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The Biology of Mantle Cell Lymphoma: Exploring the Gender Difference in Mantle Cell Lymphoma

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The Biology of Mantle Cell Lymphoma : Exploring the Gender Difference in Mantle Cell Lymphoma

By

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I certify that all material in this thesis which is not my own work has been identified and that no unchanged or acknowledged material has previously been submitted and approved for the award of a degree by this or any other
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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Medicine has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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Abstract

Mantle cell lymphoma (MCL) is a rare B cell neoplasm that accounts for approximately 4-8% of non-Hodgkin's lymphomas (NHLs). The median age at diagnosis is 65 years with a male to female predominance of 3:1. It has also been demonstrated that female MCL patients have a greater response to therapy, especially immunomodulatory therapy compared to male MCL patients. The concept of cancer immunosurveillance is well described and it is perceived that females mount a greater immune response compared to males. In addition, although lymphomas are generally not perceived to be hormone controlled, epidemiological studies have demonstrated lower prevalence of lymphoma in females taking exogenous oestrogen.

This aim of this thesis was to explore the gender difference observed in MCL. The study investigated the difference in the quantity of immune cells in the peripheral blood and lymph node biopsies of untreated male and female MCL patients. There was a significantly greater number of T cells in the peripheral blood of male MCL patients compared to the female MCL patients. Conversely, greater numbers of immune cells were observed in the lymph node biopsies of female MCL patients compared to male MCL patients. In addition, four NK cell activating receptors; NKp46, NKp44, NKp30 and NKG2D were examined to determine if their expression was different between the genders.

The cell mediated cytotoxic function of the immune cells (PBMCs) from male and female MCL patients and healthy controls was also examined. Interestingly the healthy controls exhibited greater cytotoxicity compared to the MCL patients. PBMCs were incubated with oestrone (female hormone in postmenopausal women), lenalidomide and IL-2 to further investigate the

effects of these on the immune cells from male and female MCL patients. Incubation with IL-2 resulted in a significant increase in the cytotoxicity activity of male MCL patients but not female MCL patients in this cohort.

The lymph node biopsies from MCL patients were examined for the presence of oestrogen receptors. Oestrogen receptor β was predominantly expressed on MCL cells in all the biopsies examined. This is an area that warrants further studies.

This thesis provides some insight into the mechanisms that may influence the gender difference observed in MCL, however further studies are needed.

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List of Abbreviations

7-AAD	7-aminoactinomycin D
α	Alpha
β	Beta
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
AraC	Cytarabine
BAFF	B cell activating factor
BCR	B cell receptor
BEAM	Carmustine, etoposide, cytarabine, melphalan
BTK	Bruton tyrosine kinase
CCND1	Cyclin D 1
CCM	Complete culture media
CCR7	Chemokine receptor 7
CCR12	Chemokine receptor 21
CDKN2A	Cyclin-dependent kinase inhibitors p16
CDKN2B	Cyclin-dependent kinase inhibitors p15
CD	Cluster of differentiation
CDK 2,4,6 etc	Cyclin dependent kinases 2,4, 6 etc
CLL	Chronic lymphocytic lymphoma
CR	Complete response
CS-HI-FBS	Charcoal dextran stripped heat inactivated foetal bovine serum
CSFE	Carboxyfluorescein succinimidyl ester
CFDA-SE	Carboxyflourescein diacetate succinimidyl ester
CTL	Cytotoxic T lymphocytes
CTLA	Cytotoxic T lymphocyte-associated antigen
CoRegs	Co-regulatory proteins
CXCR4	CXC chemokine receptor 4
CXCR5	CXC chemokine receptor 5
DAB	3,3'-diaminobenzidine
DAP	DNAX – activating protein
DBD	DNA-binding domain
Dexa	Dexamethasone
DLBCL	Diffuse large B cell Lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPN	Diarylpropionitrile
EBPs	Oestrogen binding proteins
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
ENL	Erythema nodosum leprosum
Est	Oestrone
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ERE	Oestrogen response elements
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
FasL	Fas Ligand

FCS	Forward scatter signal
FDA	Food and Drug Agency
FDC	Follicular dendritic cells
FDS	Follicle dendritic cell
FFPE	Formalin fixed paraffin embedded
FL	Follicular Lymphoma
Foxp3	Forkhead box P 3
G519	Granta 519
GITR	Glucocorticoid-induced tumour necrosis factor receptor
HEV	High endothelial venules
HD-AraC	High dose cytarabine
HIER	Heat-induced epitope retrieval
HI-FBS	Heat inactivated foetal bovine serum
HLA	Human Leukocyte antigen
HRP	Horseradish Peroxidase
hyper-CVAD	Hyper-fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone
ICAM-1	Intercellular adhesion molecule 1
IDC	Interdigitating dendritic cells
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IHC	Immunohistochemistry
Im	Intermediate
IMF	Immunoflourescence
ILT	Immunoglobulin-like transcript
IL-2	Interleukin – 2
IL-4	Interleukin – 4
IL-5	Interleukin – 5
IL-6	Interleukin – 6
IL-10	Interleukin – 10
IL-12	Interleukin – 12
IL-13	Interleukin – 13
IL-35	Interleukin – 35
ITAM	Immunoreceptor tyrosine based activation motif
KIR	Killer-cell immunoglobulin-like
LAK	Lymphokine activated killer cells
LAG	Lymphocyte activation gene
LAM	Lymphocyte associated macrophage
LBD	Ligand – binding domain
LFA-1	Leucocyte function associated antigen-1
LTA	Lymphotoxin
MA	Methotrexate
MCL	Mantle Cell Lymphoma
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major Histocompatibility Complex molecule
MICA	MHC-class I chain-related protein A
MICB	MHC-class I chain-related protein B
MIPI	Mantle cell lymphoma international prognostic index
NCR	Natural cytotoxicity receptors
NF-ĸB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NK cells	Natural Killer cells
NKG2A	Natural-killer group 2 member A
NKG2C	Natural-killer group 2 member C
NKG2D	Natural-killer group 2 member D
NKT	Natural Killer T cells
NHL	Non-hodgkins lymphoma
ORR	Overall response rate
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Progressive disease
PerCP	Peridininochlorophyll-protein
PFS	Progression free survival
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-kinase
PR	Partial response
RAE	Retinoic acid early transcript
RB	Retinoblastoma
SCID mouse	Severe combined immunodeficiency mouse
SCT	Stem cell transplant
SEM	Standard error of the mean
SFM	Serum free media
SHP-1	Src homology region 2 domain-containing phosphatase-1
STWS	Scott's Tap water substitute
TBI	Total body irradiation
TCR	T cell receptor
TRAIL	TNF-related apoptosis-inducing ligand
T regs	T regulatory cells
TTF	Time to failure
ULBPS	UL16-binding proteins
VEGF	Vascular endothelial growth factor
VLA4	Very late antigen – 4
WBC	White blood cells

Chapter 1

Introduction

1.0. Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a B cell neoplasm that accounts for approximately 4-10% of non-Hodgkin's lymphomas (NHLs) (McKay *et al*, 2012). The median age at diagnosis is 65 years with a male to female predominance of 3:1 (Chandran *et al*, 2012; McKay *et al*, 2012). Histologically it is characterised by an abnormal proliferation of mature B lymphocytes which are classically $CD19^{+}CD20^{+}CD22^{+}CD23^{-}FMC7^{+}CD5^{+}CD10^{-}$ (Pérez-Galán *et al*, 2011; Campo *et al*, 2011). In involved lymph nodes, the malignant cells can reside in the mantle zone or be found in a nodular or diffuse growth pattern which are thought to represent progressive stages of tumour infiltration (Campo *et al*, 2011; Swerdlow *et al*, 2008).

1.1. Clinical presentation and epidemiology of MCL

The majority of the patients present with lymphadenopathy which is generally widespread at diagnosis. Eighty to ninety percent present with advanced-stage disease with or without B symptoms (fever, weight loss and night sweats) (Table 1). Extranodal involvement is common, especially bone marrow (in >65%), gastrointestinal tract, liver and spleen and 20-30% of patients present with a leukemic component (McKay *et al*, 2012). A small subset of patients present with disease that is largely non-nodal and confined to the blood, bone marrow and spleen. They tend to exhibit a more indolent course than those with predominately nodal disease, although the clinical distinction cannot be relied upon to predict outcome (Hsi & Martin, 2014).

Stage	Features
I	Involvement of a single lymph node region or lymphoid structure (e.g. spleen, thymus, Waldeyer's ring)
II	Involvement of two or more lymph node regions on the same side of the diaphragm
III	Involvement of lymph regions or structures on both sides of the diaphragm
IV	Involvement of extranodal site(s) beyond that designated E
For all stages A B	 No symptoms Fevers (>380C), drenching sweats, weight loss (10% body weight over 6 months)
For stages I to III E	 Involvement of a single, extranodal site contiguous or proximal to known nodal site

Table 1: Staging classification for MCL
Table demonstrating staging classification used for MCL and non-Hodgkins lymphoma by the Ann Arbour classification. Adapted from (Armitage, 2005)

1.2. Molecular Pathogenesis of MCL

MCL is characterised by the t(11:14)(q13;q32) translocation and the overexpression of CCND1 that probably facilitates the transformation of the cells by deregulating the cell cycle (Bea, 2014). This translocation juxtaposes the CCND1 locus (encoding cyclin D1) on 11q13 to the enhancer of the IgH locus on 14q32, leading to upregulation of cyclin D1 mRNA and protein. The overexpression of cyclin D1 in MCL results in cell cycle dysregulation by facilitating G1 to S phase transition by the cyclin D1 forming a complex with the cyclin-dependent kinases CDK4 and CDK6. This complex formation initiates phosphorylation of the retinoblastoma (RB) protein (a major negative regulator of S phase entry), release of E2F transcription factors (such as cyclin E), and

formation of cyclin E/CDK2 complexes. These events produce irreversible inactivation of the RB protein and ultimately progress into S phase (Jares *et al*, 2007). Rarely some cases of MCL are cyclin D1 negative and in such cases overexpression of cyclin D2 or D3 is demonstrated, suggesting that dysregulation of other cyclins may confer an alternative mechanism for MCL development (Swerdlow *et al*, 2008; Beà *et al*, 2013; Jares *et al*, 2007).

The initial translocation event occurs at the pre-B cell stage in the bone marrow and appears to follow two molecular and clinical subtypes of MCL (Jares *et al*, 2012). The most common form of MCL, the classical subtype, is derived from mature B cells that are limited to the mantle cell zone of the follicle and carry no or limited number of IGHV somatic mutations (Navarro *et al*, 2012). This subtype expresses the neural transcription factor SOX11 which are genetically unstable and accumulate alterations in regulatory genes, DNA damage response pathways and cell survival mechanism. These alterations result in aggressive tumour behaviour. The second subtype of MCL carries the IGVH with somatic mutation. These tumour cells are genetically stable and the SOX11 expression is minimal or negative. This subtype tends to disseminate more to the peripheral blood and spleen than the lymph nodes (Fernàndez *et al*, 2010; Jares *et al*, 2012) (Figure 1).

All mature B cells and mature B cell malignancies are negative for the expression of SOX11, but its presence in MCL highlights its relevance in the pathogenesis of MCL (Mozos *et al*, 2009) suggesting additional pathways for the development of MCL. SOX11 promotes tumour growth of MCL cells in vivo and regulates a broad spectrum of transcriptional program which includes B cells development by altering the terminal B cell differentiation of MCL (Vegliante *et al*, 2014).

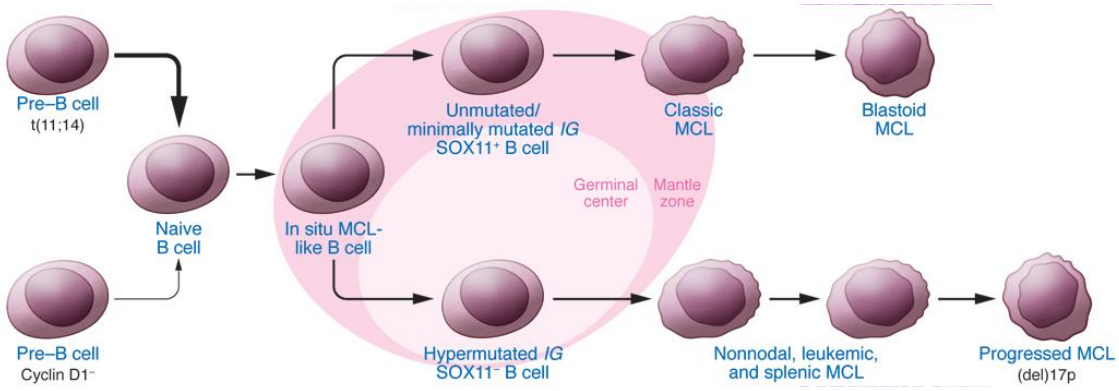


Figure 1: Two different molecular subtypes of MCL.

Two hypothetical models of different molecular subtypes of MCL. The naive B cell with the t(11;14) mutation colonizes the mantle zone of the lymphoid follicle and generates an in situ MCL lesion. Most MCLs evolve from these cells or cells in the marginal zone with no or limited IGHV somatic mutations. The tumours which express SOX11, are genetically unstable, and tend to accumulate alterations in genes dysregulating cell cycle, DNA damage response pathways, and cell survival mechanisms. Alternatively, some cells with the t(11;14) may enter the germinal center and undergo IGHV somatic hypermutations. These cells are genetically stable and do not express SOX11. The tumours derived from these cells tend to spread to the peripheral blood and spleen more than to lymph nodes. The disease seems to be stable for long periods of time, but some of these tumors may acquire mutations in genes such as TP53 that lead to disease progression. Adapted from (Jares *et al*, 2012)

Other molecular events have also been described in addition to the major t(11;14)(q13;q32) translocation in MCL. The most common alterations further deregulating cell cycle in MCL involve the *INK4a/CDK4/RB1* and *ARF/MDM2/TP53* pathways (Jares *et al*, 2012). The *CDKN2A* locus (9p21), frequently deleted in MCL, connects both pathways encoding for the CDK inhibitor *INK4a* and the positive p53 regulator *ARF*. *TP53* is commonly mutated gene in MCL (19-28%) and *RB1* is also inactivated by point mutations or gene deletions in occasional cases. Gene amplification leads to the overexpression of CDK4, MDM2 and BMI1, that in turn represses the CDKN2A locus. These mutations and alterations further dysregulate the cell cycle (Jares *et al*, 2007).

MCL has deregulated cell signaling pathways in addition to the genetic alterations that have been demonstrated in recent promising results observed in clinical trials involving inhibition of the bruton's tyrosine kinase (BTK) of the B cell receptor signaling pathway (Advani *et al*, 2013; Wang *et al*, 2013b; Shah *et al*, 2014). Furthermore, the microenvironment of MCL may also influence the development and studies have observed IL-6 influence in MCL development by activating STAT3 pathway as discussed below (Pérez-Galán *et al*, 2011; Jares *et al*, 2007; Zhang *et al*, 2012).

1.3. MCL tumour microenvironment

There has been limited data on the precise composition of the microenvironment in MCL. Extrapolating from other B cell lymphomas studies, it is somewhat possible to describe the microenvironment in MCL.

The tumour microenvironment of B cell lymphomas contains variable numbers of immune cells, stromal cells, blood vessels and extracellular matrix (Scott & Gascoyne, 2014). The extent, composition and architecture of this environment is dependent on multiple factors which include genetic aberrations that are harboured by the malignant cells and their dependence on external stimuli for survival, proliferation and immune evasion (Coupland, 2011).

Majority of MCL patients have advanced stage disease (Anne Arbour stages III/IV) and a large proportion of MCL patients have extranodal manifestation with a prevalence of circulating MCL cells in the bone marrow and gastrointestinal involvement (Campo & Rule, 2015; Shah & Rule, 2014). These clinical features suggest that MCL cells have a very active 'homing' function to lymph nodes and different tissue compartments. Lymphoma cell trafficking is a non-random, complex and regulated process which allows these cells to

migrate to tissues in response to stromal cell derived chemokine gradients (Scott & Gascoyne, 2014). B cell lymphoma cells retain the capacity of their normal counterparts for trafficking and homing (Scott & Gascoyne, 2014). MCL cells exhibit a high expression of chemokine receptors and adhesion molecules such as chemokine receptor 4 (CXCR4), CXCR5 and chemokine receptor 7 (CCR7) and similarly on the endothelial cells of the high endothelial venules allowing them to move to lymph nodes and other tumour supporting areas (Kurtova *et al*, 2009). These receptors plus the adhesion receptor; very late antigen-4 (VLA4 / CD49d), enables MCL cells to interact with accessory stromal cells, such as mesenchymal stromal cells and lymphoma-associated macrophages (LAM) (Trentin *et al*, 2004). Once the MCL cells are within the tumour supporting area, the chemokine and adhesion receptors establish contact between the LAM and stromal cells (Burger & Ford, 2011).

1.4. B cells

As MCL is a B cell disorder a brief review of normal B cells here would enable further understanding of the pathogenesis as to a degree, the composition of the microenvironment represents remnants of the normal lymphoid tissue, effaced or infiltrated by malignant cells and the host inflammatory response (Swerdlow *et al*, 2008; Scott & Gascoyne, 2014). The normal B cells originate from haematopoietic progenitor cells within the bone marrow and migrate to secondary lymphoid organs to complete their development (Cooper, 2015). This involves immunoglobulin VDJ gene rearrangement and differentiation into mature naïve B cells that express surface IgM and are often $CD5^+$ (Kraus *et al*, 2004) These naïve B-lymphocytes circulate and have a homing process toward the lymph nodes (Von Andrian & Mempel, 2003). Once they have migrated to the B cell zone of the lymph node follicle, they encounter antigens which are

presented by macrophages and dendritic cells (Bastista & Harwood, 2009). The B cells occupy the primary lymphoid follicles and follicle mantle zones until they encounter antigen whereupon they migrate into the centre of a primary follicle, undergo blastic transformation into centroblasts, and form a germinal centre (Victoria & Nussenzweig, 2012). The centroblasts matures into centrocytes within the germinal centre, resulting in somatic mutations of the immunoglobulin variable region gene and heavy chain class enabling the production of higher-affinity IgG or IgA (Victoria & Nussenzweig, 2012). Final outcome of these centrocytes is to differentiate into either antibody-secreting plasma cells, which home back to the bone marrow, or memory B cells which reside in the follicle marginal zones (Delves *et al*, 2011; Cooper, 2015).

