous or heterozygous MDR and miR-15a/16-1 mice, CD5<sup>+</sup> tumours harboured unmutated IgV<sub>H</sub> genes while CD5<sup>−</sup> NHL tumours harboured mutated IgV<sub>H</sub> genes, indicating that lymphoproliferation originated from B cells that have undergone T-cell dependent and independent antibody responses (Klein et al. 2010). In addition, whereas the CD5<sup>−</sup> NHL tumours expressed HCDR3 regions that were variable, within the CD5<sup>+</sup> B cell proliferations IgV gene usage was limited and showed similar HCDR3 regions. Therefore CD5<sup>+</sup> B cell lymphoproliferation in MDR<sup>−/−</sup> and miR-15a/16-1<sup>−/−</sup> mice can express antibodies that have stereotypical antigen binding regions, implying common or auto antigens within clonal expansion, similarly to human CLL (Ghia et al. 2008).

Klein et al. also showed that miR-15a/16-1 cluster (and not DLEU2) negatively regulated proliferation of mouse and human B cell line l83E95 derived from human 13q14<sup>−/−</sup> CLL) (Klein et al. 2010). Importantly miR-15a/16 negatively regulates Bcl-2 expression and the cluster’s downregulation results in an increase in Bcl-2, as seen in human CLL.
1.2.12.4 Bcl-2/TRA3 mouse model

TNF-receptor associated factors (TRAFs) belong to a family of adaptor proteins important in signal transduction pathways such as gene expression, proliferation and regulation of apoptosis (Zapata et al. 2004). When deregulated, TRAFs can lead to generation of autimmune and inflammatory diseases (Zapata et al. 2004). In NHL and CLL, TRAF1 is overexpressed and is associated with resistance to apoptosis (Wang et al. 1998; Arron et al. 2002; Lin et al. 2003). Zapata et al developed a transgenic mouse expressing Bcl-2 and TRAF2 mutant that lacks N’ terminus RING and zinc finger domains (TRAF2DN). These mice develop B cell leukemia and lymphoma that is similar to human CLL, although over expression of Bcl-2 alone or TRAF2DN alone does cause polyclonal B cell expansion, but does not lead to lethal malignancies (Zapata et al. 2004).

More specifically, transgenic mice overexpressing Bcl-2 and TRAF2DN develop extreme splenomegaly already evident at birth. In addition these mice die at accelerated rates compared to single transgenic (Bcl-2 or TRAF2DN alone) cohorts. Histological analysis of double transgenic mice revealed an expansion of MZ with the presence of infiltrating small B cells. In addition, double transgenic mice had infiltration of B220+ lymphocytes within major tissues and organs at much more invasive rates than single transgenic cohorts (Zapata et al. 2004). Phenotypic analysis of spleen lymphocytes from double transgenic mice revealed a significant population of $B220^{med} IgM^{hi} IgD^{low} CD21^{low-null} CD23^{null} CD11b^{low}$ B cells. CD5 was also expressed on most splenic B cells from double transgenic mice. In addition, blood counts were up to $167 \times 10^6$ B cells/ml in double transgenic mice compared to wild-type counts of $4 \times 10^6$ B cells/ml indicative of leukemia development (Zapata et al. 2004).

Analysis of double transgenic cohorts assessed for levels of expression of adhesion molecules indicative of elevated invasiveness revealed increased expression of CD54 (intercellular adhesion molecule-1), CD29 ($\beta1$ integrin) , CD49d ($\alpha4$ integrin) and CD11a (LFA-1) compared to Bcl-2 single transgenic and wild-type cohorts (Zapata et al. 2004). Additionally, analysis of IgH gene rearrangements in double transgenic cohorts revealed clonal origin of B cells. Assessment of proliferation, cell cycle and apoptosis within double transgenic cohorts revealed that B cells from transgenic cohorts had comparable proliferation rates but had much higher ability to resist apoptosis when treated with chemotherapeutic drugs compared to single transgenic and wild-type cohorts.
1.2.12.5 APRIL mouse model

APRIL is a protein that stimulates proliferation of tumour cells in vitro (Medema et al. 2003), is expressed in a number of nonhaematopoietic carcinoma cell lines and tumour samples, and in haematopoietic cells (Hahne et al. 1998; Kelly et al. 2000; Novak et al. 2002; Litinskiy et al. 2002; Pradet-Balade et al. 2002; Deshayes et al. 2004). Its role in tumour growth is highlighted by studies in colon and lung carcinomas (Rennert et al. 2000). In CLL, APRIL was found to be expressed in a small percentage of patients by one group (Novak et al. 2002), and in all CLL cases but at comparative levels to normal B cells by another group (Kern et al. 2004). Analysis of APRIL levels in sera of CLL versus normal donors through ELISA revealed elevated expression in CLL patients (Stein et al. 2002; Planelles et al. 2004)

When transgenic mice were generated overexpressing APRIL, there was evidence of thymus-independent humoral immune responses (both type 1 and type 2) (Stein et al. 2002). At 6-12 weeks, no difference in B1/B2 cell ratio was seen in APRIL transgenic mice, however as the mice aged a significantly higher percentage of B1 cells was seen in the peritoneal cavity of APRIL mice (and not spleen) compared to control littermates. APRIL mice also exhibited higher IgA serum levels, indicative of B1 cell activity. The B1 cells found within the peritoneum were also CD5+, and accumulated over time similarly to the human CLL phenotype. By 9-12 months, 40% of APRIL mice displayed enlargement of and increase in cell number of mesenteric lymph nodes (mLN) or Peyer’s patches (PP). Older APRIL mice also displayed a highly disorganized splenic architecture and B cell infiltration (Stein et al. 2002). B and T cell populations within aged APRIL mice had expanded within mLN, and there was evidence of activation. Expansion within mLN was concluded to be due to lymphocyte hyperactivation leading to hyperplasia (Planelles et al. 2004). Expanded mLN s also harboured B1 cells that were phenotypically CD5+CD19+IgMdimB220dimCD23− and therefore imply higher activation status due to downregulation of surface IgM. Furthermore, ex-vivo culture of B1 cells from APRIL mice and control littermates revealed that B1 cells from APRIL mice had a survival advantage, surviving longer ex-vivo (Stein et al. 2002).
1.2.12.6 New Zealand Black mouse model

Unlike the previous models discussed, the New Zealand Black (NZB) mouse model is not induced by expression of exogenous genes (Scaglione et al. 2007). It is an age-associated expansion of malignant CD5⁺ B1 clones (Scaglione et al. 2007). IL-10 has been associated with the expansion of these malignant B cells that first go through a monoclonal B cell lymphocytosis (MBL)-like stage before developing into a disease that resembles human CLL. Of note, MBL can be a precursor in human CLL but may also never develop into CLL (Salerno et al. 2010). NZB model also develops a mild form of autoimmunity (Scaglione et al. 2007). As in human CLL, miR-15a/16-1 tumour suppressive cluster is altered. Although within human CLL it is usually deleted, within the NZB mouse, it is mutated making it structurally unstable and unable to fully mature (Raveche et al. 2007). Interestingly, a similar point mutation was reported in 2 cases of human CLL (Calin et al. 2005). Restoring miR-16 levels in NZB mice allows for enhanced sensitivity to drug treatment (Coll-Mulet et al. 2006). Within the NZB a side population of stem-like cells was found and implicated to be the CLL progenitor cell (Tárnok et al. 2010).

1.2.12.7 miR-29 mouse model

miR-29 targets a number of oncogenes including TCL1, Mcl-1, and cyclin-dependent kinase 6 (CDK6) and is thought of as a tumour suppressor in some systems (Pekarsky et al. 2006; Zhao et al. 2010; Mott et al. 2007) and a tumour promoter in others (Gebeshuber et al. 2009; Han et al. 2010). Analysis of miR-29 expression revealed that it is up-regulated in unmutated CLL compared to mutated CLL and normal B lymphocytes (Santanam et al. 2010). Generation of a transgenic mouse over-expressing miR-29 under the Eµ promoter led to a model of CLL whereby there was evidence of an expanded CD5⁺ population. In particular, at 2 months 85% of Eµ-miR-29 transgenic mice harboured a CD5⁺ B cell population that rose to 100% at 2 years of age. Enlarged spleen and liver were a common feature of the Eµ-miR-29 cohorts and 20% developed frank leukemia and died. Some evidence of clonality of B cells was also demonstrated within the Eµ-miR-29 cohorts, however only 5 were examined and 3 showed clonal outgrowth of B cells (Santanam et al. 2010).

Histological analysis of blood smears from wild type mice and high-grade CLL Eµ-miR-29 mice revealed the presence of increased number of atypical and malignant
lymphoid cells, including smudge cells within the Eµ-miR-29 cohorts. Eµ-miR-29 also displayed distorted splenic architecture coupled with an increase in cyclin D1 expression. The authors suggest that the CLL phenotype in the Eµ-miR-29 model is not only due to accumulation, yet also to active proliferation of CD5⁺B220⁺ cells within the BM, LN and spleen. In fact, BrdU analysis of Eµ-miR-29 versus wild type mice reveals a higher percentage of proliferating B220⁺CD5⁺ B cells. The Eµ-miR-29 mice also developed hypogammaglobulinemia that is a common occurrence in CLL patients. Crossing of Eµ-TCL1 mice with Eµ-miR-29 mice revealed an even more aggressive phenotype, with an increase of 40% in CD5⁺CD19⁺ splenic B cells in the double transgenic compared to Eµ-TCL1 alone, suggesting that miR-29 can contribute to the pathogenesis of CLL independently of TCL1 (Santanam et al. 2010).

Although there are clear disadvantages in using murine models such as differences from pathogenesis of human disease and differences between microenvironment of mouse and human, they are nevertheless important tools in understanding human disease, particularly in light of the heterogeneity of the human disease. They can be used to study initiation and development of a CLL, and characterize novel therapeutic targets that may prove beneficial in treatment of the disease. Although no model to date exactly emulates human disease, all models have furthered our understanding of human CLL and may reflect the heterogeneity of CLL.

1.3 The Protein Kinase C Family

The PKC family comprises of nine closely related serine/threonine kinase isoforms that have evolutionarily conserved structural features. PKCs belonging to the mammalian family share common catalytic domains (Figure 1.8), but differ in their regulatory domain which dictates the co-factors required for their activation. The isoforms are subdivided into three main groups: conventional, novel and atypical PKCs. Conventional PKCs (α, βI, βII and γ) are activated by phospholipids, diacylglycerol (DAG) and Ca²⁺ binding (Figure 1.8). Novel PKCs (δ, ε, η, and θ) do not require Ca²⁺ for activation, but are still dependent on phospholipids and DAG. Finally, atypical PKC isoforms (ζ and ι/λ) are activated independently of Ca²⁺ and DAG (Newton 2001; Rosse et al. 2010).
Regulators of PKCs

GPCR or receptor tyrosine kinases (RTK) are activated resulting in PLC regulated generation of inositol triphosphate (IP$_3$) and DAG from cell membrane phospholipids (Griner & Kazanietz 2007). IP$_3$ releases Ca$^{2+}$ from the sarcoplasmic reticulum (or other intracellular stores) which primes conventional PKCs. Before a PKC is activated, it must undergo a process of maturation involving a series of phosphorylation steps (Figure 1.9) (Griner & Kazanietz 2007). The first phosphorylation step of a PKC is mediated by PDK1 and occurs at the activation-loop site. This first phosphorylation step exposes the turn and hydrophobic motifs within the C terminal. The PKC now autophosphorylates, leading to increased stabilization of the enzyme and maturity. The mature yet still inactive PKC is now released into the cytosol awaiting activation (Griner & Kazanietz 2007). Upon increase in levels of intracellular Ca$^{2+}$ and DAG, Ca$^{2+}$ binds the C2 domain of classical PKCs and DAG binds the C1 domain conferring a high-affinity interaction between the membrane and the PKC resulting in a conformational change that results in release of the pseudosubstrate from the substrate-binding site, thus releasing this domain for binding of actual substrates, serine/threonine.

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Figure 1.8 The structure of PKCs

1.3.1 Regulators of PKCs

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phosphorylation and activation of signaling effectors that lie downstream (Colón-González & Kazanietz 2006). Novel PKCs are not pre-targeted by Ca^{2+} but have higher affinity to DAG (Giorgione et al. 2006).

Figure 1.9 Activation of cPKCs

1.3.2 PKCs: Tumour Promoters or Suppressors?

PKCs are ubiquitously expressed and necessary for regulation of processes in normal cells such as proliferation, apoptosis, differentiation and migration (Newton 2001; Tan & Parker 2003). PKCs are not only a target of tumour promoting phorbol esters which act as DAG analogues, but have also been linked to oncogenes such as RAS, FOS and myc, placing PKCs at the core of cancer signaling pathways (Griner & Kazanietz 2007). Indeed, expression of specific PKCs is dysregulated in a number of cancers (Griner & Kazanietz 2007; Michie & Nakagawa 2005). PKCs have also been implicated in mitogenesis, survival and transformation (Griner & Kazanietz 2007). Overexpression of PKCβ₁ and PKCγ in murine fibroblasts causes a malignant transformation of these cells and enhances tumorigenesis in nude mice (Bredel & Pollack 1997). Overexpression of PKCβ₂ in colon cancer causes hyperproliferation of colonic epithelium and increased carcinogenesis (Griner & Kazanietz 2007). In fact inhibiting PCKβ with enzastaurin has shown to effectively induce apoptosis in or stop the proliferation of cancerous cells in colon cancer, T cell lymphoma, glioma and thyroid cancer (Griner & Kazanietz 2007). Therefore PKC inhibition has been a key focus in cancer therapy to date.

However, not all PKCs act as tumour promoters. Sometimes, where one isoform acts as a tumour promoter, another acts as a tumour suppressor. For example,
some isoforms like PKCε and PKCδ can have opposing roles in proliferation, survival and tumour promotion. PKCε acts as a tumour promoter, whereas PKCδ has been shown to have tumour suppressive qualities such as delivery of anti-proliferative and apoptotic signals in various cell types (Griner & Kazanietz 2007). Similarly, overexpression of PKCβII in mice stimulates the proliferation of colon epithelial cells and is an early promoting factor in colon carcinogenesis (Gökmen-Polar et al. 2001), whereas PKCα expression is usually decreased (Dupasquier et al. 2009).

1.3.3 PKCα

A single isoform can serve completely opposing roles in a system-dependent manner. For example, PKCα is upregulated in a variety of human cancers such as breast, gastric, prostate and brain cancers, implicating it as a tumour promoter in these cancers (Griner & Kazanietz 2007; Michie & Nakagawa 2005). In melanoma, PKCα activation is associated with increased tumour cell proliferation, invasiveness and decreased differentiation (Lahn & Sundell 2004; Krasagakis et al. 2004). Similarly, in breast cancer cells, PKCα expression has been linked to increased aggressiveness and invasiveness (Tan et al. 2006; Lønne et al. 2010). Moreover, PKCα and PKCβi antisense depletion studies in gastric cancer cells demonstrated reduced size and rate of tumour formation and anchorage-independent growth (Griner & Kazanietz 2007). Such studies have led to the development of pharmacological agents targeting the expression or activity of PKCα, however clinical trials results have not been encouraging (Martiny-Baron & Fabbro 2007; Tortora & Ciardiello 2003; Mackay & Twelves 2007; Cripps et al. 2002). However, the expression or function of PKCα has been found to be downregulated in thyroid, pituitary, epidermal, pancreatic, colon cancers (Kahl-Rainer et al. 1994; Alvaro et al. 1997; Detjen et al. 2000; Gökmen-Polar et al. 2001; Tibudan et al. 2002; Neill et al. 2003) as well as in CLL (Alkan et al. 2005; Abrams et al. 2007) suggesting that in these cancers PKCα may act as a tumour suppressor. Overexpression of PKCα in melanocytes results in attenuated proliferation (Bredel & Pollack 1997). In a carcinogen-induced colon cancer mouse model, expression of PKCα decreases (Gökmen-Polar et al. 2001). Similarly, within the ApcMin/+ mouse model of colorectal cancer PKCα expression is decreased (Nakashima 2002), and crossing of ApcMin/+ mice with PKCα−/− mice resulted in development of more aggressive tumours within mice that died earlier than their PKCα-proficient counterparts (Oster & Leitges 2006; Leitges 2007). Another mouse
colorectal cancer study showed that PKCα transcription is repressed by the SOX9 transcription factor in proliferating intestinal epithelial cells in vitro and in vivo (Dupasquier et al. 2009), generating the cancerous phenotype. Collectively, these studies underline the fact that a single isoform can have either tumour promoting or tumour suppressive roles, stressing the importance of differential regulation of PKC isoforms in distinct cell contexts.

Importantly, subversion of PKCα signaling through expression of dominant-negative PKCα (PKCα-KR) in lymphoid progenitor cells has led our laboratory to develop a murine model of CLL (Nakagawa et al. 2006). Therefore, within our model of CLL, PKCα acts as a tumour suppressor. The aim of this project was to investigate events downstream of PKCα subversion during the initiation and development of disease and to draw links to human CLL. The following thesis will therefore characterize a previously established CLL model in order to gain further understanding of human CLL with the following aims:

Aims:

1. Elucidate similarities between human CLL and the mCLL model;
2. Investigate properties of mCLL cells and define molecular events occurring downstream of subversion of PKCα signaling;
3. Evaluate the lineage plasticity potential of mCLL cells.
Chapter 2: 
Materials and Methods
2.1 Plasmids and cell lines

2.1.1 Plasmids

All plasmids encoding PKC mutants were a kind gift from Dr. Jae-Won Soh. Briefly, PKCα-KR was generated by introducing a point mutation in the full length PKCα cDNA at lysine (K) 368 in the ATP-binding site, changing it to arginine (R), the denomination PKCα-KR. The mutant was introduced into the backbone plasmid pHACE (Figure 2.1 top; Soh et al. 1999) at the EcoRI site. pHACE was derived from pcDNA3 (Invitrogen, Paisley, UK; summary of supplier’s addresses can be found in Table 2.1), modified to contain a C-terminal HA tag. The constitutively active PKCβI and PKCβII isoforms (PKCβI-CAT and PKCβII-CAT) were generated by deletion of the respective gene’s regulatory domain, and were introduced into the backbone pHANE (Figure 2.1 bottom; Soh et al, 1999) at the EcoRI restrictin site. pHANE was derived from pcDNA3 but modified to contain an N-terminal HA tag. The full-length, wildtype PKCβI and PKCβII (PKCβI-WT and PKCβII-WT) were introduced into the pHACE and pHACB backbone (Figure 2.1 top; Figure 2.2 top) at the EcoRI site (into pHACE) and BamHI site (into pHACB) respectively. Like pHACE, pHACB was generated by altering pcDNA3 to contain a C-terminal HA tag.

Retroviral constructs were generated by subcloning of wildtype and mutated isoforms into the retroviral backbone MIEV (Figure 2.2, bottom). MIEV contains 5’ and 3’ long terminal repeats (5’LTR, 3’LTR) flanking a cytomegalovirus (CMV) promoter and green fluorescent protein (eGFP) driven by an IRES sequence. LTRs are used by viruses to insert their genetic information into the genome of the host, whereas the IRES sequence allows for initiation of translation of messenger RNA (mRNA). PKCα-KR, PKCβI-CAT and PKCβII-CAT were cloned into MIEV at BgIII-NotI restriction enzyme sites (Figure 2.3 top). PKCβI-WT was cloned into MIEV at Xhol, NotI (Figure 2.3 bottom). PKCβII-WT was excised out of pHACB with Xhol, EcoRV and subcloned into MIEV at Xhol, SnaBI (Figure 2.4). MIEV-CyclinD1 was created by subcloning CyclinD1 from pCMV-Sport6 (Bioscience LifeScience Ltd., Nottingham, UK; Figure 2.5 top) into MIEV at EcoRI, NotI sites (Figure 2.5 bottom). All inserts were cloned into MIEV 5’ of the internal-ribosomal entry site (IRES), allowing for the bicistronic expression of the specific gene of interest with GFP and effective tracking.
2.1.2 Cell Lines

2.1.2.1 PT67 retroviral packaging cells

PT67 cells (ClonTech, Saint-Germain-en-Laye, France) are NIH/3T3 fibroblasts that express \( \text{gag}, \text{pol}, \text{and} \text{env} \) genes to continuously produce retrovirus packaged into an amphotropic envelope 10A1 capable of infecting most mammalian cells after co-transfection with a retroviral vector (Miller & Chen 1996). PT67 cells were cultured in DMEM medium (10% FBS (Invitrogen), 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, 50 \( \mu \)M \( \beta \)-ME (Sigma-Aldrich, Dorset, UK) and 2 mM L-glutamine (Invitrogen)) at 37°C in a humidified incubator containing 5% (v/v) \( \text{CO}_2 \).

2.1.2.2 GP+E.86 retroviral packaging cells

GP+E.86 cells are NIH/3T3 fibroblast cells that were modified to contain \( \text{gag}, \text{pol}, \) and \( \text{env} \) genes to continuously produce an ecotropic virus after co-transfection with a retroviral plasmid. For safety reasons, the \( \text{gag}, \text{pol} \) and \( \text{env} \) genes were initially separated onto two different plasmids and their \( \Psi \) packaging sequence was deleted, alongside the 3’ LTR to ensure replication incompetent virus (Markowitz et al. 1988). GP+E.86 cells were cultured in DMEM medium at 37°C in a humidified incubator containing 5% (v/v) \( \text{CO}_2 \).