The B cells major role in adaptive immunity is to produce immunoglobulins that recognise, bind and eliminate specific antigen (Scott & Gascoyne, 2014). Each individual B cell produces immunoglobulin of a single specificity which is also expressed on its surface in a membrane-bound form known as the B-cell receptor (BCR) (Kraus *et al*, 2004). Binding of antigen to the BCR either directly or indirectly (via $CD4^+$ helper T cells) triggers B cell activation, clonal expansion, and the secretion of soluble immunoglobulin (antibodies) into the extracellular space (Delves *et al*, 2011; Cooper, 2015). B cells that bind antigen via BCR internalize and represent the antigen on major histocompatibility complex (MHC) class II with activation of nuclear factor-ƙB (NF-ƙB) directing the primed B cell towards the T cell – B cell border (McHeyzer-Williams *et al*, 2012). The interaction of B and T cells here provides a checkpoint that selects cells with high affinity BCR for further development in the germinal centre of the B cell follicle (Cooper, 2015). Immunophenotyping, histological analysis and gene expression profile of MCL have identified characteristics that are reminiscent of

naïve B cells. Furthermore 15-40% of MCL cells also show somatic hypermutation indicating that they have passed through the germinal centre reaction possibly following T cell – B cell interaction (Jares *et al*, 2012).

1.5. T cells in MCL microenvironment

Cellular composition of MCL microenvironment has not been systematically or definitively analysed. It has been extrapolated from other B cell lymphoma studies suggesting that MCL has a similar microenvironment to follicular lymphoma to a certain extent (Scott & Gascoyne, 2014). This environment consists mostly (90-95%) of malignant MCL cells but other cells are also present such as $CD4^+$, $CD8^+$ T cells, T regulatory cells, follicular regulatory cells, macrophages, follicular dendritic cells and normal B cells are also present (Scott & Gascoyne, 2014). The expression of CD40 and responsiveness to CD40 ligand (CD154) suggest interaction with T cells which provide MCL cell survival and expansion (Burger & Ford, 2011). In addition B cell lymphomas have a high infiltration of $CD4^+$ cells which include $CD4^+CD25^+$ T cells expressing CTLA4 and Foxp3 (intratumoral T regulatory cells) and follicular regulatory T cells further supporting their survival and suppression of immunoediting by cytotoxic T cells (Yang *et al*, 2006). Interestingly the interaction of $CD4^+$ T cells with lymphoma cells encourage them to develop into T regulatory cells (Yang *et al*, 2006). This process is mediated by the expression of molecules that are involved in co-stimulation of naïve T cells, including CD70, CD80 (B7-1) and CD86 (B7-2) (Yang *et al*, 2009). Furthermore it has been demonstrated that lymphoma cells have higher expression of CD70 which supports the mechanism by which naïve T cells are encouraged to develop into T regulatory cells (Yang *et al*, 2006, 2009). In context of MCL and

its relation to T cells, a brief review of normal T cells below aids to appreciate their role in the microenvironment.

1.6. The T cells

T cell mediated immunity is central to the adaptive immune system. T cells are derived from the haematopoietic cells and each T cell lymphocyte expresses a unique T cell receptor on the surface as the result of developmental selection upon maturation in the thymus (Kared *et al*, 2014). The mature T lymphocytes leaving the thymus are also known as naïve T cells. They circulate through the blood and lymphatic system and reside in secondary lymphoid organs (spleen, lymph nodes, tonsils, gut and nasal lymphoid tissue plus mucosal lymphoid tissue) (Andersen *et al*, 2006). The naïve T lymphocytes have not encountered foreign antigens and thus are not activated. They reside in the spleen for a few hours and in the lymph nodes for approximately a day before leaving to enter another lymph node and repeating the cycle until its gets activated (Munoz *et al*, 2014; Kared *et al*, 2014). Activation of T cells is an adaptive process whereby antigenic peptides are presented to the naïve T cells in secondary lymphoid tissue by antigen presenting cells (APC) such as the dendritic cells (DC), which are the most efficient antigen presenting cells (APC). Antigens presented to T cells are recognised through the T cell receptor (TCR) leading to activation of T cell immunity (Medzhtov & Janeway, 2000; Delves *et al*, 2011).

1.7. The T cell receptor (TCR)

The TCR consists of two heterogeneous polypeptide chains, an α and a β chain, linked by a disulphide bond which is expressed on the surface and is unique to each T cell (Wucherpfennig *et al*, 2010). Both these chains possess a variable (V) region, which recognises and binds specific antigen, and a constant

(C) region, which provides effector or signalling functions once antigen is bound. The V region of each TCR is encoded by different gene segments that are assembled within the developing lymphocyte by somatic DNA recombination, a process referred to as gene rearrangement (Medzhtov & Janeway, 2000). This enables the T cells to have a vastly diverse repertoire of highly specific TCRs, thus allowing majority of the antigens encountered within an individual's lifespan to be recognised (Rossjohn *et al*, 2014).

TCR with α and β chains also have additional accessory chains which are required for optimal T cell activation following antigen binding (Gras *et al*, 2008). These accessory chains; γ, δ and ε are part of the CD3 co-receptor (commonly used surface marker in the laboratory to identify T cells) and the ζ chain which is largely intracytoplasmic (Rudolph *et al*, 2006). The αβTCR T cells are subdivided into several groups on the basis of lineage markers and functional activities. Furthermore, two major surface co-receptor molecules, CD4 and CD8, define two separate T cell lineages with different functions (Gras *et al*, 2008). The $CD4^+$ cells recognize antigens in the context of MHC class II molecules (only expressed on APC such as B cells, macrophages and DC) and produce cytokines as effector T helper cells. The $CD8^+$ lymphocytes are activated by antigenic peptides presented by MHC class I molecules (expressed on all nucleated cells) and form effector cytotoxic T lymphocytes (CTL) (Rossjohn *et al*, 2014; Andersen *et al*, 2006; Doherty & Zinkernagel, 1997).

The TCR recognizes antigenic peptides presented in the context of Major Histocompatibility Complex (MHC). MHC is a set of cell surface proteins required to recognise foreign molecules, thus determining histocompatibility. (Medzhtov & Janeway, 2000). The main function of MHC molecules is to

present and display antigenic peptides derived from intracellular proteins on the cell surface for recognition by appropriate T cells (Ku *et al*, 2009; Doherty & Ko, 1997). The presented peptide can be either self or non-self, thus regulating normal cells from being attacked by the immune system. Class I MHC molecules have a heavy polypeptide chain linked to a smaller polypeptide called a β2-microglobulin subunit so can only be recognized by CD8 co-receptors (Doherty & Zinkernagel, 1997). Class II MHC molecules consist of α and β polypeptide chains with no presence of β2-microglobulin so can be recognized by CD4 co-receptors (Neefjes *et al*, 2011). The MHC class I and II molecules are polygenic and there are three MHC class I α-chain gene, HLA-A, HLA-B and HLA-C which result in at least three different class I proteins (Pende *et al*, 2002). Similarly there are three different types of MHC class II α and β genes expressed; HLA-DQ, HLA-DP and HLA-DR, thus resulting in at least 3 different class II molecules but much more when polymorphisms are taken into account (Rossjohn *et al*, 2014; Trowsdale & Knight, 2013). The different types of class I and class II molecules all exhibit the same basic structure and all participate in presenting peptides to T cells but, because of significant differences in their peptide groove each presents a different range of peptides to the immune system (Trowsdale & Knight, 2013).

In 1996, Zinkernagel and Doherty were awarded the Nobel Prize in Medicine for demonstrating that $CD8^+$ T cells could only recognize virally infected target cells if they expressed a particular set of MHC molecules (Doherty & Zinkernagel, 1997). The MHC molecules are able to present a vast range of peptides. It is estimated that there are up to 250,000 of each HLA class I molecule on the surface of a cell (Parham & Ohta, 1996). Similarly, naïve CD4+

cells are activated by their TCR recognition of peptides presented by MHC class II, which has a more restricted expression pattern.

The classical expression of antigen by MHC class I molecules essentially have three processes; first , the proteasomal degradation of endogenous proteins to peptides in the cytosol; second the translocation across the endoplasmic reticulum and third, the presentation of the antigen by assembly of the MHC with its β_2-microglobulin molecule (Crotzer & Blum, 2009; Chapman, 2006). MHC class I also present exogenously processed peptides to cytotoxic T cells and this is referred to as the alternative pathway. Antigen Presenting Cells (APC) and dendritic cells are generally involved with the alternative pathway, presenting peptides to the effector T cells using a similar mechanism to the one described above (Shen & Rock, 2006).

The type of MHC class expression on tumour cells and cell lines can be demonstrated by flow cytometry. This is an effective method of investigating the expression of MHC-class expression on various cells (Schietinger *et al*, 2010; Manning *et al*, 2008). In relation to MCL, Drenou et al, have demonstrated reduced expression of MHC on mantle cell lymphoma cells by flow cytometry after studying mantle cell lymphoma cells from patients as part of a larger study encompassing B cell lymphomas (Drenou *et al*, 2002).

1.8. T helper cells (Th)

T helper cells play critical roles in orchestrating the adaptive immune responses. Their function is mainly through secreting cytokines and chemokines that activate and/or recruit target cells (Zhu & Paul, 2015). The activated $CD4^+$ T helper cells can be further distinguished by their cytokine profile. They are subdivided into Th1, Th2, Th17 and regulatory T cells (T regs) based on their

effector function and the production of specific cytokines (figure 2.0) (Tato & O'Shea, 2006).

The three most relevant subsets for this thesis and MCL; Th1, Th2 and T regulatory cells will be discussed in more detail below and later in this chapter.

Th1 and Th2 cells

The Th1 cells produce mainly IFN-γ, but also IL-2 and lymphotoxin-α (LTα). The Th1 cells enhance pro-inflammatory cell mediated immunity by stimulating cytotoxic T cells and have been shown to induce delayed-type hypersensitivity and B cell production of opsonizing isotypes of IgG (Williams *et al*, 2006).

The Th2 cells produce and secrete IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25 and encourage non-inflammatory immediate immune responses. They have also been shown to be essential in B cell production of IgG, IgA and IgE. IL-4 has a positive feedback effect for Th2 cell differentiation and is a major mediator for IgE class switching in B cells (Zhu & Paul, 2015). IL-5 recruits eosinophils and affects mast cells and lymphocytes. IL-9 induces mucin production in epithelial cells and IL-10 suppress Th1 cell proliferation and can suppress dendritic cell function (Trinchieri, 2007). IL-25 (also known as IL-17E) is also a Th2 cytokine. IL-25, signalling through IL-17RB, enhances the production of IL-4, IL-5, and IL-13 (Fort *et al*, 2001). IL-25 can induce the production of chemokines including RANTES (CCL5) and eotaxin (CCL11) that recruit eosinophils (Zhu & Paul, 2015).

The development of these subsets of T helper cells from naïve T cells is dependent on the particular cytokines present in the microenvironment. Th1 development prevails in the presence of IFN-γ and IL-12 whilst Th2

development predominates in the presence of IL-4. The resulting Th1 or Th2 population will subsequently produce more IFN-γ or IL-4 respectively providing a positive feedback loop for further Th1 or Th2 cell production. This enables maintenance of the $CD4^+$ T cell subtype most suited to the immune response required (Geginat *et al*, 2014; Zhu & Paul, 2015). Figure 2 below summarizes CD4 T cells, including their unique cytokine production and cytokines critical for their final development and maturation including some of their functions.

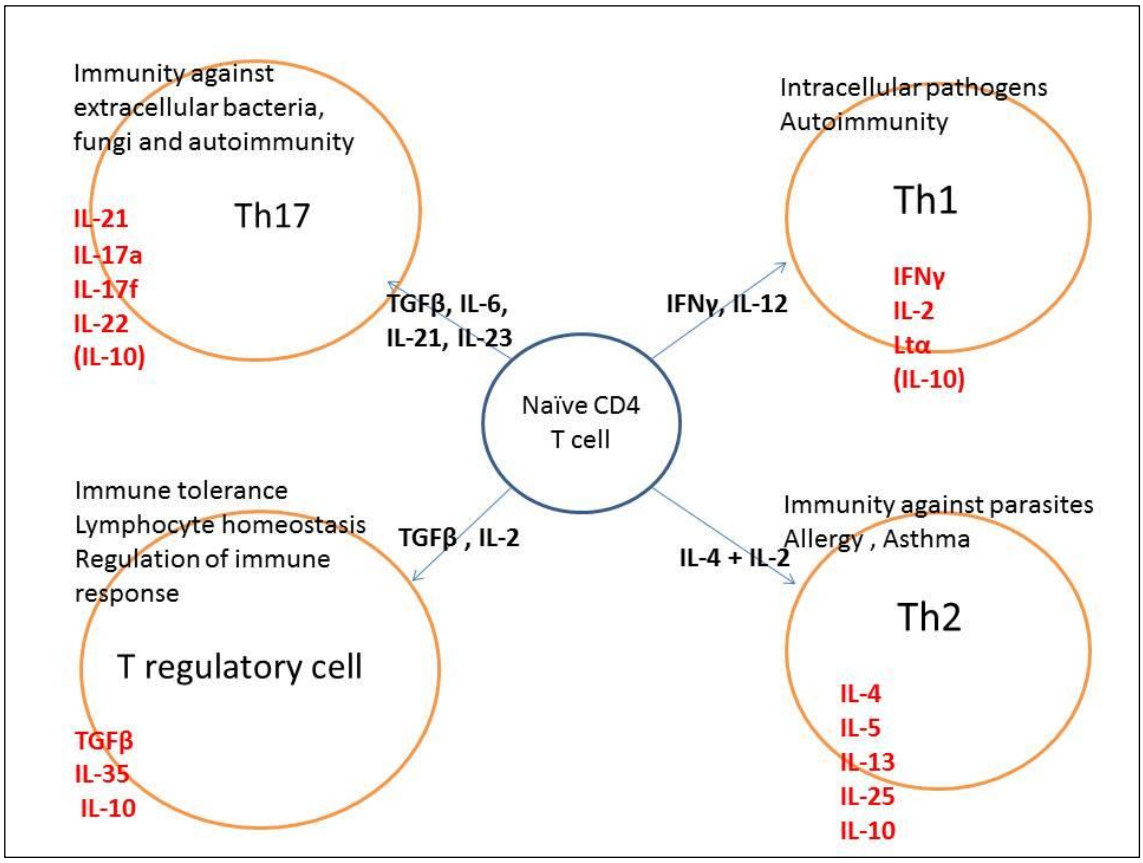


Figure 2: Summary of CD4 T helper cell fates
Their functions, their unique products (as shown in red) and cytokines critical for their final determination (shown in black bold print). Adapted from (Zhu & Paul, 2015).

Cytotoxic T cells

Cytotoxic T cells develop from naïve $CD8^+$ cells and their role is to monitor all cells of the body, ready to destroy any that are thought to be a threat to the body (Andersen *et al*, 2006). Cytotoxic T cells can be easily activated by the antigen MHC class I complex and undergo dramatic clonal expansion

(Andersen *et al*, 2006; Doherty & Ko, 1997). Cytotoxic T cells are efficient effector cells due to their ability to destroy more than one target cell, whilst sparing innocent 'bystanders'. Destruction of the target cells require cell contact to be established and antigen recognition before initiating the release of cytolytic granules into the synapse (Von Andrian & Mackay, 2000). Cytotoxic T cells do not require a co-stimulatory signal upon antigen recognition in order to kill the target cells (Andersen *et al*, 2006; Delves *et al*, 2011).

There are two major cytotoxicity pathways that have been described. One is a calcium dependent perforin/granzyme mediated apoptosis and the second is calcium independent Fas ligand/Fas-mediated apoptosis. Both pathways are initiated via the TCR signalling (Andersen *et al*, 2006).

Perforins and granzymes are transported into the target cell as one complex and granzymes induce apoptosis via the caspase-dependent and independent mechanisms. Perforin appears to enable transfer of granzyme via pores it forms. The Fas-mediated apoptosis is initiated by binding of the Fas molecule (CD95) on the target cell via the Fas ligand (CD95L) or TRAIL on the effector cells, which subsequently initiates the caspase dependent apoptosis in the target cell (Andersen *et al*, 2006).

1.9. Activation of T cells

Naïve T cells received two separate signals to become activated. The first signal is provided through engagement of the TCR with its MHC-bound antigenic peptide (Von Andrian & Mackay, 2000). The second, or co-stimulatory, signal is provided by interaction between various accessory molecules on the surface of APCs and T cells (Smith-Garvin *et al*, 2009). The best-characterised co-stimulatory pathway involves the binding of CD28 on T

cells to B7-1 (CD80) or B7-2 (CD86) on APCs although many other co-stimulatory pathways interactions, such as OX40/OX40L and CD27/CD70 have been identified (Figure 3)(Sharma *et al*, 2011). Once activated by both signals the T cells rapidly proliferate and differentiate to produce large number of effector T cells. This process is also critically aided by the production of IL-2 by the activated T cells. Conversely in the absence of co-stimulation the T cell which has recognised the antigen via the TCR only, is either clonally deleted or functionally inactive (Kared *et al*, 2014). Once activated the effector cells do not require co stimulation and can be activated by the antigen alone (Von Andrian & Mackay, 2000; Sharpe & Abbas, 2006; Kared *et al*, 2014).

Activation of T cells is restricted by inhibitory signals. This is achieved by the upregulation of cytotoxic T lymphocyte-associated protein 4 (CTLA4) following the initial stimulation of T cells by B7-CD28 interaction. CTLA4 has a greater affinity to B7 and therefore out-competes CD28 thus initiating inhibitory signals for the T cells (Figure 3B) (Sharma *et al*, 2011; Andersen *et al*, 2006). In addition other inhibitory signals are initiated by programme cell death 1 (PD1) and its interaction with PD1 – ligand on the APC (Myklebust *et al*, 2013; Greenwald *et al*, 2005).

In the context of MCL, a recent study by Wang et al, demonstrated the expression of B7-H1 on MCL which resulted in inhibition of T-cell proliferation induced by the tumour cells, impaired generation of antigen-specific T-cell responses, and mantle cell lymphoma cells being rendered resistant to T-cell-mediated cytolysis (Wang *et al*, 2013).