2.1.2.2.1 Generation of retroviral packaging lines

PT67 cells were transfected with retroviral vectors generated above (section 2.1.1) using lipofectamine or CaCl\(_2\). Virus was collected after 48 hr, filtered through a 0.45 \( \mu \)m filter, ultracentrifuged at 24 000 g for 2 hr at 4°C and used to transduce GP+E.86 cells. GFP\(^+\) GP+E.86 cells were sorted twice using BD FACSAria (BD Biosciences, Oxford, UK) and subsequently used to continuously generate virus for retroviral transduction of lymphoid progenitors (Figure 2.6).

2.1.2.3 OP9 cells

OP9 cells are a murine stromal cell line generated from newborn B6C3F2 op/op mouse calvaria (Kodama et al. 1984) with a mutation in the gene encoding for M-CSF, thus resulting in the lack of secretion of functional M-CSF (Nakano 1995). OP9 cells support the differentiation of ES cells into B cells when co-cultured with cytokine IL7 and \( \beta \)-mercaptoethanol (\( \beta \)-ME) (Nakano et al. 1994; Nakano 1995) and have since been used to differentiate FL cells into B cells under similar conditions. OP9 cells were cultured in complete medium: \( \alpha \)-MEM (Invitrogen) containing 20% foetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin, 100
µg/ml streptomycin, 50 µM β-ME, 2 mM L-glutamine, 10 mM HEPES, 1 mM Sodium Pyruvate and 10 µg/ml gentamycin (Invitrogen) at 37°C in a humidified incubator containing 5% (v/v) CO₂.

2.1.2.4 OP9-DL1 cells

OP9-DL1 cells are OP9 cells that have been modified to ectopically express the Notch ligand delta-like-1 (DL1) (Schmitt & Zúñiga-Pflücker 2002). During lymphocyte development, Notch receptors and their ligands are important regulators of T cell lineage commitment, and in particular, Notch1 has been shown to be critical in T cell development (Wang et al. 2006). Following the addition of Flt3L and IL7 (PeproTech, London, UK), OP9-DL1 cells preferentially support T cell differentiation of ES cells and FL stem cells. OP9-DL1 cells were cultured in complete medium at 37°C in a humidified incubator containing 5% (v/v) CO₂.

2.2 Tissue culture

All tissue culture was conducted under sterile conditions using a laminar air flow hood. Tissue culture consumables were purchased from Fisher Scientific U.K. and Greiner Bio-One Ltd unless otherwise stated.

2.2.1 Preparation of HPCs from FL cells

Single cell FL suspensions were created via crushing of FL extracted at day 14 of gestation and filtering through a 70 µM nylon mesh (Biodesign Inc., NY, USA) followed by a wash in DMEM media (4 min, 324g, RT). Complement-mediated lysis was carried out using anti-CD24 antibody, and incubating cells with low-lox rabbit complement (Cedar Lane, ON, Canada) at 37°C for 30 min. Thereafter, a discontinuous density gradient centrifugation over Lympholyte-Mammal (Cedar Lane) was performed for 30 min (623g; RT) in order to extract all viable CD24-low-HPCs. Viable HPCs were then washed in DMEM medium for 5 min (324g) once before subsequent use.

2.2.2 Retroviral transduction of HPCs

Retroviral GP+E.86 packaging lines expressing PKCα-KR (GP+E.86-PKCα-KR) or MIEV empty retroviral vector (GP+E.86-MIEV) were plated at 60% confluence one day prior to retroviral transduction in 6-well plates. Packaging lines were incubated with mitomycin C (10 µg/ml) for 3 hr at 37°C, and subsequently washed twice with DMEM. Single cell suspensions of prepared FL were re-suspended in complete medium supplemented with 10 ng/ml polybrene, 5 ng/ml IL7 and Flt3L.
and were co-cultured with packaging cell lines for overnight transduction prior to being placed on an *in vitro* B cell generation system (OP9).

### 2.2.3 *In vitro* B cell generation system

OP9 stromal cells were plated at 60% confluence one day prior to FL co-culture. FL:OP9 co-culture was maintained in complete medium supplemented with 10 ng/ml IL7 and Flt3L with a change of media every second day, and a change of OP9 layers every fourth day. After day 5 of FL:OP9 co-culture Flt3L was removed from the medium (Figure 2.7 top).

### 2.2.4 *In vitro* T cell generation system

OP9-DL1 cells were plated at 70% confluence one or two days prior to FL co-culture. Pro-B cells or HSCs were sorted from FL:OP9 co-cultures and subsequently co-cultured with DL1 cells in complete medium supplemented with 5 ng/ml IL7 and Flt3L. Medium was changed every 2 days and OP9-DL1 layers were replaced once per week (Figure 2.7 bottom).

### 2.2.5 Isolation of HSC, pro-B and T cells from FL in vitro cultures

FL cells were transduced overnight with MIEV or PKCa-KR retroviral packaging lines in complete medium and thereafter labelled with fluorochrome conjugated antibodies (B220-PE, CD45-PerCP, Sca1-PE-Cy7, CD117-APC, CD19-APC-Cy7) and a lineage cocktail of biotin-conjugated antibodies (CD3ε, CD4, CD8, CD11b, Gr1, NK1.1 and Ter119) and visualized by streptavidin (SA) -conjugated Pacific blue (Invitrogen) (refer to Table 2.2 for list of FACS antibodies used). HSC populations were sorted by gating on: GFP^+^Lin^-^CD45^-^B220^-^CD19^-^CD117^{hi}Sca1^{hi} and subsequently co-cultured with OP9 cells in complete medium supplemented with 10 ng/ml IL7 and Flt3L for 9 days. Late pro-B populations were then sorted from day 9 HSC:OP9 co-cultures by gating on: GFP^+^Lin^-^CD45^-^B220^-^CD19^-^CD117^+^ and subsequently cultured on OP9-DL1 stromal layers in complete medium supplemented with 5 ng/ml IL7 and Flt3L. B lineage cells (CD19^-^B220^-^Lin^-^) were sorted from both MIEV and PKCa-KR cultures and CD19^+^B220^-^CD90.2^+^CD25^+^Lin^-^ cells were sorted from PKCa-KR-cultures.

### 2.2.6 Drugs and Inhibitors

Details of the stock concentrations, manufacturers and storage conditions of all drugs and inhibitors used are listed in Table 2.8.
Phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) is a polyfunctional
diterpene phorbol that is an activator of PKC (Saitoh & Dobkins 1986) and is
known as a widespread tumour promoter in cancer (O’Brien et al. 1975).
Ionomycin (Iono) (Sigma-Aldrich) is an ionophore that is used to raise intracellular
Ca\(^{2+}\) levels and is often used in combination with PMA to stimulate the activation
of PKCs. Hispidin (Sigma-Aldrich) is a fungal metabolite that inhibits PKCβ\(_I\) and β\(_II\) isoforms. Enzastaurin, a synthetic bisindolylmaleimide, inhibits PKCβ by binding
to the ATP-binding site and was a kind gift from Eli Lilly (Eli Lilly and Co. Ltd,
Hampshire, UK). Rapamycin (rapa) is an inhibitor of mammalian mTOR by
associating with its intracellular receptor FKBP12 and was purchased from
Calbiochem. Okadaic Acid (OA) is a potent inhibitor of protein serine/threonine
phosphatase A (PP2A) and was also purchased from Merck (Merck Chemicals
Ltd., Nottingham, UK).

### 2.2.6.1 In vitro drug treatment

B cells were carefully harvested from FL:OP9 co-cultures and centrifuged over
Lympholyte-Mammal prior to drug treatments. 1 x \(10^6\) cells per condition were
plated in complete medium and treated with drug or vehicle control for 24 or 48 hr.

### 2.2.6.2 In vivo drug treatment

Enzastaurin was re-suspended in D5W (5% dextrose in water) and dosed by oral
gavage (0.2 ml per dose) with 80 mg/kg daily in order to approximate the plasma
concentrations achieved clinically as suggested by Eli Lilly. Mice were dosed for
14-21 consecutive days and maintained on a normal diet.

### 2.3 In vivo models

#### 2.3.1 RAG\(^{-/-}\) and ICR mice

All animal work was carried out in accordance with regulations set by the Animals
Scientific Procedures Act 1986 (ASPA, 1986). Imprinting control region (ICR)
mice purchased from Harlan UK Ltd (Harlan UK Ltd., Blackthorn, UK) and RAG\(^{1/-}\)
mice were used, and were maintained at the University of Glasgow Central
Research Facilities. ICR mice are an outbred strain of Swiss mice established by
Hauschka and Mirand in 1973 (Eaton et al. 1980) and are often used for
oncological research. RAG\(^{1/-}\) mice have a blockade in lymphocyte differentiation
at the pro-B to pre-B cell stage in development, similar to that described in severe
combined immunodeficient (SCID) mice. RAG genes are also responsible for
TCR rearrangement, and therefore RAG1−/− mice have no mature B and T lymphocytes (Mombaerts et al. 1992), and an impaired immune system.

In preparation for isolation of FL derived HPCs, timed pregnant mice were generated, and FL was extracted at day 14 of gestation.

2.3.2 *In vivo* adoptive transfer

Lymphocytes were isolated from retrovirally transduced HPC FL cultures by centrifugation over Lympholyte-Mammal. Briefly, HPCs were isolated from day 14 gestation FL (as described in section 2.2.1), retrovirally transduced overnight (as described in section 2.2.2), collected, washed and re-suspended in 5 ml, underlaid with Lympholyte-Mammal and centrifuged for 30 min (623g; RT). Cells were isolated from the interphase, washed twice in PBS and re-suspended in PBS at a concentration of 1×10^6 cells/ml. Neonatal RAG1−/− mice were injected intraperitoneally (i.p.) with the cell suspension at a desired concentration of cells and monitored for the development of disease. Mice were sacrificed by cervical dislocation 4-8 weeks post-injection and organs (BM, LN, spleen), blood, peritoneal wash and any present tumours were harvested for processing and analysis or storage for later analyses.

2.3.2.1 Processing of blood and organs from injected RAG1−/− mice

Lymphoid organs were removed from injected RAG1−/− mice and placed into separate tubes containing 2 ml PBS. The organs were crushed in order to generate a cell suspension. Cells were filtered through a 70 µM nylon mesh (Biodesign Inc., NY, USA) and pelleted by centrifugation for 5 min at 400 g at RT. The cell pellet was re-suspended in 5 ml PBS and underlaid with 2.5 ml Lympholyte-Mammal (as described in Section 2.1.1) in order to remove red and dead cells. Cells were re-suspended in FACS buffer and stained with antibodies for FACS analysis.

2.4 Normal human B cells and CLL blood samples

Normal B lymphocytes were isolated from buffy coat samples acquired from healthy individuals through the Scottish National Blood Transfusion Service (SNBTS), with approval from SNBTS ethics committee. Samples were received within 24 hr after bleeding of donor, after standard virology screening was completed. B cells were isolated using MACS human CD19 MicroBeads according to the manufacturer’s protocol (Miltenyi Biotec, Surrey, UK). CLL lymphocytes were isolated from peripheral blood of patients with a clinically confirmed diagnosis
of B-CLL, after informed consent. Patients had not received treatment within the preceding three months. CLL B cells were isolated using Rosettesep™ human B cell enrichment cocktail (Stemcell Technologies, Grenoble, France) according to the manufacturer’s protocol. After separation, B cell purity was >90% assessed by FACS in both normal and CLL samples. For a list of clinical characteristics CLL samples used see Table 2.3. CLL patients were anonymised and linked through acquisition of a field of clinical data.

2.5 Western Blotting

B lineage cells were removed from OP9 co-cultures and placed on empty 6-well plates in complete medium for 2 hr in order to separate B cells from adherent OP9 layers. 1 x 10^7 cells were washed twice in ice-cold PBS with phosphatase inhibitors (Roche, West Sussex, UK) and re-suspended in 100 µl lysis buffer (20 mM Tris pH7.5, 137 mM NaCl, 10% glycerol, 1% NP40) containing protease inhibitor cocktail (Roche, West Sussex, UK) and phosphatase inhibitor cocktail (Roche) on ice for 30 min. Lysates were then spun at 14 000 rpm for 30 min at 4°C and supernatant collected.

Quantification of lysates was conducted with a bichinchoninic acid (BCA) Protein Assay Kit (Pierce, Northumberland, UK). Bovine albumin serum (BSA) protein standards were prepared in sterile water at concentrations of: 2000, 1500, 1000, 750, 500, 250, 125, 26 and 0 µg/ml and stored at -20°C until use. 2 µl of BSA protein standard or sample lysate was pipetted per well of 96-well plate in triplicate and 200 µl of 50:1 solution of kit reagent A:B was pipetted to each well and the plate was incubated at 37°C for 30 min. Absorbance was read at 562 nM on a Spectramax M5 plate reader (MDS Analytical Technologies, Berkshire, UK) and analysed with SoftMax Pro 5.2 software (MDS Analytical Technologies).

Equal amounts of protein (typically 10-20 µg) were incubated with NuPage LDS Sample buffer (10 % (w/v) Glycerol, 1.7% (w/v) Tris-Base, 1.7% Tris-HCl, 2% (w/v) lithium dodecyl sulfate (LDL), 0.15% (w/v) EDTA, 0.019% Serva Blue G250 and 0.063% Phenol Red (pH 8.5) (Invitrogen) and sample reducing agent (Invitrogen) at 72°C for 15 min. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 4-12% NuPAGE Novex Bis-Tris precast gel (Invitrogen) (unless otherwise stated), using MOPS SDS Running Buffer buffer (50 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 50 mM Tris-Base, 3.5 mM SDS and 1.0 mM EDTA (pH 7.7)) supplemented with NuPAGE
antioxidant (Invitrogen). Samples were run alongside SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) at 150 volts (V) for 1 hr.

Gels were then transferred onto an Immun-Blot polyvinyliden difluoride (PVDF) membranes (BioRad Laboratories, West Sussex, UK) using a 1 x solution of 20 x NuPAGE transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1.0 mM EDTA, 50 µM Chlorobutanol (pH 7.2)) supplemented with 20% (v/v) methanol (Sigma-Aldrich). Briefly, the PVDF membrane was soaked in 100% methanol for 1 min, rinsed in distilled water (dH2O) and equilibrated in transfer buffer prior to assembly of gel/membrane sandwiches using 1.0 mm gel blotting paper (Whatman plc, Kent, UK) and sponges in an XCell II™ Blot Module (Invitrogen). Transfer was performed at 30 V for 1 hr.

Blots were washed with 1 x TBST (20 mM Tris HCl pH 7.4, 150 mM NaCl, 0.01% Tween 20), blocked in TBST containing 5% BSA (Sigma-Aldrich) or 5% milk for 1 hr at RT, and incubated with primary antibody overnight at 4ºC. Thereafter, the blots were washed 4 times with TBST and incubated with horseradish-peroxidase (HRP)-labelled secondary antibodies for 1 hr at RT. After 4 washes with TBST, the blots were developed with Immun-Star™ Western C™ HRP chemiluminescent kit for one minute, and imaged with the Molecular Imager® ChemiDoc™ XRS system (BioRad Laboratories). Western blot antibodies were purchased from Cell Signaling Technology unless otherwise stated. Refer to Table 2.4 for a list of antibodies used.

2.5.1 Membrane stripping and re-probing

When multiple proteins or proteins of similar sizes were viewed on the same gel, PVDF membranes were stripped. Blots were incubated for 5 - 10 min in mild stripping buffer (200mM Glycine, 01% SDS, 1% Tween 20), 2 x 10 min in PBS, 2 x 5 min in TBST and subsequently re-blocked in 5% BSA (TBST) or 5% milk (TBST) for 1 hr at RT prior to incubation with primary antibody.

2.6 Genomic DNA extraction

Cells were sorted, pelleted by centrifugation at 4°C and re-suspended in PBS. DNA was isolated using the Easy-DNA kit according to the manufacturer's protocol (Invitrogen) and then dissolved in DNase-free H2O.
2.6.1 Genomic DNA PCR

For each reaction, DNA from 1 - 5 x 10^3 cells was used. DNA was amplified in a PCR reaction with a hot start at 94°C for 2 min, 35 cycles of: 10 sec denaturation at 94°C, 30 sec annealing at 59°C, 2 min extension at 68°C; and a final extension at 68°C for 6 min. TCR D_β-J_β, Ig_β D-J and Ig_β V-J primers used for gene rearrangement analysis have been previously described (Pennycook et al. 1993; Michie et al. 2001). Genomic DNA from wildtype mouse splenocytes was used as the positive control, H_2O as the negative control, and β2M was used as the loading control. Refer to Table 2.5 for primer sequences and PCR conditions. All primers were made by Eurofins MGW Operon (London, UK). Products were separated by 1% agarose gel electrophoresis and visualized by addition of Ethidium Bromide (EtBr) and imaging with the Molecular Imager® ChemiDoc™ XRS system.

2.7 Total RNA extraction

RNA was purified using RNeasy mini kit columns (Qiagen, West Sussex, UK) according to the manufacturer’s protocol. Samples were re-suspended in RNase-free H_2O and quantified with a spectrophotometer (Nanodrop ND1000 Spectrophotometer; Labtech International Ltd, East Sussex, UK).

2.7.1 Measuring mRNA levels using qRT-PCR

Up to 1 µg RNA was used as a template per 20 µl reverse transcription reaction using the First Strand cDNA Synthesis Kit for RT-PCR (Roche, West Sussex, UK). Quantitative PCR was performed using Taqman® Gene Expression Assays (Applied Biosystems, Warrington, UK; see list of assay IDs in Table 2.6). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene unless otherwise stated. 1 µl cDNA was used per 20 µl PCR reaction containing 1 x TaqMan® Gene Expression Mastermix (AmpliTaq Gold® DNA Polymerase (Ultra Pure), Uracil-DNA glycosylase, dNTPs with deoxyuridine triphosphate, ROX™ Passive Reference; Applied Biosystems). All reactions were performed in technical triplicates and at least three biological replicates using the 7900HT Fast Real-Time PCR system (Applied Biosystems) programmed to complete 40 cycles as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. After normalization to the endogenous control gene GAPDH, levels of gene mRNA expression in each sample were determined by the 2^-ΔΔCT method of relative quantification (Schmittgen & Livak 2008).
2.8 Cell proliferation by 5-bromo-2'-deoxyuridine (BrdU) incorporation

Cell proliferation was indirectly measured by monitoring of DNA synthesis by relative incorporation of BrdU using the Cell Proliferation ELISA, BrdU (colorimetric) kit as per manufacturer’s protocol (Roche). Briefly, cells from OP9:FL co-cultures were passed through lympholyte mammal to isolate live lymphocytes and consequently plated in 96-well plates at a confluence of $1 \times 10^4 - 5 \times 10^4$ cells/well in complete medium +/- drug treatments. Prior to the termination of a time point of an experiment, cells were labelled with 10 µl of 100 µM BrDU and cultured for an additional 2 hr. Plates were then centrifuged at 300g for 10 min at RT and medium was removed by flicking. Cells were consequently dried with a hair-dryer for 15 min and fixed with 200 µl/well of FixDenat reagent (ethanol based) for 30 min at RT. FixDenat reagent was then removed by flicking and peroxidase-conjugated anti-BrdU antibody was added to the wells and cells incubated for 90 min at RT. Anti-BrdU antibody was removed by flicking and wells washed three times prior to addition of 100 µl per well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Absorbance was read at 370 nM and 492 nM on a Spectramax M5 plate reader and analysed with SoftMax Pro 5.2 software.

2.9 Assessment of surface antigen expression

Cells were harvested by gentle pipetting and washed in flow cytometry (FACS) buffer (Hank’s Balanced Salt Solution (HBSS) supplemented with 1% BSA and 0.05% sodium azide (Sigma-Aldrich)). Cells were then incubated with anti-CD16/CD32 antibody (1/200 dilution) to block Fc receptors and non-specific binding. Cells were washed again in FACS buffer and stained with fluorochrome-conjugated antibodies (in 100 µl FACS buffer) at 4°C in the dark for 30 min. When biotin antibodies were used, cells were incubated with fluorescent-conjugated SA for 30 min at 4°C in the dark. Cells were then washed twice (in FACS buffer), re-suspended in 100 µl FACS buffer and passed through a 70 µM nylon mesh prior to FACS analysis. FACS analysis was performed using a FACSCanto II flow cytometer (BD Biosciences), data was acquired using BD FACSDiva (BD Biosciences) software and analysed using FlowJo (Tree Star Inc., Ashland, USA) software.

2.10 Analysis of intracellular proteins by flow cytometry

ZAP70 protein levels were measured by intracellular flow cytometry. Cells were washed once in FACS buffer, fixed using Cytofix/Cytoperm (BD Biosciences)
solution, and incubated for 20 min at 4°C. Cells were washed twice in BD Perm/Wash solution (BD Biosciences) and subsequently stained with PE-conjugated ZAP70 (ZAP70-PE) antibody. Cells were acquired on FACSCanto II flow cytometer as mentioned above (section 2.9).

2.11 Detection of apoptosis using Annexin V

Following drug treatments, 1 x 10^6 cells were washed once in ice-cold PBS, and re-suspended in 100 µl HBSS (Sigma-Aldrich) containing 5 µl Annexin V APC and 5 µl Viaprobe (BD Biosciences) per test, and incubated for 15 min in the dark at RT. To stop the reaction, 400 µl HBSS was added to the samples, and the cells were analysed on a FACS Cantoll flow cytometer. When autofluorescent drugs such as enzastaurin and hispidin were used, diamidino-2-phenylindole (DAPI, 10 µg/ml final concentration) was used instead of Viaprobe, and was added just prior to FACS analysis. Control tubes containing unstained cells, Annexin V or DNA stain (Viaprobe/DAPI) single-stained cells were recorded to set FSC/SSC voltages and compensation. FCS analysis was performed as described above (section 2.9). Viable cells were considered to be Annexin V^-/DNA stain^- (Figure 2.8).