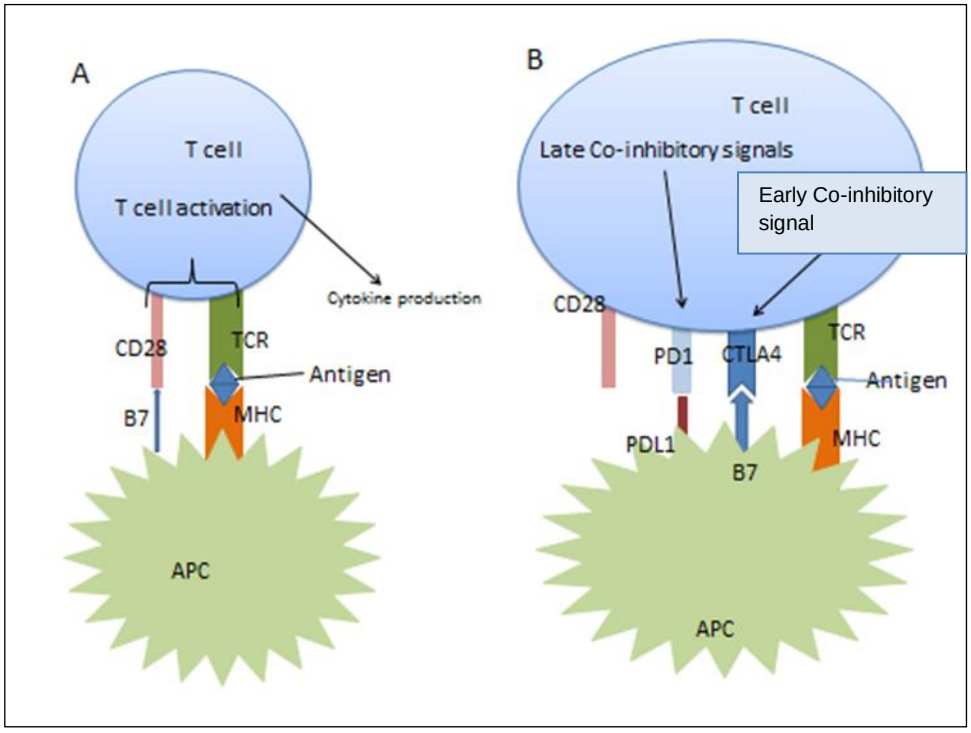


Figure 3: T cell activation

A: The initial T cell activation begins with TCR and interaction with antigen bound on the MHC found on the antigen presenting cell (APC), but additional co-signals are provided by the interaction between CD28 and B7.

B: T cell activation is limited by cytotoxic T lymphocyte-associated protein 4 (CTLA4) which is upregulated following initial activation. CTLA-4 has a higher affinity to B7 and out-competes CD28 for binding to B7 on the APC. Later inhibitory signals as provided through programmed cell death 1 (PD1) which binds to PD1 ligand (PD1L) and other regulators are also present such as T cell immunoglobulin and mucin domain containing protein 3 (TIM3) and V domain immunoglobulin suppressor of T cell activation (VISTA). Adapted from (Sharma *et al*, 2011).

1.10. The regulatory T cells (T regs)

The majority of the T regulatory cells appear within the $CD4^+$ T cell subset and their primary role is to suppress the immune response mounted against self and foreign antigens. Therefore they are critical in the maintenance of peripheral tolerance and prevention of autoimmune disease (Corthay, 2009). The exact mechanism by which T regulator cells exert their function is not fully described however immunosuppressive cytokines such as TGF-β, IL-10 and IL-35 play an important role. Presently, the T regulatory cells have the following functions described (Corthay, 2009):

- Prevention of autoimmune diseases by establishing and maintaining immunologic self-tolerance via inhibitory cytokines such as IL-10, TGF-β, IL-35 (Collison *et al*, 2007).
- Suppression of allergy and asthma.
- Metabolic disruption e.g. via IL-2 consumption.
- Induction of maternal tolerance to the foetus.
- Suppression of pathogen-induced immunopathology
- Modulation of dendritic cell maturation and / or function.

T regulatory cells are classified into two types; natural and induced. The natural T regulatory cells develop in the thymus express surface CD4 and CD25 (IL-2Rα), and are positive for the intracellular transcription factor forkhead box P3 (Foxp3) that is essential for their development, survival and function (Curotto de Lafaille & Lafaille, 2009). They represent approximately 10% of the $CD4^+$ population (Kelley & Parker, 2010). Inducible T regulatory cells are generated in the periphery from $CD4^+CD25^-Foxp3^-$ cells in response to certain antigenic stimuli and in the presence of TGF-β and IL-10, or in the absence of co-stimulation especially in the mucosal tissues (Josefowicz *et al*, 2012; Curotto de Lafaille & Lafaille, 2009). Furthermore, within the population of the inducible T regulatory cells there is additional heterogeneity which include the T regulatory 1 (Tr1) and T helper 3 (Th3) cells which depend on IL-10 and TGF-β for their suppressive actions respectively (Broere *et al*, 2011).

The inhibitory effect exerted by all T regulatory cells can also be via the TCR stimulation. Upon activation their function may be mediated via direct cell contact through inhibitory molecules such as cytotoxic T lymphocyte-associated antigen 4 (CTLA4) or via secretion of IL-10 and TGF-β (Lindqvist & Loskog, 2012). IL-10 can suppress Th1 and Th2 cells by influencing the production of

IL-2 (Steinbrink *et al*, 2002), TNF-α and IL-5 and indirectly by down-regulating MHC and co stimulatory molecules on the APC (Lindqvist & Loskog, 2012).

T regulatory cells are identified by the following markers; $CD4^+$, $CD25^+$, CTLA-4, glucocorticoid-induced tumour necrosis factor receptor family related gene (GITR), lymphocyte activation gene-3 (LAG-3), CD127 and Foxp3. However, these markers are not specific for T regulatory cells as all of them are either up or down regulated depending on the activation of the $CD4^+$ cells and are also present on other cells such as $CD8^+$ T cells (Corthay, 2009). Nonetheless, Foxp3 remains a reliable marker for T regulatory cells and $CD127^{low / dim}$ can be used as an alternative marker for Foxp3 if cell permeabilisation is to be avoided (Simonetta *et al*; Liu *et al*, 2006). CD127 is an IL-7 receptor which is down regulated in a subset of $CD4^+$ cells in the peripheral blood. Lui et al, have demonstrated that majority of these cells are $Foxp3^+$ including, those expressing low levels or no CD25. A combination of CD4, CD25 and CD127 resulted in highly purified population of T regulatory cells and hence allowed the CD127 biomarker to identify T regulatory cells (Liu *et al*, 2006).

1.11. Macrophages in MCL microenvironment

Macrophages have been postulated to have an important presence in MCL tissue. However their specific role in the pathogenesis remains to be described (Burger & Ford, 2011). High numbers of macrophages have been shown to correlate to more aggressive disease characteristics and it has been suggested that they are central to MCL survival (Kurtova *et al*, 2009). Macrophages present antigens to B cells and the rapid in vivo delivery of immune-complexes to marcophages in the lymph node subcapsular sinus including their capture by

follicular B cells has been demonstrated by multiphoton intravital microscopy of lymph nodes (Junt *et al*, 2007). They also express TNF family members, B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) that provide survival signals to MCL cells via the receptors BCMA, TACI and BAFF-R on MCL cells (Rodig *et al*, 2005) thus providing additional support for growth and survival. This mechanism of action with normal B cells and malignant cells by macrophages could be similar to events that occur in the microenvironment of MCL with macrophages and malignant MCL cells (Burger & Ford, 2011).

1.12. Stromal Cell in MCL microenvironment

Another potentially important cell is the mesenchymal stromal cell (MSC), which is also found in the microenvironment of MCL. In lymphomas stromal cells create a microenvironment that initially attracts malignant cells, allowing for early survival and subsequently contributing to tumour growth (Gascoyne *et al*, 2010). MCL cells adhere to MSC cells by adhesion receptors CXCR4 and VLA-4. Blocking of CXCR4 (by plerixafor a CXCR4 antagonist) and VLA-4 (by natalizumab a VLA-4 antagonist) disrupts the adhesion interactions and releases the MCL cells from their microenvironment thus making the cells more susceptible to anti-MCL therapy (Kurtova *et al*, 2009; Burger & Ford, 2011). MCL cells have a high affinity for MSC and have been shown to induce drug resistance probably because of the interaction with the MSC cells resulting in a protective environment (Kurtova *et al*, 2009). Cross talk between stromal and MCL cells involve many soluble mediators including interleukin (IL)-6, IL-7, IL-4, IL-8, vascular growth factor, CXCR4 and insulin growth factor 1 (Coupland, 2011). Zhang et al demonstrated survival and chemotherapy resistance with IL-6 in MCL. They exposed MCL cells to neutralizing autocrine IL-6 or IL-6 receptor antagonist which resulted in MCL cell growth inhibition and enhanced

rate of spontaneous apoptosis and sensitivity to chemotherapy drugs (Zhang *et al*, 2012). This provides further evidence of stromal's interaction with malignant MCL cells.

1.13. Other survival and growth signals in MCL microenvironment

Again the exact characteristics and details of survival and growth signals have not been fully detailed in MCL. However it has been demonstrated that B cells require an intact B cell receptor (BCR) for survival (Kraus *et al*, 2004; Burger & Buggy, 2013). It is a key factor in promoting clonal expansion in various malignancies such as MCL, chronic lymphocytic leukaemia and diffuse large B cell lymphoma via activation of downstream kinases (Pérez-Galán *et al*, 2011; Pighi *et al*, 2011; Ponader & Burger, 2014). MCL cells have been found to have constitutive activation of Spleen tyrosine kinase (Syk) and PKCβ II which are signal components downstream of BCR (Herman *et al*, 2011). Pighi et al, demonstrated BCR associated kinases including Syk and Btk as the most abundant phosphor-proteins in MCL by phospho-proteomic analysis (Pighi *et al*, 2011), suggesting that BCR signalling plays a central role in disease maintenance and progression, although the mechanism and possible antigens involved in BCR stimulation remains obscure.

The BCR is made up of antigen-specific membrane Ig paired with Ig-α / Ig-β heterodimers (CD79α/CD79β). Once the antigen has engaged the BCR it induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmatic tails of Ig-α and Ig-β with subsequent activation of tyrosine kinases the Src-family kinases (Lyn, Fyn, Blk and Lck) and the Syk as shown in figure 2. These then activate Btk downstream, which in turn activats phospholipase-Cγ (PLCγ), enabling calcium mobilization and activation of the

NF-kB and MAP kinase pathways promoting cell proliferation and survival (Burger & Buggy, 2013; Shah *et al*, 2014; Advani *et al*, 2013). New targeted drugs have been recently developed that block the downstream Btk and inhibit cell survival and proliferation (Wang *et al*, 2013b). Additionally it has been demonstrated that there is an initial lymphocytosis due to the 'off target' effects on the adhesion receptors such as CXCR4 and CXCR5. This causes the interference of the external stimuli from BCR and disruption of the lymphoma-stromal interaction in the microenvironment thereby releasing the MCL cells out of the 'fertile' microenvironment to less suitable environment which results in a short survival (Advani *et al*, 2013; O'Brien *et al*, 2014; Shah *et al*, 2014; Chang *et al*, 2013).

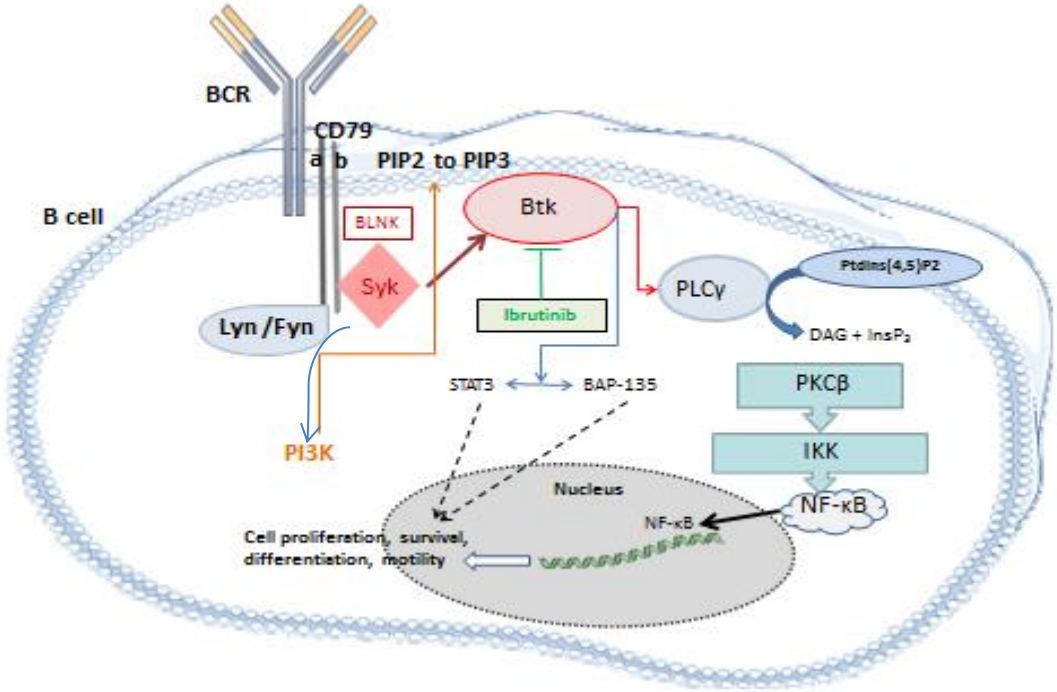


Figure 4: A simplified B cell receptor signalling pathway
The BCR is activated as an antigen is attached, initiating the phosphorylation of CD79a and CD79b (within the cytoplasmic tails of their immunoreceptor tyrosine kinase activation motifs (ITAMs), by the tyrosine kinases Lyn and Fyn. Btk, BLNK, PI3K amongst others are recruited to the membrane and Syk in turn is recruited and phosphorylates Btk and multiple other substrates including PI3K. Phosphorylated Btk activates signals downstream to induce activation of NF-κB which is involved in B cell proliferation and survival. Btk also activates other molecules which are involved in B cell survival and proliferation. The irreversible inhibition of Btk by ibrutinib stops the Btk's downstream cascade of signals which in turn inhibits or affects B cells proliferation, motility, differentiation and survival. Adapted from (Shah *et al*, 2014).

Immununity and immune cells as shown above have a significant role in tumour microenvironment as discussed above (Dunn *et al*, 2002). The role of immune surveillance on tumour cells is further discussed below.

1.14. Immune surveillance of tumours

The immune system has three primary roles in the prevention of tumours (Dunn *et al*, 2004):

1. Elimination of virus infected cells to protect the host from virus-induced tumours for example secondary lymphomas driven by Epstein Barr virus (EBV) infections and HTLV driven T cell lymphomas.
2. Removal of pathogens in a timely manner to prevent an inflammatory environment conducive to tumour genesis.
3. Identification and removal of cancerous cells or stressed cells on the basis of their expression of tumour specific antigens or molecules induced by stress.

Immune surveillance is a process by which the immune systems identifies cancerous or precancerous cells and eliminates them before they cause harm. This concept that was first proposed half a century ago and has now been further refined by incorporating evidence of tumour genesis in a functioning immune system resulting in a more complete explanation termed as tumour immunoediting (Dunn *et al*, 2004).

The immunoediting concept is divided into three phases; elimination, equilibrium and escape (Dunn *et al*, 2004). The elimination phase has the same concept that is described in immune surveillance whereby the immune system detects and eliminates cancerous cells. This phase can be complete with removal of all cancerous cells or incomplete with the removal of only a small proportion of cancerous cells. The incomplete removal of cancerous cells results in a temporary state of equilibrium whereby the cancerous cells remain dormant or continue to evolve by facilitating further DNA changes and modulating the antigens they present. At this stage the immune system is sufficiently keeping the cancerous cells in control, but if the immune system fails to completely eliminate these cells, this can result in cancerous cells developing variants by selection that are able to resist, avoid or suppress the immune system and

eventually lead to the escape phase and proliferation of the cancer (Swann & Smyth, 2007).

The evidence of immunoediting is present in both animal and human models of cancer (Dunn *et al*, 2002). In mouse models the elimination of specific components of the mouse's immune system via gene-targeted mice has led to these mice developing cancers (Shankaran *et al*, 2001). For example, mice lacking in T cell and NK cell cytotoxic effector pathway due to the lack of perforin, which is used by cytotoxic T cells and NK cells to form membrane pores on target cells have been shown to develop spontaneous lymphomas with time (Street *et al*, 2002). The lymphomas that developed in these mice were transplanted into wild type (WT) immunocompetent mice and were rejected by their intact immune system, particularly cytotoxic T cells (Smyth *et al*, 2000). Furthermore B cell lymphomas in mice lacking both perforin and β_2-microglobulin are rejected by either NK cells or T cells following transplantation to WT mice supporting the notion of MHC-class I molecule expression on cancer cells will influence and mediate the effector cells involved (Street *et al*, 2004). In addition there have been many other studies using gene targeted mice to knock out certain aspects of the immune system including loss of immune cells and production of cytokines to demonstrate and support immune surveillance. This has been reviewed in more detail elsewhere detailing evidence of immunoediting in mice models (Swann & Smyth, 2007).

In humans, a number of clinical observations have provided supporting evidence of cancer immune surveillance. In immunosuppressed patients there is higher risk of malignancy. Immunosuppression to prevent transplant rejection is associated with up to a hundred fold increase in certain malignancies among

which lymphomas predominate (Penn, 1988; Opelz & Döhler, 2004). Additionally immunocompromised patients with acquired immunodeficiency such as in HIV patients are more prone to cancers (Ponce *et al*, 2014; Yanik *et al*, 2013).