2.12 Cell Cycle Analysis using Propidium Iodine (PI) staining

All solutions used for PI staining were ice cold. 1 x 10^6 cells were harvested and suspended in 1 ml PBS and centrifuged for 5 min at RT. After aspiration of PBS, cells were re-suspended in 500 µl PBS and fixed by adding 4.5 ml 70% (v/v) ethanol. Cells were centrifuged (400g for 5 min), washed in 5 ml PBS and re-suspended in DNA staining solution (20 µg/ml PI in PBS containing 0.2 mg/ml Dnase free RNase) for 30 min at RT in the dark (Riccardi & Nicoletti 2006). FACS data was acquired using a FACSCantoll flow cytometer. Figure 2.9 illustrates an example of cell cycle analysis pattern.

2.13 Sorting of Cells

Cells were harvested, washed once in PBS, once in sorting buffer (HBSS; 1 % BSA), incubated with anti-CD16/CD32 antibody as described above for 10 min on ice, and then incubated with appropriate antibodies on ice in the dark for 30 min. Cells were then washed and re-suspended in 500 µl sorting buffer in order to dilute out sodium azide present in antibody solutions, and passed through a sterile 70 µm nylon mesh prior to sorting. All sorting was performed using a BD FACSＡria and data acquired using FACSDiva software.
2.14 Immunohistochemistry

Paraffin embedded sections were cut into 3 µm slices and placed on slides (Leica Microsystems, Buckinghamshire, UK) that were dewaxed with 2 washes with xylene, followed by 4 washes in 100% alcohol (to remove excess xylene), and finally water. Heat antigen retrieval was carried out by submerging slides into hot EDTA solution (10 mM Tris, 1 mM EDTA, pH 8.0) in a pressure cooker and subsequently microwaved on high for 3 min, and 6 min thereafter. The solution was cooled with water and slides washed in 1 x Envision™ Flex wash buffer (DAKO UK Ltd, Cambridge, UK). All endogenous biotin was blocked using Avidin/Biotin Blocking Kit (Vector) and sections were blocked with a 3% $\text{H}_2\text{O}_2$ peroxidase blocking solution. Thereafter sections were blocked with 5% horse serum (in DAKO wash buffer), incubated with primary antibody for 30 min, washed, and incubated with biotin-labelled secondary antibody (in all cases apart from biotinylated B220). The sections were washed again in DAKO wash buffer incubated with the avidin-biotin-complex (ABC) using the Alkaline Phosphatase or HRP Vectastain ABC kit (DAKO UK Ltd). Finally substrate was added (either ImmPACT™ diaminobenzidine (DAB) or Peroxidase Substrate) for 5 min, washed with DAKO buffer, washed with $\text{H}_2\text{O}$, stained with haematoxylin solution and dipped into 1% HCl-alcohol solution, then Scot’s Tapwater, 0.5% Copper Sulphate solution, and finally $\text{H}_2\text{O}$ prior to mounting. Tissue sections were scanned with SlidePath Digital Pathology Solutions system and uploaded to the University of Glasgow SlidePath server. Refer to Table 2.7 for a list of antibodies used.

2.15 Data and statistical analysis

All results are shown as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software Inc., CA), using the Students unpaired t-test.
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<td>Enzo Life Sciences Ltd.</td>
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<td>Maxted Rd, Hemel Hempstead, West Sussex, HP2 7DX, UK</td>
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<td>Carl Zeiss Ltd.</td>
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- neg = ZAP-70 negative, pos = ZAP-70 positive
- n/a = not available
- nil = no abnormality detected by FISH
- Chromosomal deletions are indicated by (-) and trisomy denoted by (+)
### Table 2.4 Western Blot Antibodies

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<td>5 % BSA</td>
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Table 2.5 DNA primer sequences

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<tr>
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<td>Jβ reverse</td>
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Table 2.6 RNA gene expression assays

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2° antibody

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## Table 2.8 Drugs

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Figure 2.1 Vector map of pHACE and pHANE constructs

pHACE and pHANE are mammalian expression vectors containing a CMV promoter, Kozak translational initiation sequence, ATG start codon, EcoRI cloning site and a stop codon. In addition, pHACE has a C-terminal HA epitope tag whilst pHANE has an N-terminal HA epitope tag.
Figure 2.2 Vector map of pHACB and MIEV constructs

pHABE is a mammalian expression vector containing a CMV promoter, Kozak translational initiation sequence, ATG start codon, BamHI cloning site, C-terminal HA epitope tag and a stop codon. MIEV is a retroviral vector containing a CMV promoter and 5' IRES, allowing for the bicistronic expression of the gene of interest along with GFP.
Figure 2.3 Vector map of MIEV-PKCα-KR, MIEV-PKCβI-CAT, MIEV-PKCβII-CAT and MIEV-PKCβI-WT

MIEV-PKCα-KR plasmid was generated by subcloning of full length open reading frame of PKCα with a point mutation at the ATP binding site out of pHACE and into MIEV at BglII, NotI sites. MIEV-PKCβI-CAT and MIEV-PKCβII-CAT were generated by subcloning of cDNA fragments encoding the catalytic domains of PKCβI or PKCβII out of pHANE and into MIEV at BglII, NotI sites. MIEV-PKCβI-WT plasmid was generated by subcloning of full-length open reading frame of PKCβI out of pHACE and into MIEV at XhoI, NotI sites.
**Figure 2.4 Vector map of MIEV-PKCβ_{II-WT}**

MIEV-PKCβ_{II-WT} plasmid was generated by digesting full-length open reading frame of PKCβ_{II} out of pHACB with Xhol, EcoRV and subcloned into MIEV at Xhol, SnaBI sites.
Figure 2.5 Vector map of pCMV-Sport6 and MIEV-CyclinD1

pCMV-Sport6 is a mammalian expression vector containing a CMV promoter, and a multiple cloning site in which Cyclin D1 was cloned in at Sall, NotI sites. MIEV-CyclinD1 was generated by subcloning of Cyclin D1 into MIEV at EcoRl, NotI sites.
Figure 2.6 Generation of retroviral packaging lines
Retroviral packaging lines were generated by transfecting DNA into PT67 cells using CaCl$_2$ or Lipofectamine, collecting and concentrating the virus by ultracentrifugation, and subsequently transducting GP+E.86 cells. GFP$^+$ GP+E.86 cells were sorted twice and grown for at least two weeks before HPCs were transduced by co-culture with GP+E.86 cells.
Figure 2.7 *In vitro* OP9 and OP9-DL1 system

HPCs were prepared from d14 gestation FL and were co-cultured on OP9 cells with growth factors to generate B cells (OP9 system). In order to generate T cells, pro-B cells from FL:OP9 co-cultures were sorted and subsequently co-culture with growth factors and OP9-DL1 cells that express Notch ligand allowing for Notch ligation.
Figure 2.8 Apoptosis assessed by Annexin V/Viaprobe
Cells were stained with Annexin V and Viaprobe as described in Section 2.8.3 and gated as shown above. Annexin V^-Viaprobe^- gate represents viable cells, Annexin V^-/Viaprobe^- gate represents early apoptotic cells, and Annexin V^+/Viaprobe^- gate indicates late apoptotic cells.
Figure 2.9 Cell cycle analysis using PI
PI incorporation was analysed by FACS and a typical histogram is shown above. SubG₀ population represents apoptosing cells; G₀ represents quiescent cells; G₁ represents cells that are growing and preparing chromosomes for replication; S phase indicates DNA synthesis; G₂ phase is where mitosis preparation occurs; M phase is where mitosis occurs.
Chapter 3

Over-expression of PKCα-KR in murine haematopoietic progenitor cells results in the generation of a CLL-like disease marked by the upregulation of PKCβII.
3.1 Introduction
The use of murine CLL models has proven to be very beneficial in gaining understanding of the human disease. In fact, the importance of oncogenes like TCL1 was highlighted by transgenic mouse models expressing TCL1, indicating that deregulation of this gene may be a key event in the pathogenesis of CLL (Herling et al. 2005; Hamblin 2010; Gorgun et al. 2009). Similarly, deregulation of human PKCα in a murine model was shown to generate a disease similar to human CLL (Michie & Nakagawa 2006; Nakagawa et al. 2006). This chapter aims to further develop the PKCα-KR CLL mouse model, investigating events that occur downstream of PKCα deregulation.

3.2 Aims and Objectives
The specific aims of this chapter were:

i. To recapitulate the CLL mouse model;
   a. *In vitro* and
   b. *In vivo*

ii. To assess the similarities between human CLL and that generated within the PKCα-KR mouse model;

iii. To investigate therapeutic targets in the PKCα-KR mouse model by treatment with drug.
3.3 Results

3.3.1 Introduction of dominant negative human PKCα into murine HPCs results in a generation of cells with increased survival capacity and surface CLL phenotype expression profile.

In order to reinforce previous findings that loss of PKCα may act as an oncogenic trigger within murine CLL (mCLL) (Nakagawa et al. 2006; Michie & Nakagawa 2006), HPCs were isolated from FL of day 14 gestation ICR wildtype mice and retrovirally transduced to express vector only (MIEV) or PKCα-KR containing vector. The resultant cells were maintained in an *in vitro* B cell generating system containing stromal cells (OP9) with the addition of IL7 and FLT3L (Figure 3.1A). The PKC mutants used in this study which were constructed as described in Section 2.1.1, contain the bicistronic expression of GFP alongside the PKC gene of interest, thereby allowing GFP fluorescence to be monitored by FACS. The PKC constructs are also tagged with HA at the C or N terminus which can be validated by Western blot using an anti-HA antibody. HA was detected in lysates of PKCα-KR containing B cells and not in MIEV control B cells (Figure 3.1B).

MIEV and PKCα-KR cultures were maintained in the *in vitro* B cell generating system and GFP expression was assessed by FACS (Figure 3.2). At day 1, the PKCα-KR transduced cells were less bright in GFP fluorescence and contained significantly less GFP⁺ cells (22%), compared to MIEV control that were brighter and already mostly GFP⁺ (80%). The difference in overall fluorescence intensity of GFP between the cultures is likely due to the presence of the CMV promoter driving IRES and GFP and lack of a 2 Kb gene (PKCα-KR) upstream of GFP within the MIEV vector (see Figure 2.2). At day 10, there was a notable increase in the percentage GFP⁺ cells in the PKCα-KR cultures (68.3 % ± 13.3% n = 4) and by day 17, both MIEV and PKCα-KR cultures contained virtually no GFP⁻ cells (<5 %). The increase in GFP⁺ cells indicated a possible growth advantage of GFP⁺ cells over GFP⁻ cells within the PKCα-KR culture.

In order to assess the surface phenotype of MIEV and PKCα-KR cells, FACS analysis was carried out at day 10 and 17 of OP9 co-culture using cell surface markers indicative of B cell commitment (CD45 and CD19) and CLL phenotype (CD19, CD23, CD5). At day 10, both cultures contained almost 100% CD45⁺/CD19⁺ cells (Figure 3.3A) as expected within the *in vitro* B cell generating system. When CLL surface markers were assessed, PKCα-KR cultures
expressed a notably higher level of CD19, CD5 and CD23 compared to MIEV control. Also, IgM surface expression was downregulated within the PKCα-KR expressing B cells (Figure 3.3B and C). Collectively, these findings indicate that murine PKCα-KR expressing B cells resemble human CLL B cells by surface marker expression profile.

3.3.2 PKCα-KR cells express key CLL markers indicative of a poor prognostic outcome

Once it was established that murine PKCα-KR expressing B cell phenotypically resemble human CLL B cells, the expression of key genes and proteins associated with CLL pathogenesis were assessed within the PKCα-KR cultures including Bcl-2, aicda (encodes for AID), ZAP70 and activated NFκB.

At day 6 and 13 of OP9:FL cultures, Bcl-2 transcript levels were significantly higher in PKCα-KR expressing cells compared to MIEV control (Figure 3.4A left). Similarly, PKCα-KR cells express significantly higher mRNA transcripts of aicda, another key CLL gene, similar to a more aggressive, unmutated form of human CLL (Heintel et al. 2004) (Figure 3.4A right). In accordance with human unmutated CLL cells, PKCα-KR cells also express higher ZAP-70 protein levels as determined by Western blot and intracellular FACS analysis (Figure 3.4B, 3.4C). Constitutive activation of the NFκB pathway has become one of the hallmarks in CLL (Frenzel et al. 2011). Phosphorylation of NFκB-dependent genes are associated with anti-apoptosis (Bcl-2, XIAP), cell proliferation (cyclins) and metastasis (VEGF) and collectively contribute to a worse overall prognosis (Pepper et al. 2009). Similarly to human CLL, the NFκB pathway also seems to be active in PKCα-KR expressing cells as phosphorylation of IκBα protein, a classic activation marker of NFκB, is higher in PKCα-KR expressing cells compared to the MIEV control (Figure 3.4B left).

3.3.3 RAG1<sup>−/−</sup> mice injected with PKCα-KR-HPCs exhibit shortened lifespan

In order to evaluate the effects of subversion of PKCα signaling in vivo, neonatal RAG1<sup>−/−</sup> mice were subjected to i.p. in vivo adoptive transfer of MIEV or PKCα-KR retrovirally transduced HPCs (see section 2.3.2) and monitored for development of disease. Four to six weeks post injection, PKCα-KR injected mice exhibited signs of distress marked by significant weight loss and development of subcutaneous tumours at injection sites. At this time, mice were sacrificed and time of death recorded. PKCα-KR-HPC injected mice had a significantly shorter lifespan than
their MIEV-HPC injected counterparts (Figure 3.5). Importantly, lifespan was inversely dependent on number of PKCα-KR-HPCs injected.

3.3.4 Evidence of expansion of haematopoietic lineage cells with CLL phenotype in lymphoid organs and blood of RAG1−/− mice injected with PKCα-KR cells

At signs of distress, mice injected with PKCα-KR or MIEV HPCs were sacrificed and organs (BM, spleen, LN) and blood were processed (as described in section 2.3.2.1) and analyzed by FACS to determine the percentage of GFP+ haematopoietic (CD45+) cells and B cells (CD19+). There was a significant increase in the haematopoietic lineage within the PKCα-KR-HPC injected mice evident in the blood, spleen and BM (Figure 3.6). In addition, there was a significant increase of B cells within the spleen, BM and LN of PKCα-KR-HPC injected mice (Figure 3.6).

Blood and spleen of mice injected with PKCα-KR or MIEV HPCs were also subjected to FACS analysis to evaluate expression CD19, CD23, IgM and IgD (Figure 3.7). Concurrent with in vitro surface phenotype of PKCα-KR expressing cells, B cells within the blood and spleen of RAG1−/− mice injected with PKCα-KR-HPCs expressed surface IgM and IgD at lower levels than their MIEV-HPC injected counterparts (1.1% vs 76.2% of IgM+ IgD+ B cells). Co-expression of CD19 and CD23 revealed consistently lower expression of CD23 on CD19+ cells within the MIEV cohorts (Figure 3.7).

3.3.5 PKCα-KR injected RAG1−/− mice develop splenomegaly and disorganized splenic architecture

Analysis of the gross structure of spleen from PKCα-KR HPC-injected neonatal RAG1−/− mice revealed enlarged spleens and disorganized splenic architecture assessed by H & E staining of splenic tissue sections (Figure 3.8 and 3.9) indicative of tumour formation. As expected, MIEV HPC-injected harboured average sized spleens and displayed an organised splenic architecture. Splenic tissue sections were also stained with antibodies specific for B cell (anti-B220) antigens and GFP to assess the location of GFP+ B cells within the spleen. B cells were located within the follicles of the spleen in an organized fashion in MIEV injected mice, whereas PKCα-KR injected mice displayed disrupted follicular development (Figure 3.10; see appendix 3-A for staining controls). Of note, before B220 was used to identify B cells within splenic sections, co-expression with CD19
was verified by FACS (see Figure 3.10B). Very few GFP+ cells were observed within the spleen tissue sections from MIEV injected RAG1−/− mice compared to a higher number of GFP+ cells within the PKCα-KR injected mice (Figure 3.10). It appears that there are many GFP+ cells that are not B cells within the PKCα-KR injected mice, although their identities were not established.

3.3.6 Downregulation of PKCα signaling results in a gradual and sustained upregulation of PKCβII

Although the overall PKC kinase activity is reduced early on in the PKCα-KR expressing B cell cultures compared to their MIEV counterparts, at later stages (day 17), PKC kinase activity is elevated in the PKCα-KR cultures (see Appendix 3-B; Nakagawa et al. 2006). The elevation in overall PKC kinase activity within the PKCα-KR expressing B cells is coupled with an upregulation of particularly PKCβII protein, where as PKCβI protein expression remains constant (Figure 3.11A) and PKCβ mRNA (Figure 3.11C) expression specifically during the later stages of culture, similar to that noted in human CLL cells (Abrams et al. 2007a; Buschenfelde et al. 2009). Of note, the primers and probes for determining mRNA expression are not specific to a particular isoform of PKCβ, but detects both isoforms. The upregulation in PKCβII coincides with an increase in transcript levels of VEGF which regulates and is regulated by PKCβII, and ERK signaling which, like PKCβII, is important in CLL B cell survival (Krysov et al. 2012; Calpe et al. 2011)(Figure 3.11A; 3.11D). In vivo, PKCβII expression is concentrated in B cell areas within spleens of MIEV and PKCα-KR injected RAG1+/− mice (Figure 3.12).

3.3.7 Introduction of PKCβII into HPCs provides a survival advantage to B cells

In order to determine whether PKCβII alone is sufficient to transform HPCs into CLL cells, PKCβI-WT, PKCβI-CAT, PKCβII-WT and PKCβII-CAT were cloned into MIEV backbone and virus was generated as described before (section 2.1.1).

HPCs were isolated from d14 gestation FL and retrovirally transduced to express MIEV, PKCα-KR or PKCβ genes and subsequently co-cultured on OP9 in a B cell generating in vitro environment. Cells were harvested at day 1, 14, 17 and 22 of co-culture and assessed by flow cytometry to determine the percentage of GFP+ cells within the culture (Figure 3.13). As seen previously, MIEV cells transduced at a higher efficiency than the vectors containing a gene of interest. Also, as seen before, the PKCα-KR culture showed a rapid increase in the percentage of GFP+
cells over time, overtaking the culture completely by day 14. All of the PKCβ constructs had a much lower transduction efficiency compared to MIEV and PKCα-KR. Of interest, only the PKCβII transduced HPCs expanded over the time course of the experiment increasing from 7.84% GFP+ at day 1 to over 40% GFP+ at day 22 (Figure 3.13), indicating that these cells have a survival advantage.

To determine whether PKCβII transduced B cells harbour CLL phenotypic surface markers as seen in the PKCα-KR cultures, B cells from day 22 FL:OP9 co-cultures were harvested and stained with CD45, CD19, CD5 and CD23 antibodies and analysed by flow cytometry. When assessing CD19 and CD5 positivity (Figure 3.14), only the PKCα-KR expressing cells displayed the CLL phenotype, with 31% of cells staining for both CD5 and CD19. When CD23 expression was assessed (Figure 3.15), 15% of PKCα-KR cultures were highly co-expressing CD23 and CD19, similarly to what was seen previously. Interestingly, a small but distinct population of cells (2.75%) co-expressing CD23 and CD19 was evident within PKCβII-WT cultures. Collectively these results indicate that at this stage of the in vitro culture, PKCβII is alone not sufficient to transform HPCs into B cells resembling human CLL cells by phenotypic surface protein analysis, as seen within the PKCα-KR cultures, although it is possible that the transformation toward a CLL-like cell may just take longer.

3.3.8 Inhibition of PKCβ does not cause preferential apoptosis of murine CLL cells

To address whether inhibition of PKCβII can result in apoptosis of PKCα-KR expressing cells, B cells were harvested from MIEV and PKCα-KR FL:OP9 co-cultures, centrifuged over lympholyte mammal to remove dead cells and OP9 stroma, and subsequently treated with pan PKCβ inhibitors hispidin and enzastaurin. Hispidin is an older agent (in comparison to enzastaurin) that targets mainly PKCβ, but also β-secretase, protein tyrosine phosphatase, α-glucosidase, aldose reductase and some NFκB pathways (Gonindard et al. 1997; Park et al. 2004; Lee et al. 2010; Huang et al. 2011; Wu et al. 2011). Enzastaurin is a selective PKCβ/PI3K inhibitor (Rizvi et al. 2006; S. Ma & Rosen 2007; Chen & LaCasce 2008; Willey et al. 2010). We used the drugs in the µM range based on previously published work within the field. At 24 and 48 hr, cell viability was assessed by FACS by annexin V/DAPI staining. Treatment with hispidin (Figure 3.16) resulted in concentration-dependent apoptosis of both MIEV and PKCα-KR
cells expressed as percentage of annexin V− cells, similar for both cultures at 24 hr, and a slightly higher for MIEV culture at 48 hr. Enzastaurin treatment (Figure 3.17) resulted in only a moderate drop in cell viability (around 20-30%) at 24 hr. At 48 hr, the drug was more potent, however even at 50 µM, the percentage of viable cells did not drop below 40%.

To confirm results obtained with annexin V/DAPI, induction of cell death was also evaluated by considering the sub-G0 phase of cell cycle. PI analysis was conducted on hispidin and enzastaurin treated MIEV and PKCα-KR cultures and apoptosis was assessed by observing the percentage of cells in sub-G0 phase of cell cycle. As seen with apoptosis measured by annexin V/DAPI, PI analysis indicated no preferential induction of cell death in either culture (Figure 3.18).