The number of T cells and NK cells present in patients with lymphoma, have been associated with prognosis. In Follicular lymphoma which is a B cell lymphoma, a study reported by Shafer et al, demonstrated an inferior overall survival of patients with follicular lymphoma who exhibited low NK cell count ($CD3^-CD56^+$ +/- CD16) in the peripheral blood (Shafer *et al*, 2013). In addition the number of absolute lymphocytes have also been observed to predict outcomes in follicular lymphoma (Siddiqui *et al*, 2006). More recently Nygren, et al reported on the number of T cells surrounding MCL in lymph node biopsies. They observed a lower number of T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$) within the biopsies correlated to poorer outcomes (Nygren *et al*, 2014).

Multiple myeloma has been studied to demonstrate evidence of immunoediting due to the presence of the premalignant state known as monoclonal gammopathy of undetermined significance (MGUS). MGUS can be easily identified and interestingly T cells from the bone marrow of patients with MGUS mount strong responses to autologous premalignant cells, which is not detected in patients with multiple myeloma (Dhodapkar *et al*, 2003). Furthermore, certain low grade lymphomas can remain stable for long periods of time without progression such as follicular lymphoma and *in-situ* mantle cell lymphoma supporting the possibility of the immune system maintaining some equilibrium (Hsi & Martin, 2014; Swann & Smyth, 2007; McKay *et al*, 2012).

In general there is varying evidence supporting immunoediting in humans. Further evidence to support the involvement of the immune system in eliminating cancerous cells has been demonstrated by immunomodulatory drugs such as lenalidomide which is discussed later (Acebes-Huerta *et al*, 2014; Richardson *et al*, 2010; Dauguet *et al*, 2010). These are a few examples from many studies that support the concept of immunoediting and immune surveillance in cancer.

1.15. Immune escape of tumours

Although the immune system prevents or restricts tumour growth, it is eventually overwhelmed or evaded allowing the tumour to proliferate. There are a number of mechanisms by which the cancerous cells can escape elimination by the immune system some of which include, modified antigens presented by cancerous cells leading to T cell inactivity (Schwartz, 2003), expression of T cell inhibitory molecules such as B7-H1, HLA-G or HLA-E by cancerous cells, such as the expression of B7-H1 in mantle cell lymphoma (Wang *et al*, 2013a), tumour antigen loss or modification of MHC molecules (Drenou *et al*, 2002) and presence of suppressive T regs (Swann & Smyth, 2007).

As previously discussed, loss or reduction of MHC class I and II is commonly observed across many lymphoma types. Certain mechanisms have been proposed in relation to the reduction in MHC expression. One such event is the deletion of chromosome 6 resulting in reduced MHC class II at a protein level which has been evident in B cell lymphoma found in the central nervous system and testes (Riemersma *et al*, 2000). In addition, high grade B cell lymphomas lose MHC II as part of their partial differentiation towards the plasma cell stage (Wilkinson *et al*, 2012). Next generation sequencing efforts in B cell lymphomas

have demonstrated recurrent mutation in β_2-microglobulin, a part of the MHC class I molecule that is necessary for cytotoxic T cells to recognize tumour cells. Large proportion of lymphomas show disruption of β_2-microglobulin surface expression with half of them containing genetic mutation or deletions (Challa-Malladi *et al*, 2011). Although the lack or reduced MHC class I would be expected to escape cytotoxic T cell attack, NK cell would normally be expected to detect these cells. However these tumours show loss of CD58, a ligand for C2-receptor which is involved in the recognition and destruction of cells by both NK and cytotoxic T cells (Scott & Gascoyne, 2014).

The increase in number of T regulatory cells and tumour associated macrophages in the tumour microenvironment of lymphomas have shown to be associated with worse outcomes and tumour progression (Ame-Thomas *et al*, 2012; Qian & Pollard, 2010). T regulatory cells as discussed above have been shown to inhibit proliferation of the infiltrating cytotoxic T cells. During tumor initiation, macrophages create an inflammatory environment that is mutagenic and promotes growth. As tumours progress to malignancy, macrophages stimulate angiogenesis, enhance tumour cell migration and invasion, and suppress antitumor immunity which can be typically observed in a subtype of macrophages termed as M_2 (Qian & Pollard, 2010; D Foey, 2015). In the context of MCL the importance in pathogenesis and/or disease progression has not been systematically studied or characterised as described earlier. It has been shown that higher number of macrophages have been linked to more aggressive disease (Argatoff *et al*, 1997). These findings suggest that macrophages may play an important (yet ill defined) role as lymphoma-associated macrophages as described in other lymphomas and cancers (Farinha *et al*, 2005; Condeelis & Pollard, 2006).

Cancer cells can become resistant to cytotoxicity due to the mutation in the gene encoding for FAS and TRAIL receptors and expansion of immature myeloid cell population can lead to significant T cell suppression. Therefore cancerous cells can alter the tumour microenvironment and skew the immune response such that tumour growth, rather than elimination is favoured (Swann & Smyth, 2007). The interaction between tumours and the immune system is extremely complex and although there is greater understanding of this process there are still many aspects that remain unexplained.

1.16. Treatments options for MCL

There is no standard curative therapy for MCL. Although, almost all patients respond to initial therapy the majority have incomplete responses and often relapse. The natural course of MCL is characterised by multiple relapses with increasing chemo-resistance over time. The clinical course and response to therapy is heterogeneous. Some patients having a very aggressive presentation and succumb to MCL in a relatively short period of time, whilst other patients have an indolent clinical course. Most clinical trials demonstrate a median duration of response of 1.5 to 3 years and the median overall survival (OS) of 4-6 years (Vose, 2013). The choice of initial treatment is largely made based on the patient's age and fitness (Campo & Rule, 2015), however there are 10-30% of the patients presenting with clinically indolent MCL where initial therapy can be safely deferred without negatively impacting on their OS which can be up to 12 years (Martin *et al*, 2009; Eve *et al*, 2009; Hsi & Martin, 2014).

1.17. First-line therapy for young and fit patients

For young fit patients (generally <65 years) with no significant morbidity the aim should be to proceed with cytarabine (AraC) based high-dose therapy followed

by consolidation with an autologous stem cells transplant in first remission (Shah & Rule, 2014; Campo & Rule, 2015; McKay *et al*, 2012). Ara-C has been shown to have excellent cytotoxic activity in MCL, with improved overall response (ORR) and complete responses (CR) (Khouri *et al*, 1998; Ritchie *et al*, 2007; Forbes *et al*, 2013; Geisler *et al*, 2012b). High dose-AraC (HD-AraC) was first used in the dose intense hyper-CVAD regimen (hyper-fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone [steroids are used in the treatment of lymphomas due to their activation of apoptosis by death-inducing genes and repression of transcription factor activity, thereby inhibiting the transcription of growth / survival genes (Greenstein *et al*, 2002)]) with good results (Khouri *et al*, 1998) and as MCL is a CD20 expressing neoplasm, the addition of rituximab, an anti-CD20 monoclonal antibody, to this regimen produces higher CR rates of 87-92% (Ritchie *et al*, 2007). CD20 is a pan B cell specific differentiation antigen that is expressed on mature B cells and in most B-cell non-Hodgkin's lymphomas, but not on early B-cell progenitors or late mature plasma cells (Tedder *et al*, 1988). CD20 is part of a multimeric cell-surface complex that regulates calcium transport and is involved in the regulation of B-cell activation and proliferation (Beers *et al*, 2010). However, studies involving disruption of the gene encoding CD20 in mice do not demonstrate a critical function for this molecule in B-cell development or the generation of immune responses (Uchida *et al*, 2004). Rituximab is a chimeric monoclonal antibody (incorporating human immunoglobulin G1 heavy-chain sequences and murine immunoglobulin variable regions) that recognizes the human CD20 antigen (Reff *et al*, 1994). Rituximab can cause the death of lymphoma cells by several mechanisms which include redistribution of CD20 into large lipid rafts in the plasma membrane and to have a strong complement-

dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity but has a minimal direct antitumor effect. However the exact mechanism is still debated (Weirda *et al*, 2010). Other mechanism of action on lymphoma cells is by binding of the antibody to CD20 which may activate the complement cascade through C1q, leading to cell death or deposition of complement proteins on the cell membrane, a phenomenon known as complement-dependent cytotoxicity. Antibody-coated cells may be killed by immune cells expressing Fcγ receptors through antibody-dependent cell-mediated cytotoxicity. Finally, antibody binding to CD20 may have direct antiproliferative effects or may actively induce cell death (apoptosis)(Maloney, 2012). These effects appear to be additive and possibly synergistic when anti-CD20 antibody therapy is combined with chemotherapy.

However, R-hyper-CVAD regimen is associated with significant toxicity (Merli *et al*, 2012). Subsequent studies incorporated the HD-AraC derived from hyper-CVAD regimens. The *MCL2* study (Geisler *et al*, 2012b),used R-CHOP (rituximab (R), cyclophosphamide, vincristine, doxorubicin and prednisolone) alternating with R + HD-AraC followed by autologous stem cell transplant. ORR was 96% and CR was 54%. The 6 year overall, event-free and progression free survival (PFS) was 70%, 56% and 66% respectively. Other combination chemotherapy regimens which include R and / or HD-AraC are shown below in Table 2.

Induction	Consolidation	N	OR (CR) %	Median response	Median OS	Ref
R-Hyper-CVAD / R-MA	-	97	97(68)	64% - 3 yrs FFS	82% 3-year	(Romaguera *et al*, 2005)
R-Hyper-CVAD	-	49	86 (55)	4.8 yrs PFS	6.8 yrs	(Bernstein *et al*, 2013)
R-Hyper CVAD	-	60	83 (72)	61% 5yrs PFS	73% 5yrs	(Merli *et al*, 2012)
Hyper-CVAD/HD MTX & cytarabine	TBI, high dose CY, autologous/ allogenic SCT	45	93.5/38	72% 3yr EFS	92% 3yr	(Vandenberghe *et al*, 2003)
R-CHOP x 3 + HD-Ara-C x 1	BEAM ASCT	60	70/64	36% 4-yr FFS	66% 4 yr FFS	Van't Veer et al 2009
R-maxi-CHOP x 3 / R-HD-Ara-C x 3	BEAM / BEAC followed by R-in vivo purged ASCT	160	96/54	56% 6 yr EFS	70% 6 yr	(Geisler *et al*, 2012a)
R-CHOP vs CHOP	Dexa BEAM, TBI HD CY followed by ASCT vs IFN maintenance	62/63 vs 60	97/34 – 92/33 vs 75/7 -75/8	PFS: 20 vs 19 months, TTF : 28 vs 14 months	76% vs 76% 2 yrs, 59% vs 46% 5yr	(Dreyling *et al*, 2014)
R-CHOP x 6 vs R-CHOP x 3 / R-DHAP x 3	RCHOP : DexaBEAM, HD CY, TBI followed by ASCT RCHOP/RDHAP : HD-Ara-C Melphalan- ASCT	497	P-I 90/24 vs 94/39 After ASCT 97/63 vs 97/65	TTF: 49months vs NR : RD after ASCT 48months vs NR	79% vs 80% 3 yr	(Hermine *et al*, 2012)

R-DHAP –rituximab, dexamethasone, cytarabine, ciplatin ; MA-methotrexate; TBI-total body irradiation; SCT –stem cell transplant; CY-cyclophosphamide; IFN- interferon alpha; OR-overall response; CR-complete response; PFS-progression free survival; OS-overall survival; Ref – reference; FFS-failure free survival; TTF-time to failure; BEAM-carmustine, etoposide, cytarabine, melphalan; Dexa- dexamethasone; ASCT – autologous Stem cell transplant; P-I – post induction; HD AraC- high dose cytarabine; N- number of patients; NR- not reported.

Table 2: Intensive frontline therapy in MCL

Table illustrates current intensive front-line therapies available for MCL. Table lists clinical trials conducted for these intensive treatments.

1.18. First line therapy for elderly or unfit patients

The majority of patients diagnosed with MCL are elderly (figure 1.1). High dose cytarabine and consolidation with an autologous stem cell transplant is not suitable for these patients due to the increased toxicity (McKay *et al*, 2012). There is no generally accepted standard front line therapy for this group of patients. However, incorporating Rituximab to standard chemotherapy regimes provides better results with improved OS (median survival 27 vs 37 months in chemotherapy alone versus rituximab and chemotherapy, respectively)(Schulz

et al, 2007; Griffiths *et al*, 2014). Overall, this group of patients receive a combination of rituximab with chemotherapy as shown in Table 3.

Chemotherapy	N	Median age	ORR(CR) %	Median PFS Months/ %	Median OS	Ref
R-Chlorambucil	20	64yrs	95 (90)	84%	3yr 95%	(Sachanas *et al*, 2011)
R-B vs. R-CHOP	94	70yrs	93 (40) Vs. 91 (30)	22 Vs 35	N/A	(Rummel *et al*, 2013)
R-CHOP Vs R-FC	560	70yrs	87/50 vs 78/52	R vs IFN : 57%vs26% at 4 years	64 vs 40 (R vs IFN; 87%vs 57%- 4 years)	(Kluin-Nelemans *et al*, 2012)
R-B-AraC	40	70yrs	100/95 for treatment naïve 80/70 for relapsed	95% 2 year – untreated 70% 2 year relapsed treated	NR	(Visco *et al*, 2013)

N – number of patients; ORR-Overall response rate; CR-complete response rate; PFS- progression free survival; OS-overall survival; Nr – not reached; na – not applicable; IFN – interferon-alpha; R-rituximab; FC- fludarabine and cyclophosphamide; R-B – rituximab and bendamustine; R-CHOP- rituximab and cyclophosphamide, vincristine, doxorubicin and prednislone; AraC – cytarabine. N/A – not available; yrs- years

Table 3: Therapy for elderly and unfit patients
Table illustrates therapy options for elderly and unfit patients.

1.19. Therapy in relapsed or refractory MCL and novel agents

There is no standard treatment for patients which relapse. Alternative combinations of immuno-chemotherapy may be used to those that have been used in front line therapy. In the majority of cases the responses and remission are inferior to front line therapy. In some younger and fit patients allogenic stem cell transplant can be considered and a recent study reported on a 2 year OS of 46% in patients who have relapsed after an autologous stem cell transplant, with patients relapsing after 1 year from an autologous stem cell transplant having better outcomes (Dietrich *et al*, 2014).

As with other haematological malignancies novel agents have transformed the treatment of MCL by targeting certain key molecules and/or signalling pathways involved in tumour genesis. There are four novel agents that have been licensed in relapsed and refractory MCL (Table 4). Ibrutinib is the most recent drug to gain its licence in both USA and Europe for the treatment of relapsed MCL. It was approved in the USA by the Food and Drug Adminstration (FDA) for use after 2nd line therapy in relapsed MCL. Ibrutininb is an orally available small molecule that inhibits the Bruton tyrosine kinase (Btk). Btk itself is a member of the TEC family of non-receptor tyrosine kinases (Burger & Buggy, 2013). It is a key signal protein lying downstream of the B- cell receptor (BCR) where it is recognised to be activated by BCR-linked signal molecules such as LYN and SYK (Baba *et al*, 2000). It is also activated by other important signals encountered by the B lymphocytes, including growth factors, chemokines, cytokines, integrins and Toll like receptors placing it at a key point in the control of signals between the B lymphocyte and its microenvironment. Btk is principally found within the cytoplasm and activation causes translocation to the membrane. Once activated Btk phosphorylates phospholipase Cγ2, triggering secondary messengers that activate NF-ƙβ. These signal process is critical for the regulation of apoptosis, promotion of cell survival and further B-cell development (Niemann & Wiestner, 2013; Rinaldi *et al*, 2006; Mohamed *et al*, 2009). Therefore inhibiting Btk will lead to early cell death by switching off and altering the survival pathway. The pivotal phase II trial recruited 111 patients with relapsed/refractory MCL. The ORR was 68% with 47 and 21% achieving partial response (PR) and complete response (CR) respectively. The PFS was 13.9 months in this cohort of patients (Wang *et al*, 2013b). Ibrutinib is the most active single agent in relapsed / refractory MCL (Wang *et al*, 2013b).

Interestingly, off target effects of ibrutinib can affect the Interleukin 2-inducible kinase (ITK) resulting in the inhibition of this kinase. This causes inhibition of Th2 cells after TCR stimulation which is specific for Th2-polarised CD4 T cells which inhibits the T cell response to parasites such as leishmaniasis (Dubovsky *et al*, 2013). However, Ibrutinib also affects other TEC family kinases (TFK) which includes Btk, ITK, BMX and RLK. Ibrutinib inhibits TFK in other cells such as macrophages, neutrophils, dendritic cells and mast cells, therefore effectively inhibiting their function resulting in susceptibility to infections and lower counts of these cells (Berglof *et al*, 2015). Ibrutinib is a new drug and many 'off' target effects are slowly coming to light as further studies and more clinical experience is gathered.

Bortezomib is a highly selective and reversible inhibitor of the 20S proteasome. In the context of MCL it has the capacity to block proteasomal degradation of IκBα (thus inhibiting NFkB activation) as well as the cyclin-dependent kinase inhibitor p27 (Piperdi *et al*, 2011; Pham *et al*, 2003). The PINNACLE trial, in which bortezomib gained its FDA approval in USA, recruited 155 relapsed refractory MCL and reported a 33% ORR with 8% CR with 6.5 months PFS (Goy *et al*, 2009).

Temsirolimus is a selective inhibitor of the mammalian target rapamycin (mTOR) kinase which forms part of the pathway that regulates the translation of cyclin D1 from cyclin D1 mRNA. The OPTIMAL study recruited 162 relapsed refractory MCL patients and had a ORR of 22% and CR rate of 2% with duration of response of 7 months (Hess *et al*, 2009).

Treatment	No. Patients	ORR	CR	Median DOR (months)	Median PFS (months)	Median OS (months)
Ibrutinib	111	68%	21%	17.5	13.9	Not reached
Bortezomib	155	33%	8%	9.2	6.5	23.5
Lenalidomide	134	28%	8%	16.6	4	19
Temsirolimus	122	22%	2%	7.1	4.8	12.8

CR: Complete Response. DOR: Duration of Response. ORR: Overall Response Rate. OS: Overall Survival. PFS: Progression-free Survival.

Table 4: Single agent therapies approved for relapsed and refractory MCL

In contrast to the above agents that target specific molecules, pathways or cellular components important in MCL tumour genesis, the immunomodulatory drug lenalidomide is believed to act by multiple mechanisms targeting both the malignant cell and tumour microenvironment. Lenalidomide got FDA approval on the back of the EMERGE trial which recruited 134 relapsed and refractory MCL patients and reported an ORR and CR of 28% and 8% respectively with a PFS of 16.6 months (Goy *et al*, 2013).