3.3.9 Inhibiting PKCβ results in an attenuation of proliferation of murine CLL cells

Although initially CLL was considered a disease of the accumulation of abnormal B cells that resist apoptosis, CLL is now considered a disease not only of accumulation, but also proliferation (Messmer et al. 2005; Chiorazzi 2007). The murine PKCα-KR expressing cells exhibit notably higher proliferation rates than their MIEV counterparts (see Figure 3.2 and Chapter 4). Since PKCβII is highly upregulated in the PKCα-KR cultures, it was of interest to investigate whether inhibition of PKCβ could result in the attenuation of proliferation within these highly-proliferative cultures. Therefore, MIEV and PKCα-KR OP9:FL cultures were maintained as before, and 1 x 10^4 cells were plated (per well of 96 well plate) in the presence or absence (DMSO only) of hispidin and enzastaurin and labelled with BrdU two hours prior to endpoint (24 or 48 hr). As expected, the PKCα-KR expressing cells proliferate at a significantly higher rate than MIEV (**p<0.005 for 24 hr and ***p<0.001 for 48 hr) as seen in the untreated cultures (Figure 3.19). The addition of hispidin at 10 µM resulted in a significant drop in proliferation levels of specifically the PKCα-KR containing cultures at both 24 and 48 hr (Figure 3.19). Similarly, inhibition of PKCβ with enzastaurin treatment at 10µM resulted in an even more dramatic drop in proliferation rates of particularly PKCα-KR containing cultures.
3.3.10 Inhibition of PKCβ results in an accumulation of cells in G₀/G₁ phase of cell cycle

In order to confirm results obtained by BrdU analysis of MIEV and PKCα-KR cultures treated with PKCβ inhibitors, cell cycle analysis was conducted on these cultures at 24 and 48 hr after drug (hispidin and enzastaurin) treatment. Treatment of cells with hispidin for 24 hr (Figure 3.20) resulted in a significant increase in the percentage of cells arrested in G₀/G₁ phase of cell cycle at 20 μM hispidin for PKCα-KR expressing cultures and at 30 μM hispidin for the MIEV counterpart. A significant decrease in the percentage of cells in S phase of cell cycle was only observed at 50 μM hispidin for both PKCα-KR and MIEV cultures at 24 hr (Figure 3.20). The percentage of cells in G₂/M phase of cell cycle at 24 hr post hispidin treatment significantly decreased more prominently in MIEV cultures rather than the PKCα-KR cultures (20 μM for MIEV vs. 30 μM for PKCα-KR). At 48 hr, MIEV cells were more sensitive to hispidin as the percentage of G₀/G₁ arrested cells significantly increased for MIEV cultures at 10 μM compared to 30 μM for PKCα-KR cultures. Similarly, percentage of cells in S phase significantly decreased for MIEV cultures at 10 μM compared to 30 μM in PKCα-KR cultures. Finally, a significant decrease in the percentage of cells in G₂/M phase was seen at 30 μM for both MIEV and PKCα-KR cultures.

Treatment with enzastaurin resulted in a significant increase in the percentage of cells arrested in G₀/G₁ phase of cell cycle at 10 μM enzastaurin particularly for the PKCα-KR expressing cultures at both 24 and 48 hr (Figure 3.21). A significant drop in percentage of cells in S phase of cell cycle was only observed at 48 hr at 10 μM within the PKCα-KR expressing cultures. Finally the percentage of cells in G₂/M phase of cell cycle significantly decreased in the PKCα-KR expressing cultures treated with 10 μM enzastaurin at both 24 and 48 hr. Collectively, these data indicate that the PKCα-KR expressing cultures are sensitive specifically to more selective PKCβ inhibitor enzastaurin, as treatment with enzastaurin resulted in an accumulation of non-dividing cells within G₀/G₁ phase of cell cycle, and a decrease in the amount of cells preparing for and going through cell division (S and G₂/M).
3.3.11 Enzastaurin decreases amount of CLL-like GFP+ cells \textit{in vivo}

In order to determine whether enzastaurin has an anti-proliferative effect of CLL-like cells \textit{in vivo}, neonatal RAG1\(^{-/-}\) mice were injected with PKC\(\alpha\)-KR retrovirally transduced HPCs as described above (section 2.3.2). Four weeks post injection, mice were either treated with vehicle only or 80 mg/kg enzastaurin twice daily for two weeks. At this time, mice were sacrificed and organs subjected to FACS analysis in order to determine apoptosis \textit{in vivo} using annexin V/Viaprobe and CD19 staining. A significant increase in the percentage of apoptosing GFP\(^{+}\) CLL-like cells was noted upon treatment of mice with enzastaurin (Figure 3.22A). In addition, there is an evident decrease in spleen size (Figure 3.22B) post enzastaurin treatment.

3.3.12 PKC\(\alpha\) is downregulated in transcript levels and protein levels in human CLL compared to normal human B cells

In order to determine whether the findings in the murine PKC\(\alpha\)-KR model translate to human CLL, it was necessary to consider RNA and protein levels of PKC\(\alpha\) in human CLL compared to normal B cells. Therefore normal B cells and human CLL cells were isolated from buffy coats and whole blood respectively (as described in section 2.4) and subjected to qRT-PCR analysis and Western blot. PKC\(\alpha\) is down-regulated at the transcript level in most of the CLL samples examined (in 64\% of cell samples tested), although the mean level of PKC\(\alpha\) transcript levels is higher in CLL compared to normal B cells (Figure 3.23A left). As described previously (Abrams et al. 2007; Abrams et al. 2010), CLL cells express high levels of PKC\(\beta\) mRNA (Figure 3.23A right). When levels of protein were examined, it was evident that PKC\(\alpha\) protein was downregulated in CLL cells compared to normal B cells (Figure 3.23B). PKC\(\alpha\) was downregulated at the protein level in 75\% of samples tested (n=16). However, no clear relationship was found between prognostics (age, treatment, Binet stage, cytogenetic abnormalities, mutational status of IgV\(_{H}\) genes, ZAP70 expression) and PKC\(\alpha\) expression.

3.4 Discussion

Further classification of the PKC\(\alpha\)-KR murine CLL model has demonstrated its strength as a translational model because of its similarities to human CLL. The introduction of kinase inactive PKC\(\alpha\) into early murine HPCs results in a transformation event leading to a survival advantage. The low percentage of
PKCα-KR retrovirally transduced cells soon overtake the in vitro B cell culture, resulting in a homogeneous GFP+ population.

B lymphocytes from CLL patients also strongly and constitutively express the CD23 antigen which is an important prognostic marker (Fournier et al. 1992; Goller et al. 2002; Sarfati et al. 1996). Surface IgM is characteristically low in expression on CLL cells, despite normal transcription and intracellular synthesis. This poor surface expression has been accounted for by a defect in the ability of the CLL cells to assemble BCR chains, resulting in unprocessed µ chains (Payelle-Brogard et al. 2003). In normal human B cells, repeated BCR simulation leads to anergy and CD5 expression which are both important in CLL. In addition, CD5 phosphorylation has been linked to enhanced signaling of anti-apoptotic pathways such as Bcl-2, NFkB, Wnt, Stat, TGFβ, VEGF, MAPKs and various chemokines and cytokines leading to increased cell survival (Gary-Gouy et al. 2007). Indeed, PKCα-KR expressing B cells phenotypically resemble human CLL cells by surface protein expression whereby they are CD19hiCD23hiCD5+IgMlo.

Bcl-2 is an anti-apoptotic protein that is a key player implicated in the resistance of CLL cells to chemotherapy and resistance to external microenvironmental signals that direct healthy B cells to undergo programmed cell death (Adams & Cory 2007). Like human CLL cells, PKCα-KR expressing cells express Bcl-2 at high levels, possibly indicating other intrinsic properties similar to human CLL such as a defect in apoptosis.

Another gene that bears high importance in human CLL is aicda. Aicda gene encodes AID which is essential for immunoglobulin SHM and CSR (Xu et al. 2007). CLL patients can either express mutated or unmutated variable IgVH genes, and this mutational status represents an important prognostic factor whereby unmutated VH carrying patients exhibit a poorer prognosis. CSR also predominates within the unmutated VH group, however CLL B cells can undergo CSR without SHM (Oppezzo et al. 2003). Unmutated CLL B cells have been shown to constitutively express AID which may promote genetic changes that may lead to a more aggressive disease (Leuenberger et al. 2009; Oppezzo et al. 2003; Albesiano et al. 2003; Reiniger et al. 2006; McCarthy et al. 2003; Heintel et al. 2004). Expression of AID is also associated with proliferating cells. Importantly, PKCα-KR cells express significantly higher mRNA transcripts of aicda and are therefore similar to a more aggressive, unmutated form of human CLL. Hi Ki67 positivity within B cell proliferation centres was demonstrated to be positively
associated with AID expression in human CLL (Leuenberger et al. 2009). In our model it is plausible that the higher proliferative capacity of PKCα-KR cultures can be linked higher expression of aicda.

Patients harbouring an unmutated form of IgV\textsubscript{H} genes also express ZAP70 at high levels. In fact, ZAP70 is expressed in over 90% of unmutated CLL cases and is associated with an increased cell survival and inferior clinical outcome (Wiestner et al. 2003; Crespo et al. 2003). In accordance with human unmutated CLL cells, PKCα-KR cells express higher ZAP70 protein levels. The survival of CLL cells is also dependent on the NFκB pathway, which is constitutively active in CLL (Herreros et al. 2010). The phosphorylation status of IκBα, which binds and sequesters NFκB dimer to prevent binding of DNA and activation of transcription, is often used to indicate NFκB activity (Shih et al. 2011). IκBα is phosphorylated by IκB kinases (IKKs) and consequently targeted for proteosomal degradation, allowing for NFκB heterodimers to enter the nucleus and initiate transcription of key genes essential for survival and function of mature B cells (Pasparakis et al. 2002; Gilmore 2006). As in human CLL, the NFκB pathway also seems to be active in PKCα-KR expressing cells as phosphorylation of IκBα protein is higher compared to the MIEV control.

The aggressive characteristic of the PKCα-KR expressing cells is even more so evident when these cells are observed \textit{in vivo}. Indeed, adoptive transfer of PKCα-KR-HPCs into RAG1\textsuperscript{−/−} recipient mice quickly causes tumorigenesis, marked by enlarged spleens with disorganized B cell areas. This results in a shortened lifespan of PKCα-KR-HPC injected mice compared to their MIEV counterparts. The PKCα-KR expressing cells quickly proliferate within the mice evident from the increase in the percentage of GFP\textsuperscript{+} CD45\textsuperscript{+} cells, and more specifically CD19\textsuperscript{+} B cells within the blood, spleen, BM and LN, resembling human CLL. Of note, when spleen sections of PKCα-KR-HPC injected mice were examined by IHC, it was evident that not all GFP\textsuperscript{+} cells were B220\textsuperscript{+}, and therefore implies an expansion of other lineages that can result from PKCα subversion in HPCs (see Chapter 5).

PKCα has been implicated as a tumour suppressor previously, whereby its expression/activity is downregulated (Gökmen-Polar et al. 2001; Kahl-Rainer et al. 1994; Detjen et al. 2000). Similarly within the PKCα-KR mouse model, PKCα acts as a tumour suppressor in B lymphocyte progenitors. Although initially, overall PKC kinase activity is lowered within the PKCα-KR expressing cells, at later stages of culture overall PKC kinase activity is elevated, coupled by a dramatic
elevation in PKCβII expression specifically. *In vivo*, PKCβII is expressed within the B220+ B cell areas, however comparative quantification of protein *in vivo* between MIEV and PKCα-KR HPC injected mice was not possible because of the limitations set by this technique. Of note, upregulation in PKCβII expression and activity has been strongly linked to poor prognosis in human CLL and disease progression (Abrams et al. 2010; Buschenfelde et al. 2009; Abrams et al. 2007). Similarly, within the TCL1 transgenic mouse model, PKCβ was shown to be essential for the development of CLL because PKCβ deleted TCL1 transgenic mice did not develop a CLL disease (Holler et al. 2009).

It is therefore possible that the downregulation of tumour suppressor PKCα is linked to the upregulation of PKCβII and that PKCβII aids in the poor prognosis associated with the PKCα-KR mouse model. In fact PKCβII activation has been shown to be stimulated by oncogenic pathways such as VEGF signaling which can drive its expression, contributing to the malignant phenotype of CLL cells (Abrams et al. 2010). In the PKCα-KR mouse model, VEGF transcript levels are elevated compared to control and could possibly correlate to increased PKCβII signaling, however further experiments need to be conducted to validate this relationship.

It is clear that introduction of PKCβII into HPCs provides them with a survival advantage because HPCs transduced with PKCβII expand over time compared to untransduced HPCs within the same culture. This survival advantage, however, is not enough to immediately transform HPCs into B cells that phenotypically resemble human CLL cells, as seen with introduction of PKCα-KR into HPCs. Perhaps PKCβII-transformed HPCs take longer to transform and thus analysing them at a later time point would be beneficial. The current data, however, suggest that the transformation of HPCs toward CLL-like cells does not depend on a single genetic event, but rather a combination of different events.

The rationale behind targeting PKCβ in CLL is clear. Targeting BCR signaling may prove beneficial because the mutational status of the IgVH and BCR downstream signaling molecules like ZAP70 are indicative of poor prognosis in CLL patients. In fact, treatment of primary CLL cells with enzastaurin *in vitro* results in apoptosis regardless of mutational status (Holler et al. 2009). *In vivo*, one study comprised of seven patients showed an ORR of 14.3% and a progression-free survival of 308 days (Forsyth et al. 2009). Within our PKCα-KR model, treatment with pan PKCβ inhibitors hispidin or enzastaurin did not preferentially induce apoptosis of PKCα-KR expressing cells *in vitro*, however it did
slow down their proliferation as assessed by a decrease in BrdU incorporation post treatment, most dramatically with enzastaurin treatment. The decrease in proliferation was coupled with an arrest in cell cycle post drug treatment, marked by an increase in percentage of cells in G₀/G₁ phase of cell cycle, and a decrease in percentage of cells in S and G₂/M phases, particularly with enzastaurin treatment. There was however, a relevant lack of effect of hispidin treatment on cell cycle as compared to BrdU, highlighting a difference in the two assay readouts. In vivo, treatment with enzastaurin resulted in a decrease in the percentage of GFP⁺ CLL-like cells and a decrease in spleen size. These results were encouraging, but do need to be repeated for validation purposes.

Finally, PKCα seems to act as a tumour suppressor in human CLL because it is downregulated at the transcript and protein levels in most human CLL cases investigated. Therefore, understanding the molecular events that lead to its down-regulation could prove beneficial in developing new therapeutic approaches for the treatment of human CLL.
Figure 3.1 Isolation of HPCs from FL, retroviral transduction and in vitro B cell generating system

A FL was excised from day 14 gestation mice and single cell suspensions were prepared by crushing of FL and filtering through a 70 µm nylon mesh. The cells were incubated with anti-CD24 antibody and rabbit complement to allow for complement-mediated CD24<sup>+</sup> cell lysis. Viable CD24<sup><i>lo</i></sup> cells were isolated by Lympholyte-Mammal gradient centrifugation and subsequently retrovirally transduced over night on mitomycin C treated (10 µg/ml) GP+E.86 retroviral packaging lines producing either vehicle only (MIEV) or PKCα-KR virus. The cells were collected from the packaging lines, washed, and subsequently co-cultured on OP9 cells with 10 ng/ml IL7 and FLT3L. Medium was replenished every second day and OP9 layers were replenished every fourth day. B The construct containing PKCα-KR is tagged with HA at the C-terminus, therefore allowing PKCα-KR expression to be monitored by Western blot. B cell lysates from MIEV and PKCα-KR cultures were prepared and examined for HA expression.
Figure 3.2 PKCα-KR expressing cells exhibit a growth advantage over their MIEV counterparts.

The PKCα-KR construct bicistronically expresses GFP at the C-terminus, thus GFP fluorescence can be monitored by FACS. Cells were obtained from FL:OP9 cultures at day 1, day 10, day 17 and consequently analysed by flow cytometry to determine the percentage of GFP+ cells over the duration of the culture. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) prior to evaluation of GFP fluorescence.
Figure 3.3 PKCa-KR expressing cells phenotypically resemble human CLL cells by surface protein expression

A Representative FACS plot of FL cultures around day 10. Cells were live and size gated (FSC vs SSC) and CD45 and CD19 analysis shown. B Cells from day 10 and 17 FL:OP9 MIEV and PKCa-KR cultures were stained with anti-CD19, anti-CD5, anti-CD23 and anti-IgM antibodies in order to determine levels of surface protein expression. These are representative plots of over 50 separate biological replicates. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and CD5 vs CD19, CD23 vs CD19, IgM vs CD19 analysis shown. C FL cultures were subjected to FACS analysis. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and GFP, CD19, CD23, CD5 and IgM expression shown as an average of mean fluorescence intensity (MFI). GFP p < 0.0001 (n = 6); CD19 p = 0.0068 (n = 5); CD23 p = 0.0058 (n = 3); CD5 p = 0.0125 (n = 4); IgM p = 0.0003 (n = 3). Data are represented as mean (± SEM) of biological replicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.4 PKCα-KR expressing cells have higher levels of Bcl-2, aicda, ZAP70 and activated NFκB
A RNA was isolated from MIEV and PKCα-KR cultures and subjected to qRT-PCR in order to determine transcript levels of CLL-associated genes Bcl-2 and aicda relative to housekeeping gene GAPDH. B Protein lysates were also prepared from MIEV and PKCα-KR cultures to determine levels of phospho-IκBα and ZAP70 protein by Western blot and C intracellular ZAP70 protein by FACS. Results are shown as mean ± SEM. p values were generated using the student’s unpaired t-test to compare groups (n=3).
Figure 3.5 RAG1<sup>−/−</sup> mice injected with PKCα-KR-HPCs exhibit a shortened lifespan

Neonatal RAG1<sup>−/−</sup> mice were subjected to i.p. *in vivo* adoptive transfer of MIEV or PKCα-KR retrovirally transduced HPCs. For MIEV-HPC injected mice, 1 x 10<sup>6</sup> cells were used, whereas for PKCα-KR-HPC injected mice, either 1 x 10<sup>6</sup> or 4 x 10<sup>6</sup> cells were injected. Mice were sacrificed at signs of distress and day since injection was recorded. The graph indicates a survival curve and each point marks a different mouse. n = 15 (1 x 10<sup>6</sup> PKCα-KR cells); n = 31 (4 x 10<sup>5</sup> PKCα-KR cells); n = 20 (1 x 10<sup>6</sup> MIEV cells).
Figure 3.6 PKCα-KR-HPC injected mice express expansion of haematopoietic lineage and increase in percentage of GFP+ B cells.