Lenalidomide is a second generation synthetic derivative of glutamic acid and a thalidomide analogue. Thalidomide was marketed in the 1950s as a sedative drug but became a popular treatment for morning sickness in early pregnancy due to its concomitant antiemetic properties. Its withdrawal in the 1960s was secondary to congenital malformations like phocomelia. Following an observation in 1965 that thalidomide administration improved the inflammatory lesions of erythema nodosum leprosum (ENL) in a patient suffering from sleep difficulties the use of thalidomide continued (Sheskin, 1980) and eventually gained FDA approval for its use in ENL. The research into its mechanism of action unravelled an immunological and immunomodulatory effect. Thalidomide was used in several disease states associated with immune activation and it was apparent that thalidomide was a drug with a wide range of clinical

application and was especially used to treat in multiple myeloma (Singhal *et al*, 1999).

The efficacy of thalidomide as a single agent in MCL is limited to two case studies of patients with relapsed disease who achieved durable partial remission following treatment (Damaj *et al*, 2003; Wilson *et al*, 2002). A study investigating the use of thalidomide in 19 patients with heavily pre-treated lymphoma was disappointing with only 1 response overall (in a patient with gastric marginal zone lymphoma) and no responses in 3 patients with MCL (Pro *et al*, 2004). A further study in patients with 16 relapsed MCL reported by Kaufmann *et al* demonstrated a more favourable response with thalidomide and rituximab in combination. They achieved an overall response of 81% with 31% complete responses and a median progression free survival of 20.4 months (Kaufmann *et al*, 2004). This resulted in clinical evidence of activity of thalidomide in MCL. Furthermore, Richardson et al demonstrated significant activity of thalidomide and lenalidomide in-vitro on MCL cell lines resulting in immunomodulatory anti-lymphoma activity by enhancing peripheral blood mononuclear cell mediated cytotoxicity (Richardson *et al*, 2010). Despite the benefits of thalidmode, its long-term use was also marred by significant side effects of constipation, somnolence, thrombosis and peripheral neuropathy. Subsequently, lenalidomide was developed to have enhanced immunomodulatory and anti-tumour activity compared to thalidomide whilst possessing a better side effect profile especially with regards to neuropathy and thrombosis. It evolved from adding an amino group (NH_2) at the position 4 of the phthaloyl ring and removal of carbonyl group (C=O) of the 4-amino-substituted phthaloyl ring (Bartlett *et al*, 2004)(figure 9.0).

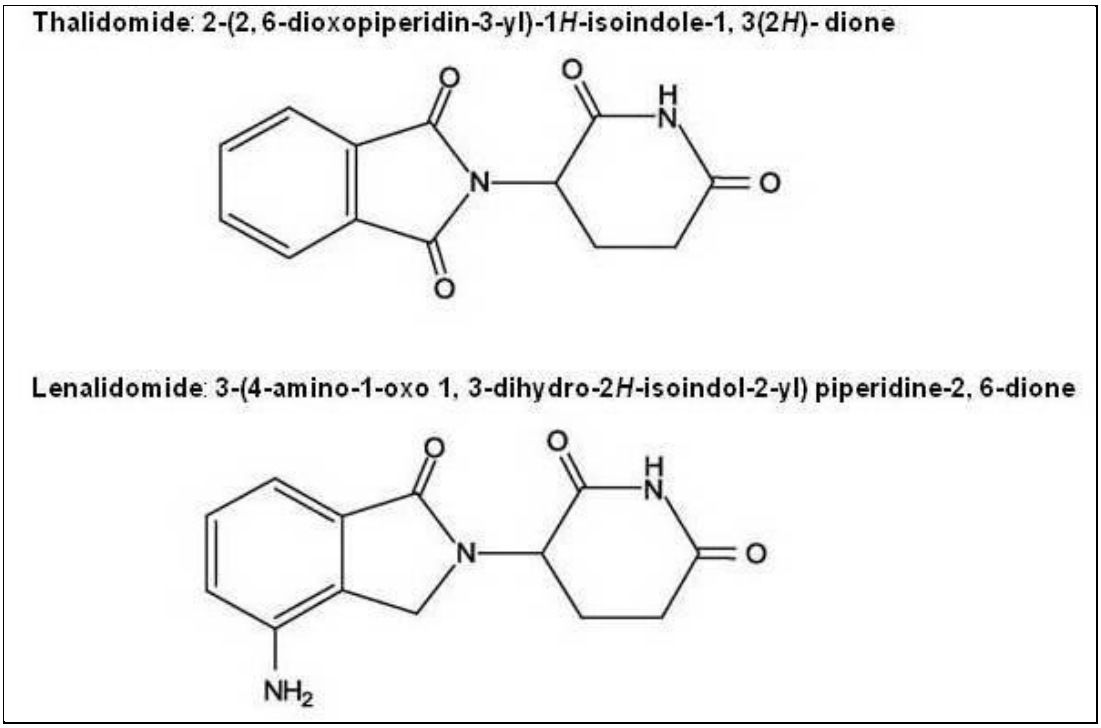


Figure 5: Chemical structure of thalidomide and lenalidomide

Lenalidomide developed from the thalidomide back bone as described above. Amino group NH2 was introduced at the position 4 of the phthaloyl ring as shown above. Adapted from (Kotla et al, 2009).

1.20. Mechanism of action of lenalidomide

In vitro and *in vivo* experimental studies have demonstrated lenalidomide to work through multiple mechanisms, including direct tumour cytotoxicity, inhibition of angiogenesis and osteoclastogenesis, and disruption of stromal cell-derived signals from the tumour microenvironment (Chanan-Khan & Cheson, 2008).

Immunomodulation

The immune system is comprised of cellular (macrophages, dendritic cells, NK cells, T cells and B cells), and humoral components (antibodies, cytokines). The

immune system can prevent development of cancers by eliminating or suppressing oncogenic viral infections, altering the inflammatory milieu conducive to tumour genesis, and by immune surveillance; identifying and destroying cancerous cells before they can cause harm (Dunn *et al*, 2004; Swann & Smyth, 2007).

The immunomodulatory properties of lenalidomide have been demonstrated by its clinical efficacy in multiple myeloma, chronic lymphocytic leukemia (CLL) and myelodysplatic syndrome by the alteration of cytokine production, regulating T cell stimulation and enhancing the NK cell cytotoxicity.

Influencing cytokine production

Cytokines are soluble proteins produced by a variety of haematopoietic and non-haematopoietic cells and are critical for the innate and adaptive immune responses. Cytokines can influence the cells by gene activation, growth, differentiation and expression of surface molecules. The harmonisation between cellular and humoral (cytokines, antibodies) interactions enables tumour elimination.

Lenalidomide inhibits the production of TNF-α, IL-1, IL-6, IL-12 and increases the production of anti-inflammatory cytokine IL-10 from human peripheral blood mononuclear cells (PBMCs) (Corral *et al*, 1999). Modulation of these cytokines within the nodal microenvironment likely influences inflammatory responses, supports tumour growth and metastasis, and contributes to chemoresistance. Zhang et al demonstrated IL-6 to be protective towards MCL cells (Zhang *et al*, 2012). IL-6 receptor ligation initiates a downstream kinase signalling cascade (such as STAT3, Ras, phosphoinositide 3-kinase/Atk) to promote tumour genesis. In some MCL cells, IL-6 secretion provides an autocrine growth signal.

Bone marrow stromal cells secrete high levels of IL-6 and PBMCs secrete both IL-6 and the soluble gp80 IL-6 receptor subunit (Zhang *et al*, 2012). Because both stromal cells and PBMCs can be found in the MCL microenvironment, they may provide a paracrine source of IL-6 for supporting MCL growth. Consistent with this hypothesis, IL-6/gp80 knockdown effectively allows chemotherapy-induced apoptosis to occur on exogenous addition of IL-6 or gp80, rather than supporting tumor growth and proliferation. Therefore the ability of lenalidomide to reduce IL-6 and this STAT3 activity may provide mechanisms for reducing signalling within the MCL microenvironment resulting in MCL growth retardation and promoting apoptosis (Chanan-Khan & Cheson, 2008).

Lenalidomide also stimulates production of IL-2, IFN-γ and TNF-α. This allows T-cell proliferation in the absence of CD28 stimulation (Hayashi *et al*, 2005a; Corral *et al*, 1999). Because T-cell receptor and costimulatory signals are required for IL-2 production, these observations suggest that lenalidomide may activate costimulatory signals normally triggered by CD28. In addition lenalidomide increases tyrosine phosphorylation of CD28 in the intracellular domain of T cells in absence of costimulatory molecules, and stimulates NF-ƙB activation downstream from CD28 (LeBlanc *et al*, 2004). It also promotes nuclear translocation and binding of nuclear factor of activated T cells and activator protein-1 to the IL-2 promotor, a process dependent on PI3K signalling that enhances IL-2 production (Hayashi *et al*, 2005a).

TNF-α is a highly pleiotropic cytokine produced by monocytes and macrophages and is important in protective immune responses against infections. However, in certain haematological malignancies high levels of TNF-α have been implicated in the proliferation of these neoplasms (Kotla *et al*,

2009). Studies have demonstrated increased levels of TNF-α in CLL patients and treatment with lenalidomide significantly reduced the TNF-α levels thus suggesting its importance in tumour genesis (Chanan-Khan *et al*, 2006).

T cell modulation

T cell activation is via peptide fragments presented by the antigen presenting cells (APCs) to the T cell receptor (TCR). However, this alone is not sufficient to generate an effective response and therefore a secondary interaction with B7 molecule on APC and CD28 on the T cell surface enables co stimulatory signal that aids in T cell proliferation, differentiation and survival with cytokine and cellular response (Sharpe & Abbas, 2006). Lenalidomide acts on the T cells via B7-CD28 co stimulatory pathway, highlighted by the ability of lenalidomide to overcome the inhibitory effects of CTLA-4-Ig (a competitive inhibitory of B7) (Leblanc *et al*, 2004). Leblanc et al demonstrated increased activity of T cells when incubated with lenalidomide in the presence of CTLA-4-Ig compared to T cells incubated only with CTLA-4-Ig (Leblanc *et al*, 2004). Although the exact mechanism for this is not clear due downstream pathways being integrally connected, however directly inducing tyrosine phosphorylation of CD28 on the T cells results in activation of downstream targets which include PI3K, MAPK and others resulting in activation of classic T cell transcription factors such as AP-1, NFAT-1 and NF-ƙB that induces secretion of IL-2, TNF-α and IFN-γ (McDaniel *et al*, 2012). Furthermore, as lenalidomide increases secretion of IFN-γ and IL-2 it leads to T cell proliferation and increase in the NK cell activity via an increase in the transcription activity of AP-1 and NFAT2, which are key regulators of the IL-2 gene (Dredge *et al*, 2002b; Hayashi *et al*, 2005b).

Influencing Natural Killer (NK) cells

NK Cells are an important part of the innate immune system. They do not require specific antigens to respond to cancerous cells and are able to kill the cells directly via the antibody dependent cell mediated cytotoxicity and natural cytotoxicity as discussed above.

Davies *et al* demonstrated increased cell cytotoxicity activity on multiple myeloma cells was largely due to the NK cells. This study demonstrated reduction of myeloma cell death on depletion of NK cells in-vitro. Furthermore incubation with thalidomide increased the NK cell numbers and IL-2 levels but the T cells numbers remained unchanged (Davies *et al*, 2001). Furthermore Hayashi et al, demonstrated an increase in NK cells when cultured with immunomodulatory drugs (thalidomide and lenalidomide) for 5 days and also enhanced ADCC activity of NK cells when incubated with immunomodulatory drugs (Hayashi *et al*, 2005b). Although these drugs stimulate NK-mediated cytotoxicity, the exact mechanism is not fully understood. It is believed the enhanced numbers and function of NK cells is secondary to the IL-2 production by the co stimulated T cells (Hayashi *et al*, 2005b; Davies *et al*, 2001). This was demonstrated by inhibition of the NK cell cytotoxicity by the IL-2 receptor being blocked by the monoclonal antibody. Additionally Hayashi et al, also demonstrated lack of phosphorylation of signalling molecules (ERK/P38MAPK/AKT/PKC) in NK cells suggesting lack of direct activation by immunomodulatory drugs (Hayashi *et al*, 2005b).

Lenalidomide as discussed above has been shown to have a significant influence on NK cells. A further expansion on the background of NK cells and

relevant activating receptors below would aid to understand their relevance with MCL and immunomodulation with lenalidomide.

The immune system can generally be divided into the innate and adaptive immune system (Medzhtov & Janeway, 2000; Gajewski *et al*, 2013). The first line defence is by the innate immune system against invading pathogens but is not antigen-specific and does not produce long-term immunological memory. The cells involved are neutrophils, macrophages and natural killer (NK) cells, all of which recognise pathogen-associated surface molecules and respond by direct elimination of the pathogen (Vivier *et al*, 2011). The adaptive immune responses are mediated by B and T lymphocytes in response to specific antigen and ultimately generate long-term immunological memory. Adaptive immunity can further be subdivided into cell-mediated immune responses, which are driven by T lymphocytes, and humoral (or antibody-mediated) responses, which are driven by B lymphocytes (Corthay, 2014; Medzhtov & Janeway, 2000). Although the two immune systems have distinctive roles there is considerable overlap between innate and adaptive immunity with the two arms working in synergy to optimise immune activity.

1.21. Natural Killer (NK) cells

NK cells are effector lymphocytes of the innate immune system that mediate two major functions; recognition and lysis of tumour and virus-infected cells and production of immunoregulatory cytokines. They develop from the same progenitor cells as T and B cells in the bone marrow and mature in secondary lymphoid tissue (Caligiuri, 2008). NK cells referred to throughout this thesis will be related to human NK cells unless otherwise stated.

NK cells are derived from the haematopoietic cells in the bone marrow; $CD34^{+}CD45RA^{+}$ progenitor cells go onto develop into NK cells which are immunophenotypically identified as $CD3^{-}CD56^{+}$. The absence of CD3 excludes T cells and CD56 is the 140-kDa isoform of neural cell adhesion molecule (NCAM) a glycoprotein found on NK cells and a minority of T cells (Lanier *et al*, 1989).

There are two phenotypically and functionally distinct subsets of NK cells distinguished by their expression of CD56 on the surface; $CD3^{-}CD56^{bright}$ and $CD3^{-}CD56^{dim}$. The $CD3^{-}CD56^{bright}$ NK cells are described to be the more immature cells predominantly located in the secondary lymphoid tissue or the decidual tissue of pregnant women and make up approximately 5-15% of the total NK cells (Fauriat *et al*, 2010). These cells are primarily cytokine producers when responding to infected or malignant cells (Fehniger *et al*, 2003), but have little or no ability to spontaneously kill target cells. The $CD3^{-}CD56^{bright}$ NK cells produce cytokines which activate nearby APCs and T cells to bridge the gap between the innate and adaptive immune systems. The $CD3^{-}CD56^{dim}$ NK cells are described to be the more mature of the NK cells and make up approximately 90% of the NK cells in the peripheral blood. They mediate a predominantly cytotoxic response which can spontaneously lyse susceptible target cells, but produce significantly fewer cytokines compared to the $CD3^{-}CD56^{bright}$ cells (Caligiuri, 2008; Takahashi *et al*, 2007). These cells have the ability to directly kill target cells through mechanisms similar to cytotoxic T cells (perforin, granzyme, FasL, TRAIL) and owing to their surface expression of CD16 (FcγRIIIa; a type III receptor for IgG), can also mediate antibody-dependent cellular cytotoxicity (ADCC). This is achieved by binding of CD16 to the Fc portion of immobilised immunoglobulin (usually IgG subclass 1 and 3) on

target cells that trigger NK cell activation, degranulation and perforin/granzyme release (Vivier *et al*, 2011; Chan *et al*, 2012).

1.22. NK cell mechanism of actions

NK cells are rapidly activated to spontaneously attack certain abnormal cells in the body, especially tumour and virus-infected cells. The NK cells predominantly target cells that have down regulated expression of MHC-class I, which is expressed on nearly every health cell in the body (Vivier *et al*, 2011). The loss or altered expression of MCH-class I is a common mechanism by which tumour cells can evade recognition by the TCR of the cytotoxic T cells and thus the NK cells can fill this potential void in the immune system by eliminating unhealthy cells that have evaded the T cells. This simplistic overview of the NK cells eliminating malignant or infected cells with lost or down regulated MHC-class I molecules forms the 'missing-self' hypothesis (Ljunggren & Karre, 1990). However, the immune process involving NK cells is far more complex, and although the detection of the expression of MHC-class I remains important in the activation of NK cells to kill target cells, NK cells also act through activating and inhibiting receptors and ligands (Vivier *et al*, 2011, 2012a). In addition to the 'missing-self' hypothesis, the balance between inhibitory and activating signals allows the NK cell to mount an immune response (Kumar & McNerney, 2005).

Activation of NK cells requires direct contact with the target cells and the formation of the immune synapse. Following initial contact, adhesion molecules expressed on the NK cell (eg. LFA-1) and target cell (eg. ICAM-1), interact with each other to further strengthen the association. NK receptors subsequently home in at the synapse site to execute a detailed surveillance of the ligands

expressed by the target cell. In the absence of inhibitory signals, activation is triggered within the NK cell that leads to cytotoxicity. Once the NK cells has completed its function it detaches from the target cell (Caligiuri, 2008; Orange, 2008).