Neonatal RAG1-/- mice were subjected to in vivo adoptive transfer of MIEV or PKCα-KR retrovirally transduced HPCs. Four to six weeks post injection, mice were sacrificed and organs and blood removed. Single cell suspensions were generated from spleen, BM and LN. FACS analysis was conducted on organs and blood stained with CD45 and CD19. Each point on the graph represents a single mouse. Averages are represented by lines and p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001) n=13 (MIEV) and n=21 (PKCα-KR).
Figure 3.7 PKCα-KR HPC-injected RAG1−/− mice exhibit CLL phenotype in vivo

Lymphocytes were isolated from blood and spleen of MIEV and PKCα-KR HPC-injected RAG1−/− mice (1 x 10⁶ MIEV cells injected; 4 x 10⁵ PKCα-KR cells injected) and subjected to FACS analysis. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and CD23 vs CD19; CD45−CD19+ cells were gated and IgM vs IgD analysis shown.
Figure 3.8 PKCα-KR-HPC injected mice exhibit splenomegaly
FL cells were prepared from wildtype mice and retrovirally transduced to express MIEV or PKCα-KR as described before. After over-night transduction, 4 x 10^5 cells were injected i.p. into neonatal RAG1^-/- mice. Four weeks post injection, mice were sacrificed and spleens excised.
Figure 3.9 PKCα-KR-HPC injected mice exhibit disrupted splenic architecture

Spleens from RAG1−/− mice injected with 4 x 10^5 MIEV or PKCα-KR HPCs were removed four weeks post injection and immediately embedded in paraffin. Three micron sections were cut and stained with H & E.
Figure 3.10 PKCα-KR-HPC injected mice display disrupted B cell follicular formation and more GFP⁺ cells within the spleen

A Paraffin embedded spleens from RAG1⁻/⁻ mice injected with 4 x 10⁵ MIEV or PKCα-KR HPCs four weeks post injection were cut into three micron sections and subsequently stained for B220 antigen and GFP using anti-B220 and anti-GFP antibodies (for isotype controls, see Appendix A). B FL cultures were subjected to FACS analysis. Haematopoietic (CD45⁺) cells were live and size gated (FSC vs SSC) and CD19 vs B220 analysis shown.
Figure 3.11 Attenuation of PKCα activity within the PKCα-KR expressing B cells leads to an upregulation of PKCβII

A Protein lysates were prepared from MIEV and PKCα-KR cultures. Proteins were separated by gel electrophoresis and immunoblotted for PKCβII, PKCβI and phospho-ERK (pERK1/2). GAPDH was included as a protein loading control. B In order to determine when PKCβII protein is upregulated within the PKCα-KR cultures, protein lysates were taken at earlier (day 8, 10) and later (day 15, 17) time points of the B cell culture. Immunoblotting for PKCβII and GAPDH (loading control) was performed. C RNA was isolated from MIEV and PKCα-KR cultures and subjected to qRT-PCR to evaluate the levels of PKCβ and D VEGFα mRNA transcripts. B and T cells sorted from wildtype spleen serve as additional controls. Results are expressed as $2^{\Delta \Delta CT}$ relative to GAPDH housekeeping gene and represent mean ± SEM (*p<0.05, ***p<0.001).
Figure 3.12 PKCβII protein is expressed *in vivo* within the B cell follicles of the spleen

Paraffin embedded spleen sections from MIEV and PKCα-KR HPC-injected mice were stained for B220 and PKCβII.
Figure 3.13 PKCβII transduced cells expand over time

Full length PKCβI and PKCβII (PKCβI-WT and PKCβII-WT) and constitutively active PKCβI and PKCβII isoforms (PKCβI-CAT and PKCβII-CAT) were cloned into retroviral backbone MIEV. Retroviral packaging lines were generated as described in Section 2.1.2.2.1 and FL HPCs were transduced overnight. FL HPCs were also transduced with MIEV and PKCα-KR for comparison. Cells were then co-cultured with OP9 and cytokines in a B cell generating system and subjected to FACS at different stages of culture (day 1, 14, 17, 22) to determine the percentage of GFP+ cells. B cells (CD45+CD19+) were live and size gated (FSC vs SSC) prior to GFP gating.
Figure 3.14 PKCβII transduced cell surface phenotype: CD5 expression

HPCs that were retrovirally transduced to express MIEV, PKCα-KR, PKCβI-WT, PKCβII-WT, PKCβI-CAT and PKCβII-CAT were subjected to FACS analysis to determine expression of CD19 vs CD5 at day 22 of FL:OP9 co-culture. FACS analysis was carried out on GFP− and GFP+ populations. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and CD5 vs CD19 analysis shown.
Figure 3.15 PKCβII transduced cell surface phenotype: CD23 expression
HPCs that were retrovirally transduced to express MIEV, PKCα-KR, PKCβI-WT, PKCβII-WT, PKCβI-CAT and PKCβII-CAT (as in Fig 3.10) were subjected to FACS analysis to determine expression of CD19 vs CD23 at day 22 of FL:OP9 co-culture. FACS analysis was carried out on GFP− and GFP+ populations. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and CD19 vs CD23 analysis shown.
Figure 3.16 Inhibition of PKCβ with hispidin does not favour apoptosis of PKCα-KR cells compared to MIEV control

Cells were harvested from MIEV and PKCα-KR FL:OP9 cultures at later stages (>14 days of in vitro culture) and treated with increasing concentrations of pan PKCβ inhibitor hispidin for 24 and 48 hr. Apoptosis was assessed by annexin V/DAPI staining. Data are represented as means (± SEM) of at least 3 biological replicates. The graphs on the left represent cell percentage; whereas the graphs on the right represent percentage of no drug control (NDC). p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.17 Inhibition of PKCβ with enzastaurin does not favour apoptosis of PKCα-KR cells compared to MIEV control

Cells were harvested from MIEV and PKCα-KR and treated with increasing concentration of more selective PKCβ inhibitor enzastaurin for 24 and 48 hr and apoptosis was assessed by FACS using annexin V/DAPI staining. Data are represented as mean (± SEM) of at least 3 biological replicates. The graphs on the left represent cell percentage; whereas the graphs on the right represent percentage of NDC. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.18 Representation of apoptosis by assessment of SubG₀ population in hispidin and enzastaurin treated cells

Cells were harvested from MIEV and PKCα-KR cultures and treated with increasing concentration of hispidin or enzastaurin for 24 and 48 hr. Cells were then washed in PBS and re-suspended in PBS containing 20 µg/ml PI in the presence of DNase-free-RNase. Cell cycle profile was assessed by FACS. Data are represented as mean (± SEM) of at least 3 biological replicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.19 Inhibition with hispidin and enzastaurin causes a decrease in proliferation of specifically PKCα-KR cultures as assessed by BrdU incorporation

Cells were harvested from MIEV and PKCα-KR cultures and treated with increasing concentrations of hispidin or enzastaurin for 24 and 48 hr. Cells were incubated with BrdU for 2 hr prior to end point, and fixed. Absorbance is represented as values read at 492 nm - 370 nm after addition of TMB substrate. Data are represented as mean (± SEM) of at least 3 biological replicates, each carried out in technical triplicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.20 Cell cycle analysis after treatment with hispidin shows no preference for PKCα-KR cultures in terms of cell cycle arrest

Cells were harvested from MIEV and PKCα-KR cultures and treated with increasing concentration of hispidin for 24 and 48 hr. Cells were then washed in PBS and resuspended in PBS containing 20 µg/ml PI in the presence of DNAse-free-RNase. Cell cycle profile was assessed by FACS. The sub-G₀ population was gated out prior to analysis. Data are represented as mean (± SEM) of at least 3 biological replicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.21 Treatment of PKCα-KR cultures with enzastaurin causes cell cycle arrest

Cells were harvested from MIEV and PKCα-KR cultures and treated with increasing concentration of enzastaurin for 24 and 48 hr. Cells were then washed in PBS and re-suspended in PBS containing 20 µg/ml PI in the presence of DNAse-free-RNase. Cell cycle profile was assessed by FACS. The sub-G₀ population was gated out prior to analysis. Data are represented as mean (± SEM) of at least 3 biological replicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 3.22 Treatment of PKCα-KR-HPC injected mice with enzastaurin results in a reduction of CLL-like cells and spleen size

RAG1⁻/⁻ mice were injected with PKCα-KR-HPCs and treated with 80 mg/kg enzastaurin or vehicle control four weeks post injection. A Cells were isolated from organs of mice and assessment of apoptosis was done by FACS using annexin V/Viaprobe. Prior to annexin V⁺ gating, cells were gated on CD19 and GFP. B Spleen size of control (RAG1⁻/⁻), PKCα-KR-HPC injected untreated, and PKCα-KR-HPC injected and enzastaurin treated mice. p values were generated using the student’s unpaired t-test to compare groups.
Figure 3.23 PKCα mRNA and protein is down-regulated in human CLL

Normal human B cells and CLL cells were isolated from buffy coats and CLL patients respectively. **A** qRT-PCR data showing expression of PKCα (left) and PKCβ (right) genes relative to GAPDH and represented as fold change over Normal B cells. **B** PKCα and PKCβII protein expression by Western blot of 3 normal and 6 CLL patients.
Appendix 3-A Isotype controls for immunohistochemistry
Paraffin embedded spleens from RAG1<sup>−/−</sup> mice injected with MIEV or PKCα-KR HPCs were stained with secondary only and isotype controls.

Appendix 3-B PKCα-KR expressing cells have decreased kinase activity in early (day 10) but not in late cultures (day 17)
Cell lysates were prepared from FL:OP9 co-cultures of MIEV and PKCα-KR retrovirally transduced cells at day 10 and 17. PKC activity was determined with ³²P PKC kinase kit. The graphs express percentage of total PKC activity in PKCα-KR expressing cells relative to MIEV expressing cells. Statistics were generated using the student’s unpaired t-test (**p<0.005). Part of this figure was published previously in Nakagawa et al, 2006.
Chapter 4

Attenuation of PKCα signaling in HPCs results in the activation of mTOR signaling and upregulation of Cyclin D1
4.1 Introduction

The mTOR pathway is responsible for cell growth and proliferation and its deregulation is associated with human disease, including cancer (Sarbassov et al. 2005). The mTOR signaling pathway mediates signals from nutrients, growth factors and energy status in order to regulate bioprocesses such as metabolism and autophagy. One of the many processes regulated by mTOR is nutrient metabolism. As cells grow, their requirement for nutrient intake is enhanced, resulting in an elevation of nutrient receptor expression. CD98 is a cell surface protein that regulates amino-acid transport. In murine fibroblasts, over-expression of CD98 is associated with malignant transformation (Shishido et al. 2000). CD71 is a transferrin receptor that is usually expressed on activated lymphocytes (among other cell lineages). CD71 binds Fe(Apo)-transferrin and delivers iron by internalization that is necessary for cellular metabolism of a growing cell (Sargent et al. 2005; Aisen 2004).

mTOR consists of two distinct complexes, mTORC1 and mTORC2. mTORC1 is comprised of mTOR, Raptor, GβL and Deptor, whilst mTORC2 is comprised of mTOR, Rictor, GβL, Sin1, PRR5/Protor-1 and Deptor (Laplante & Sabatini 2009). mTORC2 can promote cell survival by activating Akt. mTORC1 can promote cell growth if adequate nutrients, growth factors or energy are available, or can regulate catabolic processes under stress conditions. Hormones such as insulin and growth factors signal via PI3K/Akt to mTORC1, inactivating tuberous sclerosis complex 2 (TSC2) and preventing mTORC1 inhibition. In contrast, low ATP levels can activate TSC2 in order to inhibit mTROC1 signaling. Ras-related GTPase (Rag) proteins are involved with signaling to mTORC1 in regard to amino acid availability. Active mTORC1 can regulate downstream biological effects such as mRNA translation via downstream target 4EBP1. Like ERK, mTORC1 can phosphorylate translational repressor 4EBP1, thereby releasing eIF-4E and allowing it to generate a complex with eIF-4A and eIF-4G that is responsible for cap-dependent translation (Figure 4.1) (Laplante & Sabatini 2009). PKCα activates protein serine/threonine phosphatase A (PP2A) which in turn dephosphorylates 4EBP1, thus activating translational repressor 4EBP1 that binds eIF-4E, preventing it from forming the eIF-4F complex (Guan et al. 2007).
Aims and Objectives

One of the early notable features of HPCs that have been transduced with PKCα-KR mutant is their ability to expand within the culture (Figure 3.2). The attenuation of PKCα signaling leads to a survival advantage marked by an evident increase in the percentage of GFP+ cells in culture. To gain a deeper understanding of the transformation characteristics of PKCα-KR transduced cells, the specific aims of this chapter were:

i. To investigate whether attenuation of PKCα signaling in HPCs leads to increased proliferation and changes in cell cycle;

ii. To delineate the molecular mechanisms responsible for the expansion of the PKCα-KR expressing cells over their control counterparts;

iii. To determine whether the signaling pathways associated with transformation in mCLL mirror those that occur in human CLL.
4.3 Results

4.3.1 PKCα-KR expressing cells are bigger than their MIEV counterparts and have activated mTOR signaling

PKCα-KR cells appeared larger in size in comparison to their MIEV counterparts. In order to determine whether there was a size difference between the PKCα-KR and MIEV cultures, the two cultures were photographed (Figure 4.2A) and analysed by FACS (Figure 4.2B) at day 13 of FL:OP9 co-culture. The PKCα-KR expressing cells were larger than their MIEV counterparts as demonstrated in the picture (Figure 4.2A) and in the overlay histogram of forward scatter (Figure 4.2B; average of FSC mean for MIEV = 6.83 x 10^4 versus 8.91 x 10^4 for PKCα-KR, n = 6; p = 0.0049). A larger size of cell is correlated to an increased requirement for nutrients and elevation in cellular processes such as metabolism. Therefore, the expression of two nutrient receptors, CD71 and CD98 was investigated on the surface of PKCα-KR and MIEV cultures by FACS (Figure 4.3A). PKCα-KR cells expressed both CD98 and CD71 at higher levels than their MIEV counterparts at day 14 of co-culture. The increase in mammalian cell size and its growth has been attributed to the mTOR pathway (Sarbassov et al. 2005), so initial activation of the pathway was investigated by examining the phosphorylation state of p70 S6 Kinase, a common downstream target of mTOR activity. Indeed, mTOR is active in PKCα-KR cultures at day 11 and day 18 (Figure 4.3B). Phospho-p70 S6 Kinase was also present in day 11 MIEV cultures, however it was more pronounced in PKCα-KR cultures. In addition to an elevation of mTOR activity, PKCα-KR expressing cells have constitutively active ERK signaling as demonstrated by early and sustained phosphorylation of ERK1/2 (Figure 4.3B).

4.3.2 Treatment of PKCα-KR cells with mTORC1 inhibitor rapamycin does not cause preferential apoptosis compared to the control

Since it was determined that mTOR signaling is active within PKCα-KR cultures, we investigated whether inhibition of mTOR signaling by rapamycin treatment would result in apoptosis of these cells. Therefore MIEV and PKCα-KR cells were harvested from (> day 14) FL:OP9 co-cultures and treated with increasing concentrations of rapamycin in the presence or absence of OP9 stroma (Figure 4.4). The rationale behind testing drug efficacy on stroma was derived from recent studies indicating that drug resistance in vivo is correlated to the dependency of a leukaemic cell on its microenvironment that protects it from the effects of drug
seen in vitro in medium alone, rendering it chemoresistant (McCaig et al. 2011; Tabe et al. 2011). There was a significant but modest decrease in the percentage of viable cells in both MIEV and PKCa-KR cultures treated with 10 nM at 24 hr (Figure 4.4A). At 24 hr, this decrease was moderate even at highest concentration of rapamycin (300 nM) as percentage of decrease in viable cells was ≤25. The presence of OP9 stroma had a slight protective effect for both MIEV and PKCa-KR cultures. At 48 hr, a significant decrease in the percentage of viable cells was seen for rapamycin treated MIEV cultures only in the presence of stroma (Figure 4.4B left). In the absence of stroma, a significant decrease in the percentage of viable cells was seen at 10 nM rapamycin for PKCa-KR cultures, and only at 300 nM for MIEV cultures (Figure 4.4B right).

**4.3.3 Treatment of PKCa-KR cultures with mTORC1 inhibitor rapamycin results in the attenuation of proliferation as marked by BrdU incorporation**

Although treatment with rapamycin did not result in a preferential induction of apoptosis in PKCa-KR cultures compared to their control counterparts, we aimed to determine whether treatment with rapamycin would result in a decrease in levels of proliferation within these cells. Thus, MIEV and PKCa-KR cells were treated with increasing concentrations of rapamycin and proliferation was assessed by BrdU incorporation (Figure 4.5). There was a significant reduction in proliferation with addition of lowest concentration of rapamycin (10 nM) specifically in the PKCa-KR cultures. Treatment with higher concentrations of drug resulted in a further modest decrease in proliferation within these cultures.

**4.3.4 A higher percentage of PKCa-KR cells are undergoing cell division compared to MIEV cells, and are dependent on OP9 stroma**

In CLL, the microenvironment within the LN and BM has proven to be crucial for the survival and proliferation of a CLL cell. Since it was demonstrated that PKCa-KR cells proliferate more than their MIEV counterparts it was of interest to determine whether the in vitro microenvironment (stroma and cytokines) aid in the proliferation of PKCa-KR cells. Therefore, MIEV and PKCa-KR cells were cultured in medium alone (in the absence of stroma and IL7), with stroma alone, or with stroma and IL7 and cell cycle profile was analysed using FACS by PI staining (Figure 4.6A). In medium alone, in comparison to MIEV cells, PKCa-KR cells displayed a significantly lower percentage of cells in subG₀ phase and a trend of
an increase in percentage of cells in G<sub>2</sub>/M phase of cell cycle. The addition of OP9 stroma resulted in a further decrease in the subG<sub>0</sub> population (although not significant), a significant decrease in cells arrested in G<sub>0</sub>/G<sub>1</sub> phase of cell cycle, and a significant increase in cells preparing for and undergoing cell division (S and G<sub>2</sub>/M) phase. Finally, the addition of IL7 decreased the percentage of dead cells as marked by the subG<sub>0</sub> population for both the MIEV and PKCα-KR cultures. Although not significant, the addition of IL7 and OP9 to PKCα-KR cultures further decreased the percentage of cells arrested in G<sub>0</sub>/G<sub>1</sub> phase of cell cycle, and increased the percentage of cells in S and G<sub>2</sub>/M phases of cell cycle.

To determine whether the increased proliferative capabilities of the PKCα-KR cultures occurred early on in the in vitro culture, or as a later event, two early (day 9, 10) and two late (day 24, 28) MIEV and PKCα-KR cultures were cultured on OP9 with IL7 and labelled with BrdU (Figure 4.6B). It appears that the PKCα-KR cultures are proliferating more than the MIEV cultures at both early and late phases of cell culture (Figure 4.6B).

### 4.3.5 PKCα-KR cells express cyclin D1 mRNA and protein in vitro and in vivo

In the intestine, cyclin D1 was shown to be negatively regulated by PKCα (Pysz et al. 2009; Guan et al. 2007), therefore within the CLL mouse model, where PKCα signaling is attenuated, we hypothesised that cyclin D1 may be upregulated thus aiding in the proliferative characteristics of PKCα-KR cells. As such, transcript levels of cyclin D1 mRNA were determined at day 6, 10 and 17 of FL:OP9 MIEV and PKCα-KR cultures (Figure 4.7A). There was a notable increase in cyclin D1 mRNA in PKCα-KR cultures compared to MIEV and control B and T cells. In vivo, mRNA levels were determined within spleen, tumour and BM of PKCα-KR-injected mice and demonstrated to be higher than B or T controls (Figure 4.7B). Since cyclin D1 mRNA was upregulated within the PKCα-KR cultures, protein expression was evaluated by Western blot analysis in early (day 6) mid (day 13) and late (day 26) FL:OP9 co-cultures (Figure 4.7C). PKCα-KR cultures contained a higher amount of cyclin D1 protein even early on (day 6), and in correlation with the mRNA data obtained (Figure 4.7A) cyclin D1 protein levels were higher in the later cultures (Figure 4.7C). In human embryonic stem cells, cyclin D1 was shown to be regulated by miR302 that is activated by Sox2 (Card et al. 2008). Of interest, Sox2 mRNA is downregulated within the PKCα-KR cultures in vitro (Figure 4.7D).
As a result of the focus of the microenvironment in regard to CLL cell survival and proliferation, we determined whether cyclin D1 is expressed in B cells within the proliferation centres of PKCα-KR injected mice. Therefore consecutive spleen sections of MIEV and PKCα-KR-injected mice were stained for B220 and cyclin D1 (Figure 4.8). Within the MIEV spleen, cyclin D1 was found outwith the B cell follicle, whereas within the PKCα-KR spleen, cyclin D1 and B220 were co-localized within the B cell follicles as demonstrated by sequential staining of B220 and cyclin D1 respectively. It is evident that other cells within both MIEV and particularly within the PKCα-KR spleens were positive for cyclin D1, although their identities were not clarified.

In human CLL, cyclin D1 mRNA and protein is upregulated when CLL cells are co-cultured with NTL cells and NTL cells expressing CD40L (CD154) as compared to medium alone (Appendix 4-A). In addition, in human CLL LN biopsies, out of the seven samples tested, three revealed some positive staining for cyclin D1 (Appendix 4-B).

4.3.6 Over-expression of cyclin D1 does not result in an expansion of GFP$^+$ cells, however the GFP$^+$ cells do exhibit hallmark CLL markers

Since it was demonstrated that cyclin D1 mRNA and protein are upregulated early within the PKCα-KR cultures, we determined whether expression of cyclin D1 alone could transform HPCs into malignant cells by acting as an initiation factor. Therefore, cyclin D1 was cloned into retroviral backbone MIEV (see section 2.1.1) and retroviral packaging lines were generated as described in section 2.1.2.2.1. HPCs were isolated from FL as described previously, retrovirally transduced to overexpress cyclin D1, and analysed by FACS at day 1, 14, 17, and 22 (Figure 4.9). When assessing the percentage of GFP$^+$ cells, no elevation in growth or survival advantage occurs in cells that over-express cyclin D1, as indicated by lack of expansion of GFP$^+$ cells. However, when the cells were assessed by FACS at day 14 to determine the expression of surface makers CD19, CD5 and CD23 indicative of CLL, the small populations of GFP$^+$ B cells (CD19$^+$) expressed both CD5 and CD23, however the cell number was minimal (Figure 4.10). In fact, whereas 0% of B cells within MIEV GFP$^+$ and cyclin D1 GFP$^-$ populations expressed CD23, 9.6% of B cells within the cyclin D1 GFP$^+$ population expressed CD23 comparative to 7.28% within the PKCα-KR GFP$^+$ population (Figure 4.10). Similarly, whereas B cells within MIEV GFP$^+$ and cyclin D1 GFP$^-$ populations expressed CD5 at levels less than 4%, B cells within cyclin D1 GFP$^+$ populations...
expressed CD23 at levels above 20%, at even higher levels than the PKCα-KR GFP+ populations (7.4%) (Figure 4.10).

4.3.7 Within mCLL cyclin D1 is regulated through transcriptional repressor 4EBP1

In order to determine how cyclin D1 expression is regulated within the PKCα-KR model, the phosphorylation state of translational repressor 4EBP1 was assessed since published work indicates a negative regulation of cyclin D1 by PKCα through 4EBP1 in a PP2A-dependent manner (Figure 4.11) (Hizli et al. 2006; Guan et al. 2007). In addition mTOR signaling has been demonstrated to be important in 4EBP1 regulation (Gingras et al. 2001; Averous et al. 2008). Therefore, day 14 MIEV and PKCα-KR cells were treated with pan-PKC activator PMA to activate PKCα (among other PKCs) and selective calcium ionophore agent Iono, mTORC1 inhibitor rapa, and PP2A inhibitor OA. Within the untreated cultures, 4EBP1 is hyperphosphorylated in PKCα-KR cultures compared to the MIEV cultures as demonstrated by the stronger expression of the higher band of 4EBP1. Upon treatment with PMA/Iono, the middle band of 4EBP1 is lost, indicating a shift toward further 4EBP1 repression. Treatment with rapamycin causes a shift toward the lower bands of 4EBP1 (hypophosphorylated and therefore active state) in both MIEV and PKCα-KR cultures. Finally treatment with OA within the PKCα-KR cultures results in an expression of the top (hyperphosphorylated) band of 4EBP1, however it is unclear whether this band is stronger than that within the untreated PKCα-KR cultures. Treatment of MIEV cultures with OA results in re-expression of the top band of 4EBP1. Cyclin D1 expression is higher in untreated PKCα-KR cultures compared to MIEV control as demonstrated previously, and treatment with PMA and rapamycin results in a slight reduction of cyclin D1 protein levels. Activation of PKCs and attenuation of mTOR signaling results in a decrease of cyclin D1 protein.