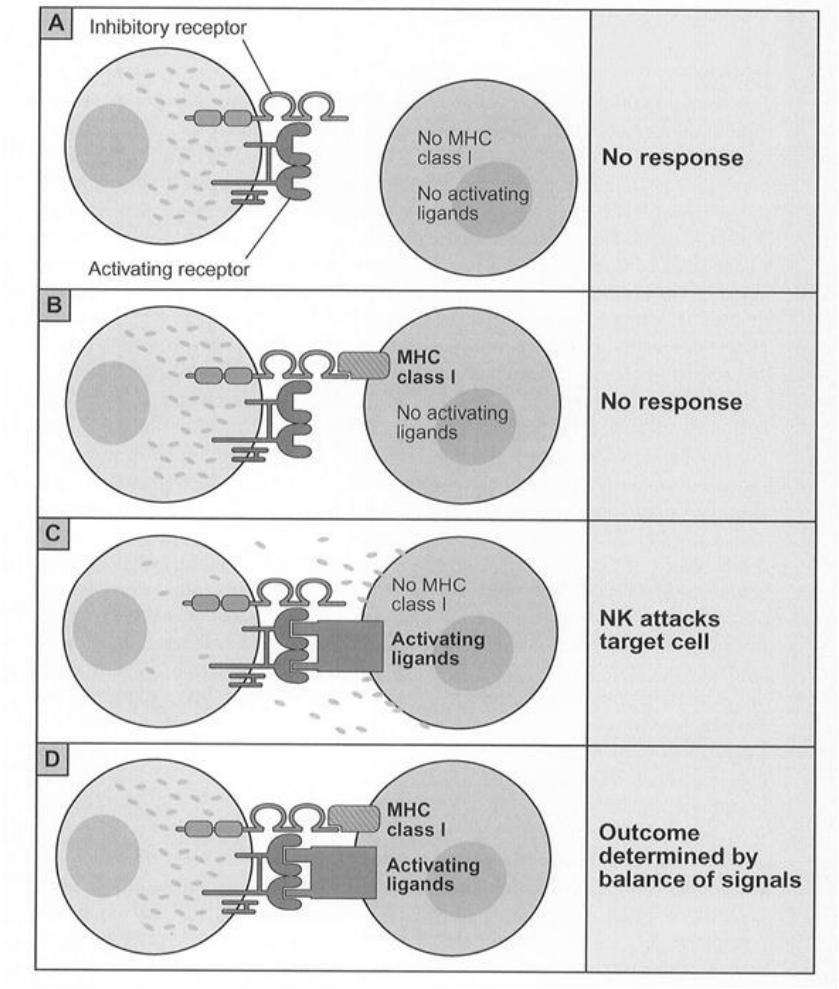


Figure 6: NK cell 'missing-self' hypothesis

[**A & B] Tolerance;** It is recognised that NK cells have both activating and inhibitor receptors which are in balance and thus NK cells may receive an activating signal which is in balance with the inhibitory signals form the ligated MHC-binding receptor resulting in no lysis of the normal healthy cell. Intact inhibitory signal will result in no response from the NK cells.

[C] Missing self; loss of MHC-binding inhibitory signals from the lack of MHC-class I expression in target tumour cells will shift the balance and activate the NK cells as the inhibitory signals are no longer held in check.

[D] Balance of signals; NK cells may be activated by stressed cells which upregulate activating ligands for NK cells thus overcoming the inhibitory signals delivered by the MHC-class 1 molecules. Therefore activation of NK cells leads to direct cell mediated cytotoxicity or indirectly through production of cytokines. However if the balance of inhibitory and activating signals are stable then it results in no response.

Adapted from (Vivier *et al*, 2011)

1.23. NK cell receptors

As described above, NK cell activation is regulated by the balance and integration of the signals triggered by ligands binding to different activating and inhibitory surface receptors. The inhibitory receptors on NK cell are mostly those that recognise MHC-class I molecules on the surface of the target cells (Seidel *et al*, 2012). Inhibitory receptors on NK cells include immunoglobulin-like transcript (ILT) 2 receptors, the killer immunoglobulin-like receptors (KIRs) and the CD94-natural-killer group 2 member A (NKG2A) receptor (Moretta & Moretta, 2004).

The activating receptors on NK cells include natural-killer group 2 member D (NKG2D), activating KIRs, NKp80, CD94/NKG2C, DNAX accessory molecule, 2B4 and natural cytotoxicity receptors (NCRs); NKp30, NKp44 and NKp46 (also known as NCR3, NCR2 and NCR1 respectively) (Koch *et al*, 2013). The major receptors responsible for NK cell activation are NKp46, NKp30, NKp44 and NKG2D. These activating receptors play an important role in NK mediated killing of most tumours and more over the surface density of these receptors correlates with the magnitude of the cytolytic activity against NK- susceptible target cells (Moretta & Moretta, 2004).

The three NKp46, NKp30 and NKp44 NCRs activating receptors and NKG2D activating receptor are the most relevant in context of MCL and will be discussed in more detail below.

The natural cytotoxicity receptors (NCRs)

The NCRs are type I membrane proteins comprising of an ectodomain with one (NKp30 and NKp44) or two (NKp46) immunoglobulin-like domains connected to

a transmembrane-spanning a-helix via a short stalk domain. NCRs belong to the immunoglobulin superfamily. They all comprise an extracellular ligand-binding domain, which binds to the cellular and exogenously derived ligands, a transmembrane domain, and a short cytosolic domain. These group of receptors lack functional intracellular signalling domains and therefore associate with appropriate adaptor proteins via a charged residue in their transmembrane domain (Hudspeth *et al*, 2013; Moretta *et al*, 2001) (figure 4.0). NCR signalling pathways involve the immunoreceptor tyrosine based activation motif (ITAM) dependent signalling molecules serving to activate several intracellular signalling pathways involving protein kinase 70 (ZAP70) and spleen tyrosine kinase (SYK) which phosphorylate transmembrane adapter molecules leading to activation of several signalling molecules like phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC) among others which are necessary for NK-mediated cytokine production and/or cytotoxicity (Koch *et al*, 2013). It has been described that the NCR can activate each other through phosphorylation of the adapter molecules in different NCRs, thus increasing their efficiency. However, the exact mechanism for activation with different ligands is not known and currently under investigation.

NKp30

NKp30 is associated with ITAM to initiate signalling. ITAM also contains the adapter proteins CD3ζ and its heterodimers have the γ-chain of the high affinity Fc receptor for IgE (FcεRIγ). In malignantly transformed cells, the NKp30 receptor recognises the tumour antigen B7-H6 and BCL-2 associated athanogene 6 (BAG 6). B7-H6 is thought to be a tumour specific antigen because it expression has not been found on healthy cells in steady state.

Detection of B7-H6 leads to eradication of the tumour cells by the NK cells (Brandt *et al*, 2009). In contrast, BAG 6 is found on the plasma cells of the immature dendritic cells and can trigger NK cells to kill these immature dendritic cells (Koch *et al*, 2013). Elimination of these immature dendritic cells is thought to select out a more immunogenic subset of dendritic cells during a protective immune response.

NKp44

NKp44 is coupled with a dimer of the ITAM-containing adaptor DNAX-activation protein 12 for downstream signalling. NKp44 is only detected after activation of the NK cells (Rosental *et al*, 2011). The extracellular domain of the NKp44 adopts a positively charged V shape groove on one site for a yet unknown ligand on tumour cell. However in the presence of tumours this receptor had an increase in expression which correlated to the level of cell mediated cytotoxicity (Moretta *et al*, 2001).

NKp46

NKp46 is deemed as the most specific marker for NK cells (Walzer *et al*, 2007). It is associated with CD3ζ and also with the γ-chain of the high affinity Fc receptor for signalling. The ectodomain of NKp46 comprises of two immunoglobulin-like domains which may be the ligand binding sites (Arnon *et al*, 2006). The identity of the ligand expressed on the tumour cells to activate NKp46 remains unknown. However, as with the other NCRs it plays an important role in tumour elimination.

NKG2D Receptor

NKG2D is a C-type lectin-like transmembrane activating receptor. It is present on the majority of the NK cells and also on cytotoxic T cells. NKG2D recognises two families of ligands; MHC-class I chain-related protein A (MICA) and B (MICB), and the UL16-binding proteins (ULBPs) (Champsaur & Lanier, 2010). These ligands are typically present on many tumours, whilst rarely expressed on healthy cells (López-Soto *et al*, 2014). Ligand engagement of NKG2D triggers the phosphorylation of the signalling adapter protein DNAX-activating protein of DAP10 and recruitment of either phosphatidylinositol-3-kinase (PI3K) or a Grb2-Vav1 complex to engage full activation (Coudert *et al*, 2005). Sole activation of NKG2D receptor in inactivated NK cells is unable to trigger activation of the NK cell and needs co-activation signal from other receptors such as 2B4 or NKp46 receptor. The result of NKG2D activation leads to NK cells' secretion of cytokines and exocytosis of cytotoxic granules (López-Soto *et al*, 2014; Lanier, 2003).

The NCRs and NKG2D have other ligands capable of activating the NK cells through these receptors. However the majority of the ligands that have been identified are associated with virally infected cells as opposed to tumour cells. Notably many tumour related ligands remain unknown and there is currently ongoing research to identify them.

Inhibition of Angiogenesis by lenolidomide

Vascular endothelial growth factor (VEGF) and its receptors are required for the formation of blood vessels during carcinogenesis (D'Amato *et al*, 1994). Thalidomide and the newer immunomodulatory drug; lenalidomide have been shown to significantly decrease the expression of angiogenic factors VEGF and Interleukin-6 (IL-6) in multiple myeloma; thereby reducing angiogenesis and hence contributing to clinical activity in multiple myeloma (Gupta *et al*, 2001). The newer IMiDs like lenalidomide were found to be 2–3 times more potent compared to thalidomide in antiangiogenic activity in various in-vivo assays (Dredge *et al*, 2002a). These properties have been found to be independent of the immunomodulation exerted by lenalidomide. The exact mechanism in MCL has not been fully elicited. However, a recent study has demonstrated that lenalidomide can reduce and inhibit the recruitment of the lymphoma-associated macrophages which are known to produce VEGF, therefore reducing lymphangiogenesis (Song *et al*, 2013; Kerjaschki, 2005)

1.24. MCL epidemiology

Like other NHLs the incidence of MCL increases with age with a median age at presentation being 65 years (Smith *et al*, 2015; McKay *et al*, 2012). This in-part could be as a result of immunosenescence, a complex process in which immunological function is impaired and or remodelled and is believed to be a major contributory factor to the increased frequency of morbidity and mortality among elderly (Caruso *et al*, 2013). This relationship with age and immunity was explored by analysing 356 healthy Japanese ranging from 20 years to 90 years. This study showed reduction of number of T and B cells and proliferation

of T cells with age. The rate of decline was greater in males compared to females (Hirokawa *et al*, 2013).

The annual incidence of MCL in UK is estimated to be up to 513 (males 331 and females 181) new cases per year (based on data from 2004-2012) and age standardised incidence rates is 0.86 per 100,000 population (males; 1.5 per 100,000 and females; 0.6 per 100,000 population)(Smith *et al*, 2015).

There is a higher incidence of MCL in males than in females (Chandran *et al*, 2012; McKay *et al*, 2012; Morton *et al*, 2006). This is in keeping with reports of higher incidence of lymphomas in men compared to women in general (Nelson *et al*, 2001; Morton *et al*, 2006). The precise reason for this is not known however epidemiological data in female has shown an association between reproductive hormonal factors and oral contraceptive to reduce the risk for NHL (Nelson *et al*, 2001; Lee *et al*, 2008a). Lee *et al* reported on an epidemiological study which demonstrated that women who were on menopausal hormone therapy for greater than 5 years were associated with a reducing risk of all NHLs (Odds Ratio (OR) 0.68, 95% CI; 0.48,0.98). Additionally they also reported on oral contraceptive pill use and pregnancy reduced the risk of all NHLs (OR 0.68, 95% CI; 0.49,0.94 and OR 0.81, 95%CI: 0.55) (Lee *et al*, 2008a). This study correlated to results from an earlier study which had demonstrated similar results. Nelson et al, reported on women who used oral contraceptives, (oestrogen based) had a significantly lower risk of NHLs (OR 0.47; 95% CI, 0.26 to 0.86) than women who had never used these compounds. They also reported on postmenopausal women who used hormone replacement therapy (primarily oestrogen) had a lower risk of NHLs compared to those that did not. Furthermore postmenopausal women were at a greater risk of NHLs compared to premenopausal (Nelson *et al*, 2001). This has alluded

to the fact that oestrogen may have a protective effect with regards to the NHLs and may be involved in a mechanism by which NHLs are either supressed or eliminated. Although the precise mechanism is not fully described several studies recently have shown oestrogens could potentially influence overall survival of NHLs in women.

Lymphomas are not generally perceived as hormone-controlled. However, as mentioned above, epidemiological studies suggest their potential role in NHLs and this has led to further studies with oestrogen and lymphoma. A study reported by Yakimchuk *et al* showed that grafted T-cell lymphomas grew faster in male mice than in female mice and that female protection was abolished by ovariectomy resulting in increasing size of the lymphoma. The tumour size could be subsequently reduced by treatment with oestrogen beta agonists such as diarylpropionitrile (DPN). This at least in part resulted in lymphoma apoptosis and inhibition of proliferation (Yakimchuk *et al*, 2011a). They also reported the expression of oestrogen receptor beta (ERβ) in the peripheral blood mononuclear cells of patients suffering from chronic lymphocytic leukaemia (CLL- B lymphocyte cell disorder). They demonstrated 69% of the patients with CLL were positive for ERβ compared to only 17% of the healthy controls ($p<0.001$). This suggested that oestrogen receptors are potentially important in this B cell malignancy and could be a therapeutic target (Yakimchuk *et al*, 2012a). It is still questionable whether hormone sensitivity of lymphomas reflect primarily the sensitivity of tumour itself or rather the sensitivity of the microenvironment or both.

Interestingly , a recent study involving 1821 patients with CLL demonstrated a better 10 year survival in female patients compared to male patients.

Additionally more female patients (83%) responded better to therapy than their male (71%; p<0.0001) counterparts (Catovsky *et al*, 2014). In addition a study reported by Eve, et al, demonstrated a significantly better response to lenalidomide in female patients compared to male patients with relapsed / refractory MCL (Eve *et al*, 2012). Lenalidomide modulates the immune system to be effective, this may suggest a better immune response in females. Female hormones may also be influential. However the authors were not able to conclude on the precise mechanism of the observed differences in the response rate of female MCL patients compared to the males. Furthermore, this pattern of response has also been described in patients with other subtypes of NHL; diffuse large B cell lymphoma and follicular lymphoma where patients treated with immunochemotherapy that is, combination chemotherapy with rituximab, the progression free survival has been observed to be significantly better in the female patients (Riihijärvi *et al*, 2011).

Further studies are needed to correlate these differences in relation to the genders and whether oestrogens have a role. To further illustrate the activity of oestrogens, a general overview of oestrogens and its receptors is portrayed below.

1.25. Oestrogen and Oestrogen Receptors

Oestrogens have widespread biological actions. There are predominately three naturally occurring oestrogens; 17-β- oestradiol (E2), oestrone (E1) and oestriol (E3) (Gruber *et al*, 2002). Oestradiol is the predominant oestrogen during the reproductive years. The primary sources of oestradiol in women are the theca and granulosa cells of the ovaries and the luteinised derivatives of these cells. The theca cells secrete androgens that diffuse to the granulosa cells to be

aromatized to oestrogens. Oestrone and oestriol are primarily formed in the liver from oestradiol. In males the aromatisation of oestrogens and androgens occur in the testes and secondary tissues (Gruber *et al*, 2002).

Serum oestradiol levels steadily rise at the onset of puberty in girls to approximately 15 to 35pg per millilitre (Gruber *et al*, 2002). During menstrual cycles oestradiol production varies cyclically with the highest rate of production and concentration being in the pre-ovulatory phase (Mihm *et al*, 2011). Oestradiol production and serum concentration are lowest pre-menstrually. In the perimenopausal period there is a steady decline in ovarian oestradiol production and in postmenopausal women serum oestradiol levels are minimal and most oestradiol is formed by extragonadal conversion of testosterone (Mihm *et al*, 2011). In peripheral tissues, the production of different oestrogens and their interconversion depend on the local expression and activity of aromatase, 17β-hydroxysteroid dehydrogenases and oestrone sulfatases (Cutolo *et al*, 2012). Oestrone is the predominant oestrogen in postmenopausal women (Table 5). During the reproductive periods control of oestrogen production is exerted by gonadotrophins, however in postmenopausal women little is currently known on the factors that control the oestrogen production (Gruber *et al*, 2002).

Phase	17-β-Oestradiol	Oestrone	Oestriol
	Serum Concentration (pg/ml)	Serum Concentration (pg/ml)	Serum Concentration (pg/ml)
Follicular	40-200	30-100	3-11
Preovulatory	250-500	50-200	-
Luteal	100-150	50-115	6-16
Premenstrual	40-50	15-40	-
Postmenopausal	<20	15-80	3-11

Table 5: Serum concentrations of oestrogens in normal women
Adapted from (Gruber et al, 2002)

There are a significant number of physiological actions of oestrogens ranging from stimulation of growth and differentiation of primary and secondary sex organs, influence of mood and behaviour, arterial dilatation, cardio-protection, maintenance of bone density to neuro-protection. The specific nuclear action of oestrogen is determined by the type of hormone and the subtype of oestrogen receptor involved (Nilsson & Koehler, 2005). Given the widespread role of oestrogens in human physiology it is not surprising that oestrogens are implicated in the development and progression of numerous diseases which include but not limited to various types of cancers (such as breast, ovarian, colorectal, prostate cancers), osteoporosis, neurodegenerative diseases, cardiovascular disease and autoimmune diseases among others. In many of the diseases oestrogen mediates its effects through the oestrogen receptors as exhibited in its normal physiological function (Nilsson & Koehler, 2005).

Oestrogens influence the maturation of both T and B lymphocytes (Grimaldi *et al*, 2001; Ackerman, 2006). They decrease B cell lymphopoiesis by down-regulating IL-7 production by bone marrow stromal cells and also reduce the B

cell receptor signal strength (Grimaldi *et al*, 2002) resulting in escape from negative selection. Exposure to oestrogen decreases the number of developing thymic $CD4^+$ / $CD8^+$ T cells and promotes T cell lymphopoiesis in the liver allowing cells to escape negative selection, potentially leading to the accumulation of autoreactive cells (Pauklin *et al*, 2009). This is supported by the increase in Bcl2 expression which enhances cells survival (Verthelyi, 2001). Oestrogens also promote Th2 responses, resulting in increased levels of IL-4, IL-5 and IL-10 (Ackerman, 2006). This also results in proliferation of B cells and their maturation. Interestingly oestrogens promote elevated levels of $CD4^+$ T cells in females and females show a pre-ponderance towards Th2 immune responses and B cell activation, whereas males predominately generate Th1 $CD4^+$ and $CD8^+$ lymphocytes (Ghazeeri *et al*, 2011). Oestrogens modify the functional capacity of T regulatory cells. They increase the expression of perforin in both the natural and induced T regulatory cells thereby enhancing cell death in the target associated effector T cells (Valor *et al*, 2011). The oestrogens also increase the suppressive function of T regulatory cells by inducing production of the regulatory cytokines IL-10 and TGF-β (Pennell *et al*, 2012). Oestrogen inhibition of T cell response is thought to be due to the oestrogen stimulation via the ERα present on the T cells resulting in an increase in the Foxp3 expression thus converting the $CD4^+CD25^-$ into the regulatory T cells largely seen in pregnant females in the uterus, but can also occur in the lymph nodes and peripheral blood (Polanczyk *et al*, 2004; Yakimchuk *et al*, 2013) . Although there is a significant influence of oestrogens on the immune cells its effects are numerous and it continues to be an area of study and research.