4.3.8 4EBP1 is hyperphosphorylated in human CLL cell line HG3 and human CLL and regulates expression of Mcl-1 and c-myc in a mTORC1 and PKC-dependent manner

Cap-dependent translation and eIF-4E have been shown to regulate Mcl-1 and c-myc expression (Benedetti & Graff 2004; Huo et al. 2011). To determine whether cyclin D1 expression is regulated through 4EBP1 as in the mCLL model, HG3 CLL cell line or human CLL cells were cultured on NTL stroma and treated with
rapamycin or PMA. Untreated HG3 cells express hyperphosphorylated bands of 4EBP1 indicating that it is inactive (Figure 4.12). Treatment with rapamycin results in the shift toward the hypophosphorylated form and a reduction in pP70 S6K demonstrating the ability of rapamycin to inhibit mTORC1 signaling (Figure 4.12). In addition Mcl-1 and c-myc are down-regulated upon rapamycin treatment. Stimulation with PMA/Iono did not alter the phosphorylation state of 4EBP1, however it did result in an increase in levels of Mcl-1, pP70 S6K and c-myc (Figure 4.12). No cyclin D1 protein was detected in this cell line under any condition. In human CLL, 4EBP1 is hyperphosphorylated and therefore inactive (Figure 4.13 left). The regulation of 4EBP1 by mTOR signaling was indicated by a marked shift toward the hypophosphorylated form of 4EPB1 upon treatment with rapamycin (Figure 4.13 left). Treatment with rapamycin also results in a decrease in pP70 S6K as expected, and a decrease in c-myc levels as seen in the HG3 cell line (Figure 4.13 right). There was no change in cyclin D1 and Mcl-1 levels upon treatment with rapamycin. Treatment with PMA resulted in a slight decrease in the inactive form of 4EBP1 (Figure 4.13 left). A decrease in cyclin D1 levels was seen with PMA treatment as in the mCLL model, however this result did not occur in another CLL sample tested. Treatment with PMA/Iono resulted in an increase in Mcl-1 and c-myc, as seen in the HG3 cell line, (Figure 4.13 right). Levels of p-ERK were evaluated in the human CLL samples and demonstrated an elevation upon PMA/Iono treatment (Figure 4.13 left). Cells treated with OA were removed from the analysis because a toxic effect was seen and thus little protein was detected.

4.4 Discussion

Co-culture of CLL cells with cytokines and stroma allows for the survival and proliferation of CLL cells in vitro (McCraig et al. 2011). In fact, it was demonstrated that the LN and BM (more prominently LN) are sites of CLL active cell proliferation (Stevenson & Caligaris-Cappio 2004). Within CLL LN and BM biopsies, a subpopulation of malignant lymphocytes are located within the proliferation centres surrounded by accessory cells such as stroma, T cells, macrophages and dendritic cells (see section 1.2.8). Similarly, within the mCLL model an early observation of the PKCα-KR cultures was their highly proliferative characteristic. It was therefore hypothesised that attenuation of PKCα signaling results in a generation of CLL cells similar to those found within the proliferation centres of lymphoid organs responsible for the expansion of the CLL clone. Therefore, we wanted to delineate
signaling molecules responsible for this proliferative capability of PKCα-KR cells, and in particular the mTOR signaling pathway.

PKCα-KR expressing cells were larger than their MIEV counterparts and expressed nutrient receptors CD98 and CD71 at higher levels. In addition, mTOR signaling was active in these cells by considering the levels of phosphorylation of p70 S6K, a common mTOR activation marker (Averous & Proud 2006). However, treatment of PKCα-KR cultures with rapamycin did not result in a dramatic preferential induction of apoptosis in these cultures compared to MIEV control cultures at 24 hr. However, the decrease in the percentage of viable cells was more significant for the PKCα-KR cultures at 24 hr. OP9 stroma had a slight protective effect on both MIEV and PKCα-KR cultures. Rapamycin has been demonstrated to be relatively unstable under tissue culture conditions (37°C, 5% CO₂), with a t₁/₂ of around 9.9 hr for degradation (Hosoi et al. 1999). Perhaps repeated treatment with consistent low concentrations of rapamycin (10 nM) may result in preferential apoptosis of PKCα-KR cultures.

mTOR is a multidomain protein that interacts with other proteins by forming complexes (Wullschleger et al. 2006). Rapamycin only targets mTORC1 therefore using a pan mTOR inhibitor such as PP242 and AZD8055 that targets both mTORC1 and mTORC2 (Huo et al. 2011) may prove to be more effective in causing apoptosis preferentially within the PKCα-KR cultures.

When cell cycle of MIEV and PKCα-KR cultures was examined in medium alone, stroma alone, or stroma with cytokine IL7, it was evident that PKCα-KR cells are more viable than MIEV cells without the microenvironment, demonstrating their survival advantage as seen in a significant decrease in the percentage of dead cells (subG₀ population). In addition, without stroma or IL7, PKCα-KR cells were still dividing, as indicated by a trend of an increase in the percentage of cells in G₂/M phase. It was evident that addition of stroma significantly decreased the percentage of non-dividing cells (G₀/G₁) and significantly increased the amount of dividing cells (S, G₂/M). With the addition of IL7, similar viability was observed in both MIEV and PKCα-KR cells, and IL7 further increased the amount of proliferating PKCα-KR cells (as marked by an increase in percentage of S and G₂/M and a decrease in G₀/G₁ phases of cell cycle). Collectively these data indicate that indeed the increased capabilities of the PKCα-KR cells to proliferate are microenvironment dependent.
The process whereby a normal cell transforms into a leukaemic cell involves the progressive acquisition of genetic abnormalities leading to inhibition of tumour suppressors and overexpression or activation of oncogenes within a certain biological system. An important group of genes, the cyclins are often deregulated in transformed cells. In particular, cyclin D1 is often overexpressed in some cancers, including breast cancer, colorectal carcinoma and lymphoid malignancies such as mantle cell lymphoma (Weinstein 2000). In these cancers, the overexpression of cyclin D1 plays a crucial role in cell transformation, leading to increased proliferation and tumorigenesis (Weinstein 2000). In CLL, cyclin D1 mRNA is expressed, whereas it is not within normal B cells (Gladkikh et al. 2010). In concordance with these findings, within the mCLL model, cyclin D1 mRNA and protein were upregulated both in vitro and in vivo. Interestingly, there was a notably higher amount of cells that stained for cyclin D1 that were not B220+, perhaps implying an accessory oncogenic role for cyclin D1 whereby it is expressed within the epithelium and cells surrounding the CLL cells. In human CLL, cyclin D1 mRNA and protein is upregulated upon co-culture with microenvironmental simulatory conditions (NTL and NTL-CD154). In addition, some human CLL LN biopises were positive for cyclin D1, indicating that the findings within the mouse model translate at least partially to the human disease, implicating that cyclin D1 could be important within the proliferation centres of the lymphoid organs in CLL.

Over-expression of cyclin D1 within HPCs did not result in an expansion of GFP+ cells as seen previously within the PKCα-KR cultures evident by a decrease in GFP+ cell population from 10.6% to 1.2%. However the small population that remained GFP+ (1.2%), did display hallmark surface CLL phenotype makers. Although this experiment was conducted only once, with more reproducibility, it could indicate that cyclin D1 is important in the transformation of normal HPCs into CLL cells. Perhaps over extended periods of time these cyclin D1 positive cells can accumulate and acquire further changes in order to generate CLL cells.

To determine how cyclin D1 is regulated, we assessed the role of Sox2, one of the main transcription factors necessary for pluripotency during early embryogenesis and embryonic stem cell maintenance (Card et al. 2008). Sox2 was downregulated transcriptionally within the PKCα-KR in vitro cultures. Sox2 activates miR-302, which in turn negatively regulates cyclin D1 post-transcriptionally (Card et al. 2008). The decreased expression of Sox2 mRNA within the PKCα-KR cultures
may be an indication of its reduced functionality, thereby its inability to activate miR-302 which represses cyclin D1, leading to an accumulation of cyclin D1. However, no protein analysis of Sox2 was conducted and further experiments should be considered before making such an assumption.

In colon cancer cells, restored PKCα expression was shown to decrease cyclin D1 levels through transcriptional and translational inhibition (Pysz et al. 2009). In intestinal epithelial cells, PKCα can inhibit cyclin D1 expression through translational repressor 4EBP1 and attenuation of cap-dependent translation initiation (Hizli et al. 2006) in a PP2A dependent manner (Guan et al. 2007). 4EBP1 is a binding partner of eIF-4E, a known target of mTOR that has been documented to have a significant role in tumorigenesis and has a key role in mRNA translation and metabolism (Gingras et al. 2001; Averous et al. 2008). Often, the genes that are extensively translated are oncogenes that aid in transformation of normal cells, such as Mcl-1 and c-myc. 4EBP1 is regulated through phosphorylation at seven sites, four of which are associated with mTOR signaling (Mothe-Satney et al. 2000; Gingras et al. 2001). In its hyperphoshorlated form, 4EBP1 is not active and thus releases eIF-4E allowing for the formation of the eIF-4E complex that is responsible for cap-dependent translation.

Within the PKCα-KR cultures, 4EBP1 is hyperphosphorylated and therefore not active. As such, it cannot repress translation of cyclin D1 which is highly expressed within the PKCα-KR cultures. PKC activation with PMA/Iono results in an activation of 4EBP1 and reduction of cyclin D1 protein levels. Pan-PKC activation includes PKCα reactivation, and thus it is plausible that reactivation leads to a downregulation of cyclin D1 as seen in other systems (Hizli et al. 2006; Guan et al. 2007). Inhibition of mTOR signaling with rapamycin treatment also results in a slight reduction of cyclin D1 levels, indicating that activated mTOR signaling within the PKCα-KR cultures is modulating 4EBP1 activity. In fact, it has been demonstrated that mTORC1 is responsible for PP2A phosphorylation which inhibits PP2A activity leading to an accumulation of hyperphosphorylated (inactive) form of 4EBP1 (Gustafson & Weiss 2010). Treatment with rapamycin, therefore, inhibits the ability of mTORC1 to inactivate PP2A resulting in hypophosphorylation and activation of translational repressor 4EBP1. A recent study shows that in mammalian cells, cell size and cell cycle progression are independent. Whereas S6K is responsible for cell growth but not proliferation, 4EBPs were demonstrated
to be essential in control of mTORC1-dependent proliferation, but not cell growth (Dowling et al. 2010). Similarly, in our mCLL model, an inhibition of mTORC1 via treatment with rapamycin results in a re-activation of 4EBP1 and an attenuation of proliferation. It is unclear whether treatment with PP2A inhibitor OA in PKCα-KR cultures results in a greater hyperphosphorylation of 4EBP1 because in the untreated PKCα-KR cultures the top band is very highly expressed, and perhaps this method is not sensitive enough to see further changes in phosphorylation state.

In human CLL cell line HG3 and primary human CLL cells 4EBP1 is hyperphosphorylated and therefore inactive. Its phosphorylation state is regulated through mTOR signaling as seen through sensitivity to rapamycin treatment. In two CLL samples tested cyclin D1 levels did not change upon treatment with rapamycin at 4 hr indicating that the protein is not regulated though mTORC1 in human CLL or that a longer time point is needed to see the effects of rapamycin treatment. Again, a pan-mTOR inhibitor could prove to be more effective here. A decrease in levels of c-myc was seen in HG3 cells and in one of the two CLL samples tested. Mcl-1 was only down-regulated in HG3 cells upon rapamycin treatment. Indeed, both Mcl-1 and c-myc are known to be regulated by eIF-4E and cap dependent translation (Benedetti & Graff 2004; Huo et al. 2011). Treatment with PMA resulted in an increase of Mcl-1 and c-myc in both HG3 cells and primary CLL cells. An increase in ERK activity was also seen in primary CLL samples that were analysed. Of note, ERK can also phosphorylate S6 kinases under specific conditions (Pende et al. 2004). These data suggest that inactivation of 4EBP1 through other PKCs or ERK could result in an increased translation or an accumulation of these proteins. A reduction in cyclin D1 protein was seen in one of the two CLL samples analysed after treatment with PMA. Further studies in primary CLL samples are required to determine whether different patients differ in cyclin D1 expression, possibly correlated with prognostic data, and whether cyclin D1 expression is regulated through mTOR and PKC signaling.
Figure 4.2 PKCa-KR cells are larger than their MIEV counterparts.  
A Cells were cultured as described previously until day 13 of OP9:FL co-culture and subsequently photographed with an inverted light microscope under 10x magnification.  
B Unstained cells were analysed by FACS, live-gated on FSC/SSC, and representative histograms were overlaid.
Figure 4.3 PKCα-KR cells express higher levels of CD98, CD71 and pP70 S6 Kinase indicative of mTOR activation.

A PKCα-KR and MIEV cells were incubated with CD98 and CD71 antibody and analyzed by FACS to determine levels of surface protein expression at day 13 (PKCα-KR late culture was day 18). Cells were first gated FSC/SSC, CD45+, GFP+ and CD19+ prior to CD98 and CD71 analysis. B Protein lysates were prepared from early, mid and late PKCα-KR and MIEV cultures prior to Western blot analysis of pP70 S6 Kinase and pERK. GAPDH was used as a loading control.
Figure 4.4 Treatment with mTORC1 inhibitor rapamycin does not cause preferential induction of apoptosis in PKCα-KR cultures compared to their MIEV counterparts.

Cells were harvested from MIEV and PKCα-KR cultures and treated with increasing concentrations of mTORC1 inhibitor rapamycin +/- OP9 for 24 hr (A) and 48 hr (B). Apoptosis was assessed by Annexin V/7AAD staining. Data are represented as means (± SEM) of 3 biological replicates. The graphs are represented as percentage of no drug control (NDC). p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, ** p<0.005, ***p<0.001).
Figure 4.5 Treatment with mTORC1 inhibitor rapamycin causes a significant attenuation of proliferation in PKCα-KR cultures.

Cells were harvested from MIEV and PKCα-KR cultures and treated with increasing concentrations of mTORC1 inhibitor rapamycin for 24 hr (A) and 48 hr (B). Cells were incubated with BrdU for 2 hr prior to the endpoint. Data are represented as means (± SEM) of 3 biological replicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001).
Figure 4.6 A higher percentage of cells are undergoing cell division in PKCα-KR cultures.

A MIEV and PKCα-KR cells were cultured in medium alone, with OP9 stroma alone, or with OP9 stroma in the presence of cytokine IL7. Cells were stained with PI as described before and assessed by FACS. For percentage of cells in G₀/G₁, S and G₂/M phases, the sub-G₀ population was gated out prior to analysis. Data are represented as means (± SEM) of 3 biological replicates. p values were generated using the student’s unpaired t-test to compare groups (*p<0.05, **p<0.005, ***p<0.001). B Cells were harvested from MIEV and PKCα-KR cultures and incubated with BrdU for 2 hr. Error bars represent S.D. of technical triplicates.
Figure 4.7 PKCα-KR cells express higher levels of cyclin D1 transcripts and protein in vitro compared to MIEV counterparts.

A RNA was isolated from MIEV and PKCα-KR cultures and subjected to qRT-PCR to determine levels of cyclin D1 mRNA at early (day 6), mid (day 10) and late (day 17) in vitro cultures. B qRT-PCR analysis of cyclin D1 transcript levels in spleen, tumour and B.M. of PKCα-KR injected Rag 1^-/- mouse. C Protein lysates were prepared from early (day 6) mid (day 13) and late (day 26) MIEV and PKCα-KR cultures to determine levels of cyclin D1 protein by Western blot. D qRT-PCR analysis of Sox2 mRNA in early (day 3, 6), mid (day 13, 14) and late (day 28) cultures. For all qRT-PCR graphs, error bars represent S.D. of technical triplicates. B and T cells isolated from wild type spleen were used as an additional control.
Figure 4.8 Cyclin D1 protein is found within B cell follicles of spleens of PKCα-KR HPC-injected mice.

Consecutive paraffin embedded spleen sections from MIEV and PKCα-KR injected mice were stained for B220 and cyclin D1 respectively.
Figure 4.9 Over-expression of cyclin D1 in HPCs does not result in an expansion of GFP+ cells.

Full length cyclin D1 was cloned into MIEV retroviral backbone and packaging lines were generated as described in (see section 2.1.1). FL HPCs were transduced with cyclin D1 and co-cultured with OP9 and cytokines in a B cell generating system and subjected to FACS at different stages of culture (day 1, 14, 17, 22). Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and percentages of GFP+ cells are shown.
Figure 4.10 Overexpression of cyclin D1 in HPCs results in generation of CLL surface phenotype.

HPCs were retrovirally transduced to express MIEV, PKCa-KR or cyclin D1 and subjected to FACS analysis at day 14 of FL:OP9 co-culture to determine expression of CD19, CD5 and CD23. Haematopoietic (CD45+) cells were live and size gated (FSC vs SSC) and CD19 vs CD23, and CD19 vs CD5 analysis shown. For cyclin D1 transduced cells, GFP+ and GFP− populations are shown.
Figure 4.11 Cyclin D1 is regulated through 4EBP1 in PKCα-KR cultures in a mTORC1 and PKC-dependent manner.

Western blot of day 14 MIEV and PKCα-KR cultures treated with 30 nM rapamycin (rapa), 100 nM PMA plus 1 µg/ml ionomycin (PMA/Iono), or 2.5 µM Okadaic Acid (O.A) for 1 hr. β tubulin was used as loading control.
Figure 4.12 Phosphorylation of 4EBP1 is regulated by mTORC1, whereas PKC stimulation leads to upregulation of Mcl-1, pP70 S6 Kinase and c-myc in CLL cell line HG3.

Western blot of HG3 cell line cultured with NTL mouse fibroblasts for 44 hr followed by treatment with 30 nM rapamycin (rapa) or 100 nM PMA plus 1 µg/ml ionomycin (PMA/Iono) for an additional 4 hr. β tubulin and actin were used as loading controls.
Figure 4.13 4EBP1 phosphorylation is sensitive to rapamycin treatment in human CLL, and PMA stimulation leads to upregulation of Mcl-1 and c-myc.

Western blot of primary CLL sample cultured on NTL cells for 44 hr followed by treatment with 30 nM rapamycin (rapa) or 100 nM PMA plus 1 µg/ml ionomycin (PMA/Iono) for an additional 4 hr. β tubulin was used as loading control.
Appendix 4-A Co-culture of human CLL cells with stroma upregulates cyclin D1 mRNA and protein.

Cyclin D1 mRNA (left) and protein (right) expression of a primary CLL sample cultured in *in vitro* with NTL cells or NTL-CD154 cells in the presence of IL4. This experiment was conducted by Dr. Emilio Cosimo.
Appendix 4-B Cyclin D1 is expressed in human lymph node of CLL patients. Paraffin embedded lymph node biopsies from CLL patients were stained with cyclin D1. Two separate patients are shown. Out of 7 tested, 3 were positive for cyclin D1. Isotype control is also depicted. This experiment was conducted by Dr. Mark Catherwood (Belfast City Hospital).
Chapter 5:

PKCα-KR B cells exhibit ability to lineage switch to T cells upon Notch-ligation
5.1 Introduction

5.1.1 Plasticity of pluripotent HSCs

Haematopoiesis is normally a highly regulated process whereby an HSC subsequently commits toward a myeloid or a lymphoid lineage in a hierarchical manner, however lineage plasticity, although rare, is not impossible. Cells can undergo transdifferentiation whereby the lineage-specific gene program of a cell is replaced by an alternate lineage-specific program. Alternatively, differentiated cells can primarily dedifferentiate into a more primitive state and proceed to redifferentiate into another lineage (Figure 5.1) (Greer 2005).

![Diagram of transdifferentiation, dedifferentiation, and redifferentiation]

**Figure 5.1 Transdifferentiation, dedifferentiation and redifferentiation**

Although ordered B cell development was thought of as unidirectional, more recently, attention has been brought to the apparent plasticity of B cells. Indeed, introduction of a nucleus from a fully differentiated B cell into an enucleated embryonic stem cell gave rise to a broad spectrum of cell lineages (Gurdon et al. 1975; Hochedlinger & Jaenisch 2002). Similarly, reprogramming of differentiated B cell into macrophages through inhibition of PAX5 activity and subsequent downregulation of CD19 can occur when expression of C/EBPα and C/EBPβ is
enforced (Xie et al. 2004). Also raf/ras oncogenes and activated M-CSF receptor are capable of reprogramming oncogene-immortalized B cell lines into macrophages (Klinken et al. 1988; Borzillo et al. 1990). Therefore, although in the most probable scenario, the developmental pathway of an HSC to a fully functional, antigen-reactive B cell is usually directional, nevertheless it is incorrect to say that it is irreversible (Honjo & Neuberger 2004). Indeed, redifferentiation can be induced by external stimuli or increases/decreases in levels of specific transcription factors that regulate cell fate (Honjo & Neuberger 2004).