1.26. The oestrogen receptors

Oestrogens induce cellular changes through several different mechanisms. Central to this mechanism is the protein to which oestrogens binds; the oestrogen receptor (ER)(figure 10.0)(Deroo & Korach, 2006). In the 'classical' mechanism oestrogens diffuse into the cell and binds to the ER which is loosely bound in its cytoplasmic or nuclear location and is attached to receptor-associated proteins. These proteins serve to stabilise the receptor when the ER is in an inactivated state or mask the DNA binding domains of the receptors. Once the oestrogen binds to the receptor, the receptor dissociates with the attached proteins and the oestrogen – receptor complex moves to the nucleus, where it binds to the DNA and initiates transcription. Transcription is catalyzed by RNA polymerase II and requires the assembly of various proteins, including the TATA-box–binding protein (TBP) and other associated factors at a TATA box(Deroo & Korach, 2006). This recruitment of the co-regulatory proteins to the promoter increases or decreases mRNA levels associated with protein production and physiological response. ER also interact with nuclear kappa B and activating protein-1 (AP1) to influence cellular proliferation (Nilsson & Koehler, 2005).

Oestrogens can also act through 'nongenomic' mechanisms either through the ER located in or next to the plasma membrane or through non-ER plasma membrane associated proteins, resulting in cellular responses such as increased levels of calcium, nitrogen oxide (NO) and activation of kinases as mentioned below. Non-nuclear actions of ER tend to have rapid results (Nilsson & Koehler, 2005). The gene activation occurs through second-messengers downstream of growth signalling pathways such as epidermal growth factor receptor (EGFR), the insulin-like growth factor (IGFR) and the G protein

coupled receptor (GPCR) pathways. This alters intracellular kinase and phosphatase activity, resulting in altered phosphorylation of ER (Weigel & Zhang, 1998). ER regulates genes via protein-protein interactions with other transcription factors, such as c-Fos/c-Jun B (AP-1) and nuclear factor-ƙB (NF-ƙB), resulting in alteration to cell division, angiogenesis, and survival (Barone *et al*, 2010). The kinases that get phosphorylated include Akt, extracellular regulated kinase (Erk) 1 / 2 mitogen-activated protein kinase (MAPK), p21-activated kinase 1 (PAK-1), PI3K and protein kinase A (PKA) (Likhite *et al*, 2006; Wang *et al*, 2002). These phosphorylation events are complex and interdependent leading to alteration in gene signalling.

The precise gene products related to ER stimulation is still being studied. However in ovarian epithelial cells it has been shown that stimulation of the ERα upregulates the anti-apoptotic Bcl-2 gene (Choi *et al*, 2001). In relation to ERβ in-vitro study has shown pro-apoptotic signalling in ovarian cancer mediated by upregulation of FasL, a pro-apoptotic protein ligand (Sapi *et al*, 2002).

In the context of lymphoid malignancy a study reported by Yakimchuk, et al demonstrated increased apoptosis when selective ERβ agonist diarylpropionitrile (DPN) is used on murine lymphoma cells in vivo (Yakimchuk *et al*, 2011a). Although ERβ has a generally pro-apoptotic effect and ERα an anti-apoptotic effect, the responses are cell and ligand dependent and correlate to a certain degree to the ER subtype expression.

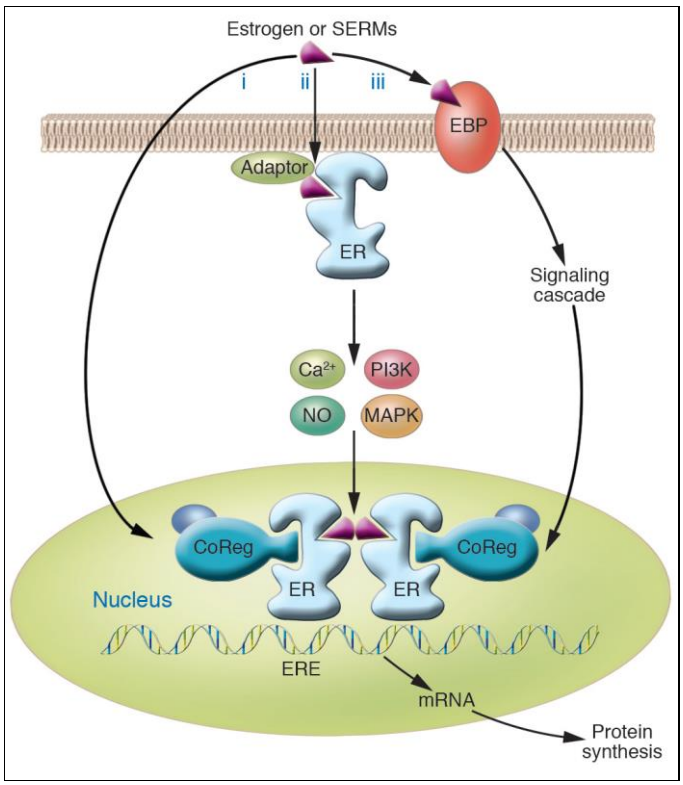


Figure 7: Diagrammatic illustration of oestrogen action with ER.

In the “classical” pathway of oestrogen action (i), oestrogen or other selective ER modulators (SERMS) bind to the oestrogen receptor (ER), a ligand-activated transcription factor that regulates transcription of target genes in the nucleus by binding to the oestrogen response element (ERE) regulatory sequences in target genes and recruiting co-regulatory proteins (CoRegs) such as coactivators. Rapid or “nongenomic” effects of oestrogen may also occur through the ER located in or near to the plasma membrane (ii), which may require the presence of “adaptor” proteins, which target the ER to the membrane. Activation of the membrane ER leads to a rapid change in cellular signaling molecules and stimulation of kinase activity, which in turn may affect transcription. Lastly, other non-ER membrane-associated oestrogen-binding proteins (EBPs) may also trigger an intracellular response (iii). Adapted from (Deroo & Korach, 2006).

There are two subtypes of oestrogen receptors; oestrogen receptor alpha (ERα) and beta (ERβ) (Mueller & Korach, 2001) and several isoforms and splice variants of each subtype are described (Deroo & Korach, 2006). The most common splice variants of ERα are expressed in multiple tissues and arise from deletions of internal exons, resulting in truncated proteins lacking segments of the DNA-binding domain (DBD) or hormone-binding (ligand-binding) domain

(LBD) of the receptor (Cheng *et al*, 2005). ERs are composed of three functional domains; the NH2-terminal domain (NTD), the DNA-binding domain (DBD) and the COOH-terminal ligand-binding domain (LBD) (Figure 8). The NTD encompasses a ligand independent activation function (AF1) domain involved in transcriptional activation of target genes and with only 16% similarity between ERα and ERβ (Jia *et al*, 2015). The DBD is highly conserved between ERα and ERβ with 97% amino acid identity and mediates sequence-specific binding of ERs to DNA sequences in target genes denoted estrogen-responsive elements (EREs). In contrast, the LBDs of ERα and ERβ show a 59% overall amino acid sequence identity yet the ligand-binding pockets of the two subtypes show only minor differences in structure. There are 3 ERα isoforms due to alterantive splicing of the ER-mRNAs. ERα∆3 lacks exon 3, which encodes part of the DNA-binding domain (Price *et al*, 2001). ERα36 lacks both AF-1 and AF-2 and the last 138 amino acids are replaced with a unique 22 sequence (Shi *et al*, 2009). ERα46 lacks the amino acid including AF-1 and was first described as a dominant negative inhibitor of ERα activity in osteoblasts (Denger *et al*, 2001). As described for ERα, wild-type ERβ (ERβ1) possesses both a DBD (C domain) and an LBD (E domain). ERβ2 codes for a variant that contains an additional 18 amino acids in the LBD, while ERβ3 lacks exon 3. ERβ4 contains both of these variations. ERβ5 lacks exon 5, and in ERβCX, the COOH-terminal 61 amino acids are replaced by a unique sequence of 26 amino acids (Moore *et al*, 1998). ERβ4 is truncated at both the N and the C termini. In humans, variants lacking exon 2, exon 4, exon 6, and exon 7 also exist (Deroo & Korach, 2006).

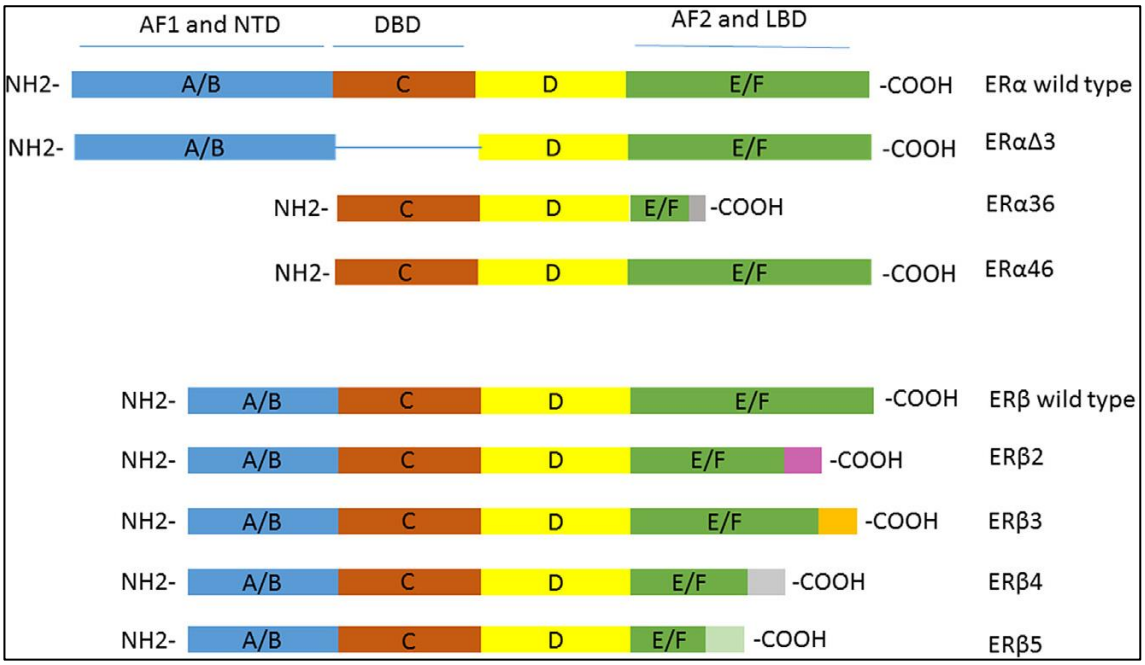


Figure 8: The Structures of the ER isoforms

The NH2- terminal domain (NTD) in blue, DNA-binding domain (DBD) in orange, and the COOH-terminal or ligand-binding domain (LDB) in green. The NTD contains a ligand-independent activation function (AF1) region which is responsible for recruitment of co-regulatory proteins. Adapted from (Jia *et al*, 2015)

ERs are members of the nuclear hormone- receptor superfamily and have several functional domains. These two receptors vary in their structure and their encoding genes are on different chromosomes. The DNA-binding domains of the two ERs are similar, but the receptors are heterogeneous in their binding domains to the ligands. As a result some oestrogens bind to the two receptors with different affinities as shown in Table 6 (Gruber *et al*, 2002).

Ligand	Oestrogen Receptor α K_i (binding affinity)	Oestrogen Receptor β K_i (binding affinity)
17-β-Oestradiol	100	100
Oestriol	14	21
Oestrone	60	37

Binding affinity K_i Values range from K_i 0 to 100, with higher values indicating greater binding affinity.

Table 6: Binding affinity of the different ligands for ERα and ERβ

The tissue distribution of ERs varies between the two receptors. Granulosa cells and developing spermatids contain mostly ERβ and this receptor is also found in other tissues such as kidney, intestinal mucosa, lung, bone marrow, bone, brain, endothelial tissue and prostate (Leitman *et al*, 2010; Shim *et al*, 2006). In contrast, endometrium, breast-cancer cells, ovarian stroma mostly contain ERα. It is thought that on both the ERs the conformation of the ligand-binding domain changes in different ways when different ligands bind resulting in variations of the transcriptional effects. For example oestradiol activates transcription when bound to ERα but exerts a suppressive effect when bound to ERβ. Although the specific effects of the various subtypes of ERs is still under investigation there is remarkable variation on its effect on various tissues and cells they are present on (Henderson *et al*, 2003; Deroo & Korach, 2006).

Furthermore, it has been suggested that ERα and ERβ elicit opposite effects on proliferation and apoptosis. Oestrogens via the ERα stimulate proliferation of the breast, uterus and developing prostate, thus increasing the risk of tumour progression in these tissues (Morani *et al*, 2006; Imamov *et al*, 2004). In contrast ERβ have exhibited a suppressive effect on proliferation of the prostate, colon, mammary glands, lung and bone marrow stem cells (Yakimchuk *et al*, 2013).

Currently there is no precise data on the exact splice variants present on lymphoma cells or MCL. The area of research is growing and of much interest.

1.27 Oestrogen Receptors on lymphocytes

Both ERα and β have been identified on immune cells. However, the expressions of the two subtypes differ in the immune subsets. $CD4^+$ T cells have a higher expression of ERα compared to ERβ and $CD8^+$ T cells have a low expression of both the ERs (Pierdominici *et al*, 2010). NK cells have both receptors expressed but in low levels (Phiel *et al*, 2005). B cells exhibit higher expression of ERβ compared to ERα which is in line with several human leukaemia / lymphoma cell lines representing chronic lymphocytic lymphoma, Hodgkin's disease, Burkitt's lymphoma and multiple myeloma (Phiel *et al*, 2005; Yakimchuk *et al*, 2013).

Thus oestrogens via their ERs have an important impact on lymphocytes, which may explain, in part, the development of gender bias in disease prevalence and immune response. The oestrogens can influence the cell signalling pathways including nuclear factor-ƙB and interferon regulatory pathways as mentioned above. Although much of the oestrogen effects on the immune system is thought to be dose dependent (Dorak & Karpuzoglu, 2012).

There are limited studies specifically in MCL in relation to oestrogens and ERs. However, due to the gender differences in the incidence of MCL and the response rate to certain therapies like lenalidmide, it is an area of increasing research.

1.28. The Main aims of this thesis

1) To investigate the quantitative difference in the immune cell subsets and expression of NK cell activating receptors between untreated male and female patients with MCL.

2) To investigate the difference in the immune response and function following exposure to lenalidomide and oestrone between untreated male and female MCL patients.

3) To look at the expression of oestrogen receptors on primary MCL cells and the distribution of selected immune cell subsets in lymph nodes of primary MCL in both male and female patients.

Chapter 2

Materials and Methods

2.0. Source of study subjects

A total number of 6 male patients (median age 64 years) and 5 female patients (median age 62 years) diagnosed with Mantle Cell Lymphoma (MCL) and 11 healthy, age matched (> 50 years) volunteers (5 males and 6 females) were selected and recruited by either myself and with the help of the haematology research team at Derriford Hospital, Plymouth, UK. Patients were recruited during their scheduled visit to the specialized Haematology Trials Clinical and age and sex matched healthy volunteers were recruited from the staff at the haematology and pathology laboratories or the haematology ward at Derriford Hospital (details of the patient are provided in Table 7 and 8). Informed consent was obtained from each patient and healthy volunteer prior to the commencement of the described studies and usage of patient materials was in accordance with the approval from the local Ethical Committee ; South West (REC ref: 09/H0203/59 - ethical approval was for 5 years from September 2009) and Cambridge (REC ref: 14/EE/1251 ethical approval from October 2014 on going with a minimum approval of 5 years). All patient material used in this study was from untreated MCL patients.

Male Patients						
Patient	**Age (years)**	**Stage of MCL**	**WBC ($x10^9$/L)**	**Lymphocyte ($x10^9$/L)**	**MIPI prognostic score**	**Clinical presentation**
MP001	59	IV	9.4	4.8	5.7- im	Indolent
MP002	56	IV	9.3	1.9	5.9-im	Aggressive
MP003	70	IV	41	37.3	6.6-high	Indolent
MP004	70	III	7.9	2.7	6.1-im	Aggressive
MP005	64	IV	18	15	6.3-high	Indolent
MP006*	77	IV	10	6.7	6.6-high	Aggressive
Female Patients						
FP001	68	IV	5.4	2.3	5.8-im	Aggressive
FP002	60	IV	10.9	4.9	5.8-im	Aggressive
FP003	43	II	7.7	2.0	4.7-low	Indolent
FP004	68	IV	20.8	19.1	6.8-high	Aggressive
FP005	62	IV	27.9	22.9	6.2-high	Indolent

MCL- mantle cell lymphoma; WBC- white blood cells: MIPI-mantle cell lymphoma international prognostic index; indolent disease- not requiring treatment > 1 year; aggressive disease- needing immediate therapy based on symptoms; * patient was recruited and only used for peripheral blood analysis of immune subsets; im- intermediate group in MIPI prognostic

Table 7: Table to show the patient characteristics

These patients were used for the experiments in the chapters 3 and 4. Staging of MCL is in accordance to the Ann-Arbour staging of lymphoma (Table 1 chapter 1) .