As mentioned previously (Section 1.1.4), PAX5 is a master B cell regulator that is present in all stages of B cell development apart from plasma cells. Of note, PAX5\(^{-}\) pro-B cells are very sensitive to environmental stimuli and their fate is heavily determined by cytokines and stroma. For example, co-culture of PAX5\(^{-}\) pro-B cells with stroma and IL7 retains their pro-B state. Alternatively, removal of stroma and co-culture with alternative cytokines promotes differentiation of PAX5\(^{-}\) pro-B cells into other lineages, such as NK cells, macrophages, dendritic cells, osteoclasts or granulocytes (Nutt et al. 1999; Schaniel et al. 2002; Carotta et al. 2006). Specifically, addition of IL15 causes a de-differentiation into the NK lineage (Carotta et al. 2006), whilst co-culture with M-CSF induces macrophage differentiation (Nutt et al. 1999). The newly formed alternate fate cells (NK or myeloid) still carry immunoglobulin DJ rearrangements reminiscent of their pro-B cell origin. In addition, in vivo adoptive transfer of PAX5\(^{-}\) cells into RAG2\(^{-}\) mice causes de-differentiation in CD8\(^{-}\) or CD8\(^{+}\) dendritic cells. Normal T cell development is also observed in the thymus and periphery (Rolink et al, 1999). Later analysis of these mice demonstrated the additional presence of myeloid cells and erythrocytes (Schaniel et al. 2002). Importantly, these PAX5\(^{-}\) pro-B cells display self-renewal and long-term reconstitution potential whereby they travel to the BM prior to re-differentiating again (Schaniel et al. 2002). Therefore deletion of PAX5 allows for the generation of cells that closely resemble pHSCs.

### 5.1.2 T cell development

T lymphopoiesis is characterised by a series of commitment events such as TCR V(D)J gene rearrangement (see Section 5.1.2.1), TCR\(\beta\) selection and positive and negative selection of thymocytes (Ciofani & Zúñiga-Pflücker 2007). These events are coupled by the ordered expression of specific phenotypic markers, namely CD4 and CD8 co-receptors. BM derived HSCs within the thymus develop into immature thymocytes which are CD4\(^{-}\)CD8\(^{-}\) (DN) (Figure 5.2A). These DN cells
are further characterised into four developmental stages that are present within the outer thymic cortex (DN1, DN2, DN3 and DN4) based on the surface expression of CD117 and CD25: CD117⁺CD25⁻(DN1), CD117⁺CD25⁺(DN2), CD117⁺CD25⁺(CD3) and CD117⁺CD25⁻(DN4) (Ciofani & Zúñiga-Pflücker 2007) (Figure 5.2B). At DN3, thymocytes rearrange their TCRβ and thus commit toward the T cell lineage. β-selection allows for a functionally rearranged TCRβ to complexes with pTα and form the pre-TCR (Kearse 2000). A dysfunctional TCRβ rearrangement results in apoptosis. Signaling via the pre-TCR complex leads to proliferation and transition into the DP stage (CD4⁺CD8⁺) which is followed by selection toward the SP (CD4⁺ or CD8⁺) stage (Ciofani & Zúñiga-Pflücker 2007). The DP stage also marks the initiation of TCRα rearrangement. CD3ε is also important at this stage in that it forms a complex with the TCR which is important in intracellular signal-transduction (Gold et al. 1986). A complete αβ-TCR/CD3 complex in DP thymocytes induces positive and negative selection resulting in mature SP thymocytes. Here, the second checkpoint occurs whereby recognition by DP cells of major histocompatibility complex (MHC) class I molecules leads to CD8 committed SP cells and recognition of MHC class II molecules leads to CD4 committed SP cells within the inner cortex of the thymus (Figure 5.2A)(Kearse 2000). Thymocytes that improperly rearrange TCRα or generate TCRα/β complex with high self-MHC affinity are negatively selected for resulting in apoptosis. Positively selected T cells can now move out of the thymic medulla and migrate into the periphery.
Figure 5.2 T cell development

A T cell development within the thymus. B Expression of surface molecules during T cell development; CD117⁺CD25⁻(DN1), CD117⁺CD25⁺(DN2), CD117⁻CD25⁺(CD3) and CD117⁻CD25⁻(DN4).

5.1.2.1 TCR rearrangement

The α and β chains of the TCR consist of variable (V) amino-terminal and constant (C) regions. Similarly to lights chains of IgM, TCRα contains V and joining (J) gene segments (V_α, J_α), whilst TCRβ contains V, J and D gene segments (V_β, J_β, D) (K. P. Murphy et al. 2008). TCR_α is located on chromosome 14 and consists of leader sequence (L) followed by 70-80 V_α segments (Figure 5.3). 61 J_α segments
are located a distance away from $V_\alpha$ segments followed by one $C$ gene. TCR$\beta$ is located on chromosome 7 and contains an $L$ sequence followed by 52 functional $V_\beta$ segments, and two distal clusters of 6 or 7 $J_\beta$ segments, each preceded with a separate $D_\beta$ segment and followed by a separate $C$ segment (Figure 5.3) (K. P. Murphy et al. 2008).

![Diagram of TCRα and TCRβ loci](image)

**Figure 5.3 Germline organization of TCRα and TCRβ loci**

During T cell development TCR gene segments rearrange in the thymus to form complete V-domain exons. For TCRα chain generation, $V_\alpha$ rearranges to $J_\alpha$ forming $VJ_\alpha$ (Figure 5.4). Transcription and subsequent splicing of $VJ_\alpha$ to $C_\alpha$ generates mRNA that is subsequently translated to TCRα protein. For TCRβ chain generation, similarly to IgM$\text{H}$, rearrangement of $V_\beta$, $D_\beta$, and $J_\beta$ forms a functional $VDJ_\beta$ V-region exon (Murphy et al. 2008). Transcription and splicing of $VDJ_\beta$ to $C_\beta$ generates mRNA that is subsequently translated to TCRβ protein. The two chains, $\alpha$ and $\beta$ join to form a $\alpha;\beta$ TCR heterodimer (Figure 5.4).
The key difference between IgM genes and genes encoding the TCR is effector function. In B cells (see Chapter 1), secreted antibodies’ heavy chain C region isotypes trigger an effector mechanism, whereas in T cells cell-cell contact and not the TCR directly mediates effector mechanisms (Murphy et al. 2008). The TCR’s main role is antigen recognition, and thus the C regions of the α and β TCR loci are less complex than IgM H.

5.1.2.2 Role of Notch in T cell commitment

T cell development is regulated via transcription factors such as GATA3, TCF1 and Notch downstream transcription regulator Deltex (Joulin et al. 1991; Matsuno et al. 1998; Liu & Lai 2005; Weber et al. 2011). Notch proteins belong to a family of type I transmembrane receptors that are involved in regulating cell fate (Deftos & Bevan 2000). Notch signaling involves the interaction between one of five Notch ligands of the Jagged (1, 2) or Delta-like (1, 3, 4) families and one of four Notch receptors (1-4) (Sandy et al. 2012). When ligand binds receptor an S2 cleavage site is exposed to a metalloprotease which cleaves Notch within or in high proximity to its transmembrane domain. An intra-membrane protease complex, or the Y-secretase complex conducts the final cleavage of Notch, releasing its intracellular domain to translocate to the nucleus allowing for interaction with the CBF1 transcription factor changing it from a repressor to an activator of gene expression.
transcription (of for example Hes1, Deltex and Notch1). In T cell development, deletion of Notch1 in BM stem cells (BMsc)s or newborn mice results in a block in T cell development and an upregulation of B cell markers on CD25^-CD44^+CD4^-CD8^- (DN1) thymic cells, highlighting that Notch signaling is necessary for commitment toward the T cell fate (Tomita et al. 1999), and lack of Notch signals results in differentiation to a B cell lineage. Introduction of BMscs expressing constitutively active Notch1 into irradiated hosts gives rise to CD4^+, CD8^+ and Thy-1^+ thymus-independent T cells, whilst no B cells were observed (Pui et al. 1999). Therefore Notch signaling plays a crucial role in T or B cell lineage commitment. In addition to its role in T cell commitment, Notch1 is important in later stages of T cell development such as αβ T cell commitment versus Υδ T cell commitment (Washburn et al. 1997), and in maturation of CD4^+ and CD8^+ SP thymocytes (Deftos et al. 1998).

5.1.2.3 In vitro system to assess T cell development

Zúñiga-Pflücker and colleagues developed an in vitro T cell system using OP9-DL1 cells. OP9-DL1 cells are OP9 cells that ectopically express the Notch ligand DL1, thus losing the ability to support B cell lymphopoiesis and acquiring the ability to support T cell lymphopoiesis (Schmitt & Zúñiga-Pflücker 2002). Within a B cell environment, subversion of PKCα signaling in HPCs resulted in transformed B cells that resemble human CLL cells (see Chapter 3; Nakagawa et al. 2006). Because lineage switching has been implicated in a number of B cell malignancies (Mikkola et al. 2002; Cobaleda et al. 2007), we were therefore interested in investigating whether PKCα-KR transformed B cells were capable of lineage trans-differentiation.

5.2 Aims and Objectives:

In order to assess whether PKCα-KR transduced B cells have the potential to lineage switch, the specific aims of this chapter were to:

i. Compare the expression of B cell specific genes between the MIEV and PKCα-KR B cells;

ii. Assess the ability of MIEV and PKCα-KR B cells to differentiate into other lineages upon Notch ligation by co-culture with OP9-DL1;
iii. Investigate whether B and T cell specific genes are differently expressed between the MIEV and PKCa-KR cultures upon Notch ligation and assess implicated molecular mechanisms.

5.3 Results

5.3.1 PKCa-KR B cells express B cell specific genes at higher levels than MIEV counterparts

In human CLL B cells, CD19 surface expression is diminished as compared to normal B cells (Ginaldi et al. 1998; Cabezudo et al. 1999; Yang et al. 2005). In contrast, one of the early notable features of PKCa-KR transduced B cells was the higher expression of surface marker CD19 (Figure 3.3 and Figure 5.5A) compared to MIEV control. Hence it was of interest to determine whether other B cell specific genes are upregulated within the PKCa-KR expressing cells, particularly in vivo because of the importance of microenvironmental interactions for B cell development. Therefore MIEV and PKCa-KR transduced HPCs were injected into neonatal RAG1-/- mice and spleens were excised 4 weeks post injection. Lymphocytes were isolated from the spleen and sorted for GFP+Lin-CD45+CD19+ B cells. Similarly B and T cells were sorted from wildtype mice to be used as additional controls. Analysis of B cell specific genes PAX5, EBF1, CD79a and T cell specific gene CD3ε was conducted with qRT-PCR and revealed that PKCa-KR cells express higher amounts of B cell specific genes PAX5, EBF1 and CD79a. As expected, only the T cells expressed CD3ε (Figure 5.5B).

5.3.2 PKCa-KR B cells have the ability to lineage switch

In order to determine whether PKCa-KR B cells have the potential to lineage switch as seen in other malignancies, MIEV and PKCa-KR transduced HSCs were isolated by cell sorting (GFP+Lin-CD45+B220-CD19-CD117hiSca1hi) and co-cultured on OP9 stroma. Thereafter, the late pro-B cell population was sorted (GFP+Lin-CD45+B220+CD19+CD117+) and subsequently co-cultured with OP9-DL1 cells that ectopically express Notch ligand DL1, and thus support T cell development (Schmitt & Zúñiga-Pflücker 2002). Unlike in MIEV-expressing CD19+ cells, co-culture of PKCa-KR-expressing CD19+ cells with OP9-DL1 resulted in the generation of cells expressing markers indicative of alternative lineages (Figure 5.6). FACS analysis of CD19, CD11b, NK1.1, CD4 and CD8 surface markers demonstrates that whereas <0.2% of MIEV cultures expressed macrophage marker CD11b, >1% of PKCa-KR cultures expressed the myeloid marker (Figure
5.6) In addition 2.7% of cells were CD19^-CD11b^- within the PKCα-KR cultures as compared to 0.8% in MIEV cultures, indicating lineage switching to other cell types. When NK cell marker NK1.1 expression was assessed, MIEV cultures contained <0.1% NK1.1^+ cells compared to 3.6% within the PKCα-KR cultures. FACS analysis of CD8 and CD4 revealed that 2.4% of PKCα-KR cells were SP CD8^+, 3.7% CD4^+ and 1.2% DP compared to no CD4 nor CD8 positive cells within the MIEV cultures. Collectively these data indicate that CD19^+ PKCα-KR cells are capable of lineage switching upon Notch ligation to macrophages, NK cells and to T cells.

5.3.3 CD19^+ PKCα-KR lineage switching cells aberrantly express T cell specific genes

Since it was demonstrated that PKCα-KR expressing B cells have the ability to lineage switch to T cells by surface marker expression analysis, we aimed to determine whether CD19^-CD4^-CD8^- T cells generated from OP9-DL1 co-cultures of CD19^+ PKCα-KR cells express T cell specific genes. Therefore CD19^-Lin^- progenitor MIEV cells and CD19^-Lin^- PKCα-KR B cells were sorted from OP9 co-cultures and subsequently co-cultured with OP9-DL1. The MIEV CD19^-Lin^- cells developed into T cells (CD4^-CD8^-) as expected, and also into B cells (CD19^-NK1.1^-), indicating that the sorted CD19^- progenitors were already primed toward the B cell lineage (Figure 5.7 left). Within the PKCα-KR sorted CD19^-Lin^- cultures, some cells retained CD19 expression whilst others switched into T cells (Figure 5.7 left). CD8^-CD4^-CD8^- T cells and CD19^-NK1.1^- B cells were subsequently sorted from the cultures using the indicated gates (Figure 5.7 left) and subjected to qRT-PCR in order to determine the expression of T cell specific genes (pTα, CD3ε), and B cell specific gene PAX5 (Figure 5.7 right). Within the MIEV cultures, the sorted T cells expressed T cell specific genes (pTα, CD3ε) and GATA3, whereas they did not express PAX5, whilst the sorted B cells expressed PAX5 and not the T cell specific genes as expected. Similarly, the lineage switched B cells (now T cells) within the PKCα-KR cultures expressed T cell specific genes, although at lower levels than T cells generated from progenitor cells within MIEV cultures (Figure 5.7 right), and did not express PAX5. Interestingly the cells that remained CD19^+ within the PKCα-KR cultures expressed low levels of T cell specific genes (Figure 5.7 right). Collectively these data demonstrate the potential of CD19^+ PKCα-KR expressing cells to lineage switch into T cells upon Notch
ligation as marked by T cell specific surface protein expression and upregulation of T cell specific genes.

5.3.4 Lineage switching of PKCα-KR CD19+ B cells occurs gradually via a transitional CD19*CD90+ stage

Since it was demonstrated that PKCα-KR expressing CD19+ B cells were capable of lineage switching into T cells upon Notch ligation, we aimed to determine when this switch occurs. Therefore retrovirally transduced MIEV and PKCα-KR HSCs were cultured on OP9 for 9 days and afterward sorted to isolate the pro-B cell population (GFP+CD19*B220*CD117*Lin-) which was subsequently cultured on OP9-DL1 stroma for an additional 10 days. Surface expression of B cell marker CD19 and T cell marker CD90 revealed a large CD19+CD90+ population within PKCα-KR cultures, indicating that lineage switching of CD19+CD90- cells toward the T cell fate (CD19*CD90+) happens through a transitional CD19*CD90+ stage (Figure 5.8). In fact, whereas the majority of sorted ProB cells within the MIEV cultures remained CD19+, 11% of sorted ProB cells within the PKCα-KR cultures expressed both CD19 and CD90 (compared to <2% within the MIEV cultures). In addition, as previously demonstrated 1.5% of sorted ProB cells within the PKCα-KR culture lineage switched into T cells as demonstrated by loss of CD19 and upregulation of CD90.

Previously it was demonstrated that PKCα-KR lineage switched B cells (now T cells) expressed T cells specific genes (Section 5.3.3). Therefore it was of interest to determine whether the upregulation of these genes occurred sequentially, in line with the transitional stage of CD19*CD90+ cells within the PKCα-KR lineage switching cultures. Therefore, CD19*CD90+, CD19*CD90+ MIEV and PKCα-KR expressing cells and CD19*CD90+ PKCα-KR cells were isolated from OP9-DL1 cultures (post 9 day OP9 culture) as indicated in Figure 5.8, and analysed by qRT-PCR to determine the expression of B cell specific genes (E2A, EBF1 and PAX5) and T cell specific genes (CD3ε, TCF1, Deltex and pTα). PKCα-KR expressing CD19*CD90+ cells expressed B cell specific genes E2A, EBF1 and PAX5 at higher levels than their MIEV CD19*CD90- counterparts (Figure 5.9), similar to that noted in Figure 5.5. The transitional CD19*CD90+ stage in both MIEV and PKCα-KR culture was marked by the decrease in B cell specific genes E2A, EBF1 and PAX5 (significant for E2A within MIEV cultures and for E2A and PAX5 within PKCα-KR cultures). The CD19*CD90+ population within the PKCα-KR expressing cells exhibit a further significant decrease in all three B cell specific genes investigated.
The sequential downregulation of B cell specific genes within the PKCα-KR transitioning cultures (CD19⁺CD90⁻→CD19⁺CD90⁺→CD19⁻CD90⁺) is coupled with a sequential upregulation of T cell specific genes CD3ε, TCF1, Deltex and pTα indicating that expression of PKCα-KR allows the CD19⁺ B cells to become sensitive to Notch signaling through DL1 ligation.

5.3.5 PKCα-KR transdifferentiated B cells (now T cells) maintain rearrangements of antibody producing Ig genes

Although it was demonstrated that CD19⁺ PKCα-KR-expressing B cells can undergo lineage switching as a result of Notch ligation through analysis of surface markers and T-lineage associated genes, to demonstrate that these T cells originated from B cells, IgH rearrangement was investigated within these cells. Therefore, CD19⁺CD90⁻, CD19⁺CD90⁺ MIEV and PKCα-KR cells and CD19⁺CD90⁺ PKCα-KR cells were sorted from OP9-DL1 cultures (after OP9 cultures as described in 5.3.4) and subjected to genomic PCR to determine D-JH and V-DJH rearrangements (indicative of B cell origin) and TCRβ rearrangement at the D-Jβ level (indicative of T cell origin). The analysis revealed that the CD19⁺CD90⁻ and CD19⁺CD90⁺ populations within both MIEV and PKCα-KR cultures had completed IgH gene rearrangement at the D-JH and V-DJH level (Figure 5.10) as expected. CD19⁺CD90⁺ PKCα-KR cells also rearranged TCRβ at the D-Jβ level confirming that they are T cells. However D-JH and V-DJH gene rearrangement was also present in the lineage switched CD19⁺CD90⁺ PKCα-KR cells. Collectively these data confirm that the lineage switched T cells had a B cell origin. Notably, no D-Jβ rearrangement occurred within the PKCα-KR CD19⁺CD90⁺ transitioning population suggesting that although these cells possess potential to convert into T cells, they have not yet fully committed to the T cell lineage.

5.3.6 Lineage switching from B cells to T cells occurs by E2A and PAX5 downregulation

Since it was demonstrated that the gradual lineage switching from B cells into T cells occurs via a downregulation of B cell specific genes and upregulation of T cell specific genes at the transcript level (Figure 5.9), it was of interest to determine if the same occurs at a functional (protein) level. Therefore, CD19⁺CD90⁻, CD19⁺CD90⁺ MIEV and PKCα-KR cells and CD19⁺CD90⁺ PKCα-KR cells were sorted from OP9-DL1 cultures (after OP9 co-cultures as described in 5.3.4) and subjected to western blot analysis to determine protein levels of E2A
protein product E47 and its downstream target PAX5, and T-cell specific protein Lck (Figure 5.11 and Appendix 5-A). Within the PKCα-KR cultures levels of PAX5 and E47 protein are decreased in the CD19^CD90^ lineage switched cultures, whilst levels of Lck are increased. Total levels of ERK and β tubulin remained constant in all MIEV and PKCα-KR cultures (Figure 5.11; Appendix 5-A).

5.3.7 ERK signaling is upregulated as B cells transition into T cells within the PKCα-KR cultures

Since it was demonstrated that lineage switching within the PKCα-KR cultures occurred via a downregulation of E2A, it was of interest to determine the signaling pathways implicated in this phenomenon. E2A proteins are known to be negatively regulated by inhibitor of differentiation (Id) protein family members (Kee 2009). Id2 and Id3 expression was analysed within the cultures to determine levels of their transcripts. Analysis shows that levels of Id2 are upregulated and levels of Id3 are downregulated within the lineage switched PKCα-KR cultures (Figure 5.12).

Since ERK-MAPK signaling has also been shown to regulate E2A gene products in T and B cell progenitors (Bain et al. 1994; King et al. 2007), pMEK and pERK levels were investigated in PKCα-KR lineage switched cultures. Western blot analysis of CD19^CD90^ MIEV and CD19^CD90^, CD19^CD90^ and CD19^CD90^ PKCα-KR cells sorted from OP9-DL1 cultures (after OP9 co-cultures as described in 5.3.4) revealed an upregulation in MEK/ERK-MAPK signaling cascade within the lineage switching cultures, whilst total ERK and MEK mirrored the loading controls (Figure 5.13).

5.4 Discussion

The idea that commitment toward a mature cell type is unidirectional and irreversible can no longer be considered a dogma. Recent studies suggest that committed B cells can reverse to other lineages (Cobaleda et al. 2007; Mikkola et al. 2002; Nakagawa et al. 2012). One study demonstrates that PAX5 inactivation gives differentiated B cells the ability to de-differentiate, and ultimately become macrophages in vitro or T cells in vivo (Mikkola et al. 2002). Another study demonstrated that conditional PAX5 deletion in mature B cells of mice allowed for de-differentiation into uncommitted progenitors in vivo and subsequent differentiation into T-cells within the thymus (Cobaleda et al. 2007). Mice with PAX5 deleted B cells also developed aggressive progenitor cell lymphomas (Cobaleda et al. 2007).
Similarly, we show here that PKCα-KR cells display the ability of lineage plasticity, whereby fully differentiated B cells can reverse their lineage into T cells via a downregulation of B cell specific genes (CD19, PAX5, EBF1, E2A) and an upregulation of T cell specific genes (pTα, CD3ε, TCF1, Deltex).

Upon notch ligation, PKCα-KR CD19+ cells can switch to macrophages, NK cells and T cells demonstrated by surface marker analysis. In order to determine whether the lineage switched CD19+ PKCα-KR expressing cells expressed T cell specific genes, mRNA from sorted B and T cells from MIEV progenitor cultures on OP9-DL1 and CD19+ PKCα-KR cultures on OP9-DL1 was analyzed by qRT-PCR. In fact, the T cells generated from CD19+ PKCα-KR cells expressed T cell specific genes pTα, CD3ε and upregulated GATA3. By co-staining with CD19 and CD90 (a commonly used T cell marker expressed on mouse thymocytes and peripheral T cells), we demonstrated that CD19+ B cells within notch-ligated PKCα-KR cultures go through a transitional CD19+CD90+ stage before fully losing expression of CD19. The transition from B cells into T cells is marked by the decrease in B cell specific genes E2A, EBF1 and PAX5, and an increase in T cell specific genes CD3ε, TCF1, Deltex and pTα at transcript levels. Importantly lineage switched PKCα-KR B cells (now T cells) maintain rearrangements of Ig genes indicative of their origin, whilst also demonstrating evidence of TCRβ rearrangement at the D-Jβ level. However, PKCα-KR CD19+CD90+ cells show no TCRβ rearrangement, suggesting that although these cells have the potential of lineage converting, they have not entirely committed to the T cell lineage or that PAX5 is still inhibiting rearrangement of the TCRβ gene.

We demonstrated that lineage conversion from B cells to T cells within the PKCα-KR cultures upon Notch ligation occurs via degradation of E2A isoform E47 at the protein level. This is not surprising because although E2A is required in the early stages of T cell development, its activity is subsequently downregulated as thymocytes mature (Barndt et al. 1999; Gretchen Bain et al. 1999; Engel et al. 2001; Pan et al. 2002; Taghon et al. 2006; Jones & Zhuang 2007). E2A degradation is coupled with PAX5 protein downregulation and upregulation of the protein tyrosine kinase Lck. Lck is important in the DN2-DN3 stage of T cell development (Buckland et al. 2000). The degradation of E2A could be a result of the increase in ERK-MAPK signaling activation within the PKCα-KR B cells as they transition into T cells in comparison to the control MIEV cells. In fact, in T and B cell progenitors, ERK-MAPK signaling can regulate E2A gene products (Bain et al.
E proteins, like E2A can also interact with antagonistic helix-loop-helix Id proteins that when activated inactivate E proteins’ DNA binding activity (Benezra et al. 1990; Kee 2009). Id protein expression is diminished as a B cell matures, correlating to Ig enhancer function (Saisanit & Sun 1995). Id over-expression \textit{in vivo} was found to repress Ig enhancers implicating their inhibitory role in B cell development (Wilson et al. 1991). Within the PKC\(\alpha\)-KR lineage switched cultures, Id2 expression was upregulated in the CD19\(^+\)CD90\(^+\) cells while absent in the B lineage cells. However, Id3 expression was reduced within the PKC\(\alpha\)-KR CD19\(^+\)CD90\(^+\) cells compared with the CD19\(^+\) cells. This is surprising because others have shown that Id3 expression is activated in thymocytes by ERK-MAPK signaling which in turn results in a decrease in E2A’s DNA binding activity (Bain 2001). In addition Lck activity was shown to be able to induce Id3 expression and decrease E12/E47 DNA binding activity (Bain et al. 2001).

Collectively these data indicate that committed CD19\(^+\) cells within the PKC\(\alpha\)-KR cultures are capable of lineage switching upon Notch ligation, by modulating E2A function. The lineage switched T cells expressed T cells specific surface markers, T cell specific genes and carried TCR\(\beta\) gene rearrangements. In addition, these lineage switched cells carried Ig\(_{\mu}\) gene rearrangements indicating their B cell origin.
Figure 5.5 PKCα-KR cells express B cell genes at higher levels than their control counterparts

A MIEV and PKCα-KR transduced HPCs were co-cultured with OP9 as previously described and stained with CD45 and CD19. Haematopoietic (CD45+) cells were live and size gated (FCS vs SSC) and an over-lapping histogram of CD19 expression is displayed. B Neonatal Rag1−/− mice were injected with MIEV or PKCα-KR transduced HPCs and spleens were excised 4 weeks post injection. Lin−CD19+GFP+ haematopoietic (CD45+) cells were sorted from spleens and subjected to qRT-PCR to determine relative levels of B cell specific genes PAX5, EBF1 and CD79a and T cell gene CD3ε. Data are expressed as means of technical triplicates ± SD and are representative of (>3) biological replicates. Sorted B and T cells obtained from wildtype ICR mice were used as controls.
Sorted MIEV and PKCα-KR transduced HSC cells (GFP^+Lin^-CD45^-B220^-CD19^-CD117^{hi}Sca1^{hi}) were cultured on OP9 for 9 days. Pro-B cells were then sorted from these cultures (GFP^+CD19^-B220^-CD117^{hi}Lin^-), subsequently cultured on OP9-DL1 for an additional 9 days and analysed for expression of CD19, CD11b, NK1.1, CD4 and CD8 surface markers.

Figure 5.6 Co-culture of PKCα-KR CD19^+ B cells with OP9-DL1 promotes differentiation into other lineages
Figure 5.7 CD19\(^+\)PKC\(\alpha\)-KR cells express T cell specific markers

MIEV and PKC\(\alpha\)-KR transduced HSCs were co-cultured with OP9 for 9 days as previously described. CD19\(^-\)Lin\(^-\) MIEV cells and CD19\(^+\)Lin\(^-\)PKC\(\alpha\)-KR cells were sorted from these cultures and subsequently co-cultured with OP9-DL1 for an additional 20 days. The resultant CD19\(^+\)B cells and CD4\(^+\)CD8\(^+\) T cells were sorted from the cultures using the gates indicated above and subjected to qRT-PCR to determine transcript levels of T cell specific genes pT\(\alpha\), CD3\(\varepsilon\) and GATA3 and B cell specific gene PAX5. HPRT was used as the housekeeping gene and results are expressed as fold change normalized to T cells. Data are expressed as mean ± SEM of 3 biological replicates. p values were generated using the student’s unpaired t-test (*\(p<0.05\), **\(p<0.005\), ***\(p<0.001\))
Figure 5.8 Lineage switching of PKCα-KR CD19⁺ occurs through a transitional CD19⁺CD90⁺ stage

Retrovirally transduced MIEV and PKCα-KR HSCs were co-cultured with OP9 cells for 9 days, proB cells were sorted and subsequently co-cultured on OP9-DL1 for an additional 10 days. Cells were then sorted according to gates demonstrated above: 1=MIEV CD19⁺CD90⁻; 2=MIEV CD19⁺CD90⁺; 3=PKCα-KR CD19⁺CD90⁻; 4=PKCα-KR CD19⁺CD90⁺; 5=PKCα-KR CD19⁺CD90⁺.
Figure 5.9 Lineage converted PKCα-KR cells downregulate B cell specific genes and upregulate T cell specific genes

RNA was isolated from cells sorted in Figure 5.8 and expression of mRNA was analysed by qRT-PCR using GAPDH/HPRT as the housekeeping genes. 1=MIEV CD19+CD90−; 2=MIEV CD19+CD90+; 3=PKCα-KR CD19+CD90+; 4=PKCα-KR CD19+CD90−; 5=PKCα-KR CD19−CD90+.

Data are expressed as means ± SEM of at least 3 biological replicates. p values were generated using the student’s unpaired t-test (*p<0.05, **p<0.005, ***p<0.001)
Figure 5.10 Lineage converted PKCα-KR B cells carry IgH gene rearrangements

Cells were cultured and sorted as in Figure 5.8 (1=MIEV CD19^+CD90^-; 2=MIEV CD19^+CD90^-; 3=PKCα-KR CD19^+CD90^-; 4=PKCα-KR CD19^+CD90^-; 5=PKCα-KR CD19^+CD90^-) and DNA was analyzed by PCR to determine IgH D-J and V-DJ and TCRβ D-J rearrangement status. β2M was used as the loading control and genomic DNA from wildtype mouse splenocytes was used as the positive control.
Figure 5.11 Lineage converted PKCα-KR B cells downregulate PAX5 and E47 and upregulate Lck

Cells were cultured and sorted as in Figure 5.8 (1=MIEV CD19⁺CD90⁻; 2=MIEV CD19⁺CD90⁺; 3=PKCα-KR CD19⁺CD90⁻; 4=PKCα-KR CD19⁺CD90⁺; 5=PKCα-KR CD19⁺CD90⁺) and protein lysates were analysed by Western blot to determine expression of PAX5, E47, total ERK and Lck. β tubulin was used as the loading control.
Figure 5.12 Lineage conversion in PKCα-KR B cells occurs by Id2 and Id3 modulation

Cells were cultured and sorted as in Figure 5.8 (1=MIEV CD19⁺CD90⁻; 2=MIEV CD19⁺CD90⁺; 3=PKCα-KR CD19⁺CD90⁻; 4=PKCα-KR CD19⁺CD90⁺; 5=PKCα-KR CD19⁻CD90⁺) and RNA was isolated. Transcript levels of Id2 and Id3 were determined by qRT-PCR and expressed as fold change relative to GAPDH and normalized to sample 3 (PKCα-KR CD19⁺CD90⁻ cells). Data are expressed as means ± SEM of at least 3 biological replicates. p values were generated using the student’s unpaired t-test (*p<0.05, **p<0.005, ***p<0.001)
Figure 5.13 PKCα-KR B cells upregulate ERK signaling

Cells were cultured and sorted as in Figure 5.8 (1=MIEV CD19⁺CD90⁻; 2=PKCα-KR CD19⁺CD90⁻; 3=PKCα-KR CD19⁺CD90⁺; 4=PKCα-KR CD19⁺CD90⁺) and protein lysates were analysed by Western blot to determine expression of pERK1/2 and pMEK1/2, ERK and MEK. β tubulin was used as the loading control.
Appendix 5-A Lineage converted PKCα-KR B cells downregulate E47 and PAX5, and upregulate Lck (example 2)

Cells were cultured and sorted as in Figure 5.8 (1=MIEV CD19⁺CD90⁻; 2=MIEV CD19⁺CD90⁺; 3=PKCα-KR CD19⁺CD90⁻; 4=PKCα-KR CD19⁺CD90⁺; 5=PKCα-KR CD19⁺CD90⁺) and protein lysates were analysed by Western blot to determine expression of E47, PAX5, total ERK and Lck. GAPDH was used as the loading control. Quantification of E47 signal strength as a ratio of loading control is shown (right) as mean ± S.D. of 4 biological replicates. p values were generated using the student’s unpaired t-test (*p<0.05).
Chapter 6:

Conclusions
6.1 Concluding Remarks

CLL is a disease marked by the accumulation of malignant B cells that harbour a characteristic phenotype of CD19+CD5+CD23+IgMlo. It is a disease of intraclonal heterogeneity and genetic homogeneity. For example clinical course is highly varied between patient groups whereby two distinctive subgroups of CLL patients exist: those that harbour mutated IgV\textsubscript{H} genes and those that harbour unmutated IgV\textsubscript{H} genes. The mutational status of IgV\textsubscript{H}, alongside surrogate markers ZAP70 and CD38 dictates prognosis, whereby an unmutated IgV\textsubscript{H} and ZAP70 and CD38 positivity indicate a worse overall prognosis. Unmutated IgV\textsubscript{H} CLL cells also have the ability to signal through the BCR at a stronger intensity than mutated CLL cells, thereby amplifying downstream signaling pathways including ERK, NFkB, mTOR, Akt and VEGF, and upregulating anti-apoptotic factors such as the Bcl-2 family.

However, even though two distinct subsets of CLL patients exist with significant heterogeneity in clinical course, there is a remarkable genetic similarity in all CLL patients, implying a homogenous disease, and a common origin to all CLL cells.

The microenvironment plays a key role in the survival and proliferation of CLL cells. In fact, CLL cells spontaneously apoptose ex vivo, but survive and proliferate with addition of stroma and cytokines (Lagneaux et al. 1999; Chiorazzi & Ferrarini 2003). The BM and secondary lymphoid tissues provide a microenvironment whereby CLL cells interact with stromal cells that protect and nurture. NLCs, BMSCs within the microenvironment interact with CLL cells, safeguarding them from drug-induced apoptosis and cytotoxic agents (Burger et al. 2000; Tsukada et al. 2002) and play important roles in chemotaxis. In addition, T cells within the microenvironment have the ability to activate CLL cells through the CD40L, and induce proliferation (Buske et al. 1997; Kitada et al. 1999; Fluckiger et al. 1992). Importantly, it is now evident that CLL is not simply a disease of accumulation, rather, a disease of active proliferation whereby CLL cells proliferate within specific compartments in the BM and LN, thereby causing the expansion of the leukaemic clone and resulting in a more aggressive CLL (Messmer et al. 2005).

Our studies have led to the development of a murine model emulating an aggressive form of CLL. Subversion of PKC\textalpha signaling in HPCs and subsequent in vitro B cell culture has resulted in the generation of B cells that phenotypically resemble CLL by the specific upregulation of surface markers CD19, CD5, CD23 and downregulation of surface IgM. Like umutated CLL, our mCLL cells upregulate
anti-apoptotic protein Bcl-2, are ZAP70+ and express AID which promotes genetic changes through SHM and CSR, collectively leading to a more aggressive disease. Among the signaling pathways upregulated within our CLL model are NFkB-, ERK- and mTOR- mediated pathways.

Injection of PKCα-KR expressing HPCs into RAG1−/− recipients results in development of disease coupled with a shorter lifespan as compared to MIEV control HPC injected mice. In addition, there is evidence of enlarged spleens harbouring disorganized architecture within the PKCα-KR HPC injected mice. An expansion of GFP+CD45+CD19+ cells is evident within the blood, spleen BM and LN of these mice in vivo (there is however, IHC evidence of an expansion of B220+ GFP+ cells in vitro and in vivo implicating that subversion of PKCα signaling can drive HPCs toward other lineages in a micro-environment specific manner). Within such a complex biological system, HPCs can develop into any lineage depending on the environment. Therefore, the expansion of GFP+ cells in vivo implies that subversion of PKCα signaling may be an oncogenic trigger for lineages other than B cells. It would be interesting to downregulate PKCα within particular lineages through transgenic murine models with a lineage-specific promoter dictating deletion of PKCα.

Downregulation of PKCα also results in a sustained upregulation of PKCβII without changes in PKCβI levels. Similarly, in human CLL, PKCβII is overexpressed and enzymatically active (Abrams et al. 2007; Abrams et al. 2010). In addition, PKCβII has been shown to be regulated by and to regulate VEGF aiding in development of the malignant phenotype of CLL (Abrams et al. 2010). In our mouse model VEGF mRNA is upregulated, however no correlative links to PKCβII have been made thus far, and further experiments are necessary to determine whether a relationship between the two exists. One study demonstrates that crossing of PKCβ−/− mice with Eμ-TCL1 mice results in the slowing down of CLL with loss of one PKCβ allele or complete abrogation of the disease with loss of both alleles (Holler et al. 2009). However CD5+ B cells were present in mice lacking PKCβ especially after TCL1 overexpression suggesting that TCL1 can compensate for loss of PKCβ and that PKCβ is not essential of the development of CD5+ B cells that may become malignant. Additionally, some mice within the PKCβ+/− cohorts developed disease, although different from that developed within the Eμ-TCL1 mice (Holler et al. 2009). The authors of this study report significant induction of apoptosis of human CLL cells with PKCβ inhibitor enzastaurin, however CLL cells
were not isolated prior to treatment, rather a mixed population of MNCs was used. On the contrary, Abrams et al found that enzastaurin had little effect on isolated CD19+ CLL cells (Abrams et al 2007). Similarly, in our study treatment with PKCβ inhibitor hispidin or with more selective PKCβ inhibitor enzastaurin even at high concentrations did not induce apoptosis preferentially within the mCLL cultures. However, treatment with hispidin, and more dramatically with enzastaurin induced an attenuation in proliferation of mCLL cells and caused them to enter cell cycle arrest, indicating that PKCβ is important for the expansion of the malignant clone within the proliferation centres.

Importantly, introduction of PKCβII in HPCs and subsequent in vitro B cell coculture resulted in an expansion of GFP+CD19+ B cells over time, however not at the same rate as HPCs harbouring PKCα-KR. In addition, a small population CD19+CD23+ cells (2.75 %) was apparent within the PKCβII cultures, collectively implicating PKCβII as important for the survival and initiation of CLL cells. Also, treatment with enzastaurin in vivo resulted in an increase in apoptosis of GFP+ CLL-like cells and a reduction in spleen size. However, limited biological replication within these experiments due to time limitations disallow for any concrete conclusions to be made.

A key signaling pathway implicated in our mCLL model is mTOR. In fact, PKCα-KR cells upregulate nutrient receptors CD98 and CD71, are larger in size then their MIEV counterparts and begin to upregulate pP70 S6 kinase early on, implicating activation of mTOR as an oncogenic event. However, treatment of mCLL cells with mTORC1 inhibitor rapamycin does not result in dramatic induction of apoptosis, however similarly to PKCβ inhibition, treatment with rapamycin results in attenuation of proliferation of mCLL cells and increase in cell cycle arrest. Therefore mTOR signaling, like PKCβII signaling may be important in the generation of a more aggressive, highly proliferative CLL. Coupled with mTOR activation, is the activation of ERK which has been demonstrated to be capable of phosphorylating S6 kinases under certain conditions (Pende et al. 2004). PKCβ has also been demonstrated to activate ERK (Guo et al. 2008; Lee et al. 2003). ERK signaling is important in cell survival and proliferation (Chuderland & Seger 2005), and can phosphorylate and subsequently activate IKKα resulting in phosphorylation of IκBα, its proteosomal degradation and activation of NFκB (Chen & Lin 2001). Indeed PKCα-KR cells exhibit higher levels of IκBα phosphorylation indicative of NFκB activation.
The oncogenic transformation of PKCα-KR expressing cells is coupled with an upregulation of cyclin D1 mRNA and protein in vitro and in vivo. It is localized within the leukemic B cell areas of the spleen of PKCα-KR injected mice, emulating a proliferation centre within human CLL. Importantly, when human CLL cells are cultured in the presence of stroma and IL4, cyclin D1 is upregulated, implicating it to be important within the microenvironment. In accordance with this finding, we found that some human CLL LN biopsies were positive for cyclin D1. Over-expression of cyclin D1 in HPCs was alone insufficient to generate a leukaemic phenotype as seen with introduction of PKCα-KR. The cells expressing cyclin D1 did not have an apparent survival advantage over untransduced cells within the same culture evident by a decrease in the percentage of GFP⁺ cells, however a small population of cells that expressed cyclin D1 also expressed hallmark CLL markers including CD23 and CD5. This implicates cyclin D1 to be important for the CLL phenotype but not the progressive nature of CLL in the mCLL model.

In mCLL, cyclin D1 was shown to be regulated through translational repressor 4EPB1 that also regulates other key genes such as c-myc and Mcl-1. 4EBP1 was found to be inactive in PKCα-KR cultures and regulated by mTOR, as evidenced by sensitivity of its phosphorylation states in response to rapamycin treatment. Similarly to the findings from mCLL, we found 4EBP1 to be inactive in human CLL, and sensitive to phosphorylation in response to rapamycin, however no relationship with cyclin D1 was established. Treatment with PMA resulted in an increase of Mcl-1 and c-myc in both HG3 cells and primarily CLL cells, coupled with an increase in ERK activity, suggesting inactivation of 4EBP1 through activation of other PKCs, such as PKCβII. This mechanism could be responsible for the accumulation of oncogeneic proteins such as Mcl-1 and c-myc.

Finally, PKCα-KR expressing differentiated B cells are capable of lineage switching into the NK, macrophage and T cell lineage upon Notch ligation. We focused on their ability to switch into T cells and show that the transition of B to T cell occurs via degradation of E2A and PAX5. B cell specific genes including PAX5, CD19 and EBF1 are downregulated whilst T cell specific genes including CD3ε, TCF1, Deltex and pTα become upregulated. Importantly the lineage switched B cells (now T cells) still retain BCR rearrangement reminiscent of their origin. Therefore, in our system, attenuation of PKCα signaling allows cells to fully differentiate down a given lineage according to internal and external environmental
stimuli, yet also allows for the ability to change lineage according to other stimuli. Although fully differentiated, our mCLL cells have preserved pluripotent quality.

Recently, Kikushige et al. have highlighted the role of stem cells in CLL (Kikushige et al. 2011). Although B and pro-B cells isolated from CLL patients failed to engraft into immunodeficient recipient mice, CD34^+CD38^−(CD90^+) stem cells isolated from CLL patients not only engrafted stably but also gave rise to myeloid and lymphoid cells. In particular, the engrafted stem cells biased toward a polyclonal pro-B cell population, and upon VDJ recombination analysis of more mature B cells, mono-and oligoclonalility was evident as well as CD5 expression, similarly to that found in monoclonal B cell lymphocytosis (MBL), a precursor to CLL. These mature B cells were also CD5^+ and exhibited a biased V-gene repertoire. Collectively the authors imply a potential origin of a CLL cell to be a leukaemic HSC, that after antigen (or autoantigen) selection develops into mono- or oligoclonal leukaemic B cell populations that along with additional genomic abnormalities develop into CLL (Kikushige et al. 2011). Given the finding that subversion of PKCα signaling in mCLL B cells allows for lineage plasticity, it would be interesting to investigate the expression and role of PKCα within different populations of human CLL cells, particularly within the CLL HSC population.
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