Male Patients						
Patient	**Age (Years)**	**Stage of MCL**	**WBC ($x10^9$/L)**	**Lymphocyte ($x10^9$/L)**	**MIPI**	**Clinical nature**
IHM-1	56	IV	9.3	1.9	5.9-im	Aggressive
IHM-2	70	III	7.9	2.7	6.1-im	Aggressive
IHM-3	77	IV	10.	3.0	6.5-high	Aggressive
Female Patients						
IHF-1	68	IV	9.0	2.6	6.1-im	Aggressive
IHF-2	60	IV	10.9	4.9	5.7-im	Aggressive
IHF-3	65	IV	7.0	2.0	5.8-im	Aggressive
MCL- mantle cell lymphoma; WBC- white blood cells: MIPI-mantle cell lymphoma international prognostic index; indolent disease- not requiring treatment > 1 year; aggressive disease- needing immediate therapy based on symptoms; im- intermediate score on MPI						

Table 8: **Patient characteristics relating to IHC and immunofluorescence**
Patient used for IHC and immunofluorescence described above were different to the cohort of patients described in table 7. This was subject to availability and access to diagnostic materials from patients. Staging of MCL described here is the same as that described above in table 7.

2.0a. General information on Materials

All sterile plastic-ware was purchased from Sarstedt (Leicester, UK) or Fisher-Scientific (Loughborough, UK), and all general laboratory reagents (eg. Phosphate buffered saline, PBS) were purchased from Sigma-Aldrich (Gillingham, UK) or Life Technologies (Paisley, UK) unless otherwise stated.

2.1. Preparation of Charcoal Dextran Stripped Heat inactivated Foetal Bovine Serum

Principle

Dextran coated charcoal is used to absorb free hormones and leave hormones that are bound to a carrier (or antibody). It is typically used to reduce oestrogen levels in foetal bovine serum and in particular dextran coated charcoal is used

to strip hormones from serum instead of charcoal alone, because there is less loss of protein.

Method

Heat Inactivated Foetal Bovine Serum (HI-FBS) (Sigma Aldrich, Gillingham, UK) used throughout was stripped of hormones with dextran coated charcoal (Sigma Aldrich, Gillingham, UK). 10g of dextran coated charcoal was added to 500mls of HI-FBS and was mixed gently for a minimum of 12 hours at 4^0C in the Innova 42 Incubator Shaker (New Brunswick Scientific, Eppendorf, Stevenage, UK). After the 12 hours had elapsed, the HI-FBS with charcoal dextran was left to stand for one hour at 4^0C before being aliqouted into 50ml polypropylene Falcon Centrifuge tubes and centrifuged at 41g (Heraeus Labofuge 400R :15cm radius, ThermoScientifc, Hemel Hempstead,UK) for 1 minute. This solution was placed in the sterile Stericup and Steritop filtration unit (Millipore, Watford, UK) which had a filtration membrane pore size of 0.22µm and under gentle suction HI-FBS with dextran coated charcoal was filtered to remove the dextran coated charcoal. The filtered charcoal stripped HI-FBS (CS-HI-FBS) was stored at -20^0C until use. The dextran coated charcoal stripping of HI-FBS was performed under sterile conditions in a class II microflow biological safety cabinet.

2.2. Preparation of cell culture reagents

1X RMPI 1640 Medium without L-glutamine and without phenol red supplemented with 10% CS-HI-FBS, 2mM L-glutamine, 100U/mL penicillin and 100µg/ml streptomycin was prepared before use and stored at 4^0C (referred to as complete culture medium [CCM] here after). Serum free media (SFM) was prepared by supplementing 1x RMPI 1640 Medium without glutamine and

without phenol red with 2mM L-glutamine, 100U/mL penicillin and 100µg/ml streptomycin. All culture reagents were prepared using heat-sterilised equipment and in a class II culture hood using aseptic techniques.

2.3. Cell Culture

Cell culture procedures were performed in a Class II microflow biological safety cabinet (Microflow II MDH M51424/2, Astec, Hampshire, UK) using aseptic techniques. All cells were incubated and grown in HERAcell 240i incubator (Thermo Scientific, Hemel Hempstead, UK) with 95% air / 5% CO_2 humidified atmosphere at 37^{o}C.

2.4. Granta 519 and K562 cell culture

2.4.1. Granta 519 cell line (Granta 519 target cells)

The Granta 519 cell line was purchased from DSMZ (Leibniz-Institute DSMZ-German Collection of Microorganisms and cell culture, Braunschweig, Germany). It was originally derived from a peripheral blood sample taken in 1991 from a 58-year-old Caucasian woman who had recently been diagnosed with stage IV cytologically high-grade B-cell. G519 cells are characterised immunophenotypically as IgM^{+} IgD^{+} $CD5^{-}$ $CD10^{-}$ $CD19^{+}$ $CD22^{-}$ $CD23^{+}$ $FMC7^{+}$ (representing differentiation-associated antigens present on mature B lymphoid cells) and carry a complex karyotype with multiple chromosomal rearrangements including t(11:14)(q13:q32). They express high levels of cyclin D1 mRNA with concurrent biallelic deletions of the genes encoding the cyclin-dependent kinase inhibitors p16 (CDKN2A) and p15 (CDKN2B), all of which is consistent with cell cycle dysregulation at the level of G1. Despite CD5 negativity of the cell line, immunophenotyping of involved bone marrow at the

time of the patient's initial diagnosis five months earlier was positive for CD5 surface expression. This factor, together with the presence of a t(11:14)(q13:q32), strongly suggests a blastic transformation of MCL and the Granta 519 cell line is thus widely accepted as such (Amin *et al*, 2003).

2.4.2. K562 cell line (K562 target cells)

K562 cell line was a kind gift from Dr. Sarah Richardson. K562 cells are the first human myelogenous leukemia line to be established. The line is derived form a 53 years old female with chronic myeloid leukemia in blast crisis. These cells are positive for the *BCR:ABL* fusion gene and are an erythroleukaemic line. They lack the MHC complex required to inhibit NK activity and also lack any trace of Epstein-Barr virus or herpes virus. K562 cell lines are widely accepted as being highly sensitive to immune cell, especially NK cell mediated death and used *in vitro* (Sirianni et al. 2005).

2.4.3. Storage and revival of Granta 519 cells and K562 cell lines

For long term storage, Granta 519 cells and K562 cells were first counted and then centrifuged (Labofuge 400R, Newport Pagnell, UK) at 670g for 5 minutes. Supernatant was discarded and the cell pellets were re-suspended in freezing media (90% HI-FBS / 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Poole, UK) to achieve a cell density of 5 - 10 x 10^6 cells/ml. 1ml of the total cell suspension was aliquoted into 1.8ml cyrovials and placed into a polystyrene container "Mr Frosty^{TM}" (ThermoScientific, Hemel Hempstead, UK) containing isopropyl alcohol and placed in a -80°C freezer for 1 day to reduce the temperature by 1 degree/min. The frozen cryovials were then transferred to a liquid nitrogen dewar (Taylor-Wharton LS300, Borehamwood, UK) for long term storage between -210^{0}C and -195^{0}C.

Revival of the frozen Granta 519 and K562 cell lines was performed by rapidly defrosting the cells by immediately incubating in a 37^0C water-bath for 1 to 2 minutes. Cells were then dispensed and seeded into T75 tissue culture flasks containing 25ml of pre-warmed (37^0C) CCM. After seeding, G519 and K562 cells were left in the incubator for 24 hours after which time the media was completely replaced with fresh CCM. Cells were subsequently re-fed with fresh CCM every 72-96 hours and discarded after they reached a maximum passage of 25. A maximum of 25 passages was chosen as higher passage potentially increased genetic mutations and could affect the cell line susceptibility to drugs and cell cytotoxicity. Both G519 and K562 cells were grown in suspension and were non-adherent cells. All cells were disinfected with Virkon 1% w/v solution (Antec international, Sudbury, UK) for a minimum of 4 hours prior to disposal.

2.5. Isolation and storage of Peripheral Blood Mononuclear Cells (PBMCs)

Approximately 24 to 30 mls of peripheral venous blood was collect in 4mls BD Vacutainer Ethylenediaminetetraacetic acid (EDTA) tubes (Becton Dickinson, Oxford, UK) from each participant for the isolation of PBMCs and for flow cytometry as described below in section 2.6.

The PBMCs were isolated within 4 hours of collecting venous blood using routine isolation methodologies. Venous blood was transferred into a sterile 50ml polypropylene Centrifuge tube (Sarstedt, Leicester, UK) and an equal volume of warmed PBS was added. This was gently mixed and layered onto 10-12ml of lymphoPrep^{TM} solution (Axis-Shield, Dundee, UK). LymphoPrep is an iso-osmotic solution with a density of 1.077 g/ml which allows cells of higher density to pass through during centrifugation. The layered blood was centrifuged (Labofuge 400R, Newport Pagnell, UK) at 670g for 30 minutes

without brakes. Following centrifugation the mononuclear cells and lymphocytes (density gradient <1.077g/ml) that were unable to pass though the medium were retained at the top. The red cells and multinuclear cells (density gradient >1.077g/ml) settled at the bottom, resulting in the separation of cells into layers as illustrated in Figure 9. The centrifugation resulted in separation into 4 layers (phases). Two thirds of the uppermost layer, composed of serum, was discarded. The opaque interface, containing mononuclear cells (PBMCs), above the LymphoPrep™, was aspirated and placed into a 15ml centrifuge tube. The PBMCs were washed twice in 10ml of PBS and in the final wash PBMCs were resuspended in 10mls PBS and centrifuged at 670g for 10 minutes followed by resuspension in 10ml of CCM.

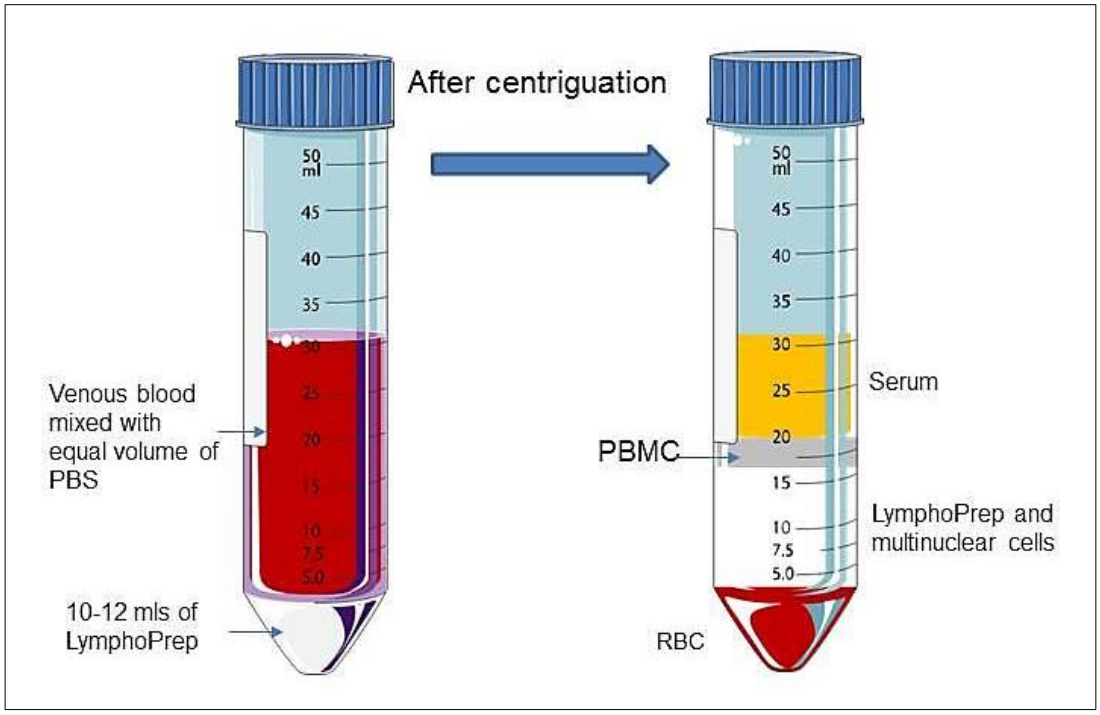


Figure 9: Diagrammatic representation of PBMC isolation

This diagram displays the layer formation with Ficoll-Haque method to isolate PBMCs from peripheral blood over LymphoPrep™ (Thorsby & Bratlie, 1970; Richardson *et al*, 2010).

Freshly separated PBMCs were counted using a Neaubaeur haemocytometer . Briefly, 10ul from the final solution was placed onto the counting chamber of the haemocytometer and all cells within the 4 quadrants were counted. This corresponded to 1 $x10^4$ cells/10ul. The number of cells counted per ml was multiplied by factor 100. 5-10 million cells/ml were then suspended in 90% CS-HI-FBS / 10% DMSO and kept in Mr Frosty^{TM} at -80^0C for 24 hours before being transferred to liquid nitrogen for long term storage.

2.6. Flow Cytometry

Principles of flow cytometry

Flow cytometry makes use of the principles of light scattering by particles crossing a beam of light, and excitation light from fluorochromes attached to specific molecules or proteins expressed by cells, to identify, analyse, and/or sort different populations of cells.

The process begins by single cells passing through one or multiple laser light beam and momentarily breaks the laser beam. The cells cause the light beam to scatter or the fluorochromes to emit light of particular wavelengths. This scattered light or fluorescence light from the fluorochrome is collected by a confocal lens and funneled onto a light detector which converts it into an electrical signal and subsequently it is digitalised to display the analysis of the cells. The light scattered in the forward direction, forward scatter signal (FSC) will give information about the size and shape of the cell. The side scatter signal (SSC) gives information about the granularity of the cell. As FSC and SSC are unique for each type of particle, the combination of the two can help identify different types of cells.

In addition, the use of fluorescent dyes that bind to extracellular or intracellular components can provide additional information about the cell. When cells, labelled with fluorescent dyes which are specific for an antibody, traverse the laser beam, the fluorescent molecules are excited to a higher energy state, causing them to emit light energy. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or 'colours') allows cells properties to be measured simultaneously and identify specific antigens on the cells to enable identification of specific cells. For example the following flourochromes; fluorescein isothiocyanate (FITC) has a emission wavelength of 512nm, allophycocyanin (APC) has an emission wavelength of 660nm and phycoerythrin (PE) approximately 700nm emission can be combined as each of their wavelength have minimal cross over on each other and emit light of different color and wavelength for the flow cytometry to identify individually without each emission overlapping / 'spilling' over each other and hence unable to read the identified the specific antigens on the cells of interest. Additionally, they have a narrow emission spectra and therefore helps to reduce this 'spill'.

When the emission spectra of flourochromes overlap the proportions of overlapping signal needs to be subtracted from the adjacent channel, a process called 'compensation'. Compensation is unique to each combination of flourochromes utilized in a single tube. To perform compensation each antibody emission is acquired by the flow cytometery individually and the spillage between the flourochromes is subtracted by the software on the flow cytometry (FACSDiva, BD Immunocytometry Systems, San Jose, CA, USA). Due to the usage of the multicolour flow cytometry (FACSCanto, BD Immunocytometry Systems, San Jose, CA, USA) (6 channel lazer) for the experiments outlined

below the inbuilt computer software mathematically creates a matrix from single flourochrome controls and optimizes the compensation. This is more accurate than the manually performed compensation in flow cytometers with multiple lazers. Compensation can be performed visually by subtracted spillage manually, but there is greater chance of false positivity as it is operator depended.

For experiments described in this thesis, flow cytometry was used for the following purposes:

1) To identify and quantitate different immune lymphocyte subsets in peripheral blood using fluorochrome-conjugated monoclonal antibodies directed against various cell surface or intracellular antigens (section 2.6.1)

2) To quantitate the mean fluorescence of activating receptors on NK cells, by using fluorochrome-conjugated monoclonal antibodies, before and after NK cell stimulation (section 2.6.1).

3) To differentiate between viable and apoptotic target (G519 cells and K562 cells) and effector cells (PBMCs and Isolated NK cells) with the use of fluorescent dyes such as propidium iodide (PI) and 7-amino-actinomycin D (7-AAD) (section 2.7).

4) To quantitate target cells (G519 cells and K562 cells) and effectors cells (PBMCs and isolated NK cells) (section 2.7)

5) To identify MCL cells in the isolated PBMC cell population from MCL patient samples (section 2.6).

2.6.1. Determination of T / NK / T regulatory cell numbers and NK cell activating receptors by flow cytometry

Flow cytometry was performed on a Becton Dickinson (BD) FACSCanto (BD Immunocytometry Systems, San Jose, CA, USA). Flow cytometry was utilised to determine the expression and quantification of T (CD3, CD4, CD8), Natural Killer (NK)(CD16, CD56)and T regulator cells (CD4 ,CD25,CD127). In addition, expression of NK cell activating receptors (NKp46, NKp30, NKp44 and NKG2D) was evaluated. All antibody reagents were purchased from BD Biosciences, Oxford, UK unless otherwise stated (Table 9). 4ml of venous whole blood collected in BD Vacutainer K_2EDTA tube (Becton Dickinson, Oxford, UK) was used for flow cytometry to identify and enumerate the above mentioned immune cells of patients in their peripheral circulation (figure 12.0 and 13.0). Results were presented as the mean of cell number with regards to quantification and mean of mean fluorescence intensity (MFI) with +/- standard error of the mean.

CD3 is a T cell co-receptor; CD4 is a glycoprotein found on surface of T cells; CD8 is a transmembrane glycoprotein co-receptor for T cells; CD45 is lymphocyte common antigen is a receptor-linked protein tyrosine phosphatase that is expressed on all leucocytes; CD16 low affinity Fc receptor; CD25 alpha chain of the IL-2 receptor; CD127 is an IL-7 alpha receptor.

Source	Specificity	Conjugate	Clone	Isotype
T cells enumeration Becton Dickinson San Jose, CA, USA	CD3 CD4 CD8 CD45-LCA	FITC APC PE PerCP	SK7 SK3 SK1 2D1 (HLe-1)	IgG_1mouse IgG_1mouse IgG_1mouse IgG_1mouse
NK cells enumertion Becton Dickinson San Jose, CA, USA	CD3 CD56-NCAM CD16-FcγRIIIA CD45-LCA	FITC PE APC PerCP	SP34 NCAM16.2 3G8 2D1	IgG_3mouse IgG_{2b}mouse IgG_1 mouse IgG_1 mouse
NK cell activating receptors analysis Becton Dickinson San Jose, CA, USA	CD3 CD56-NCAM CD45-LCA CD335-NKp46 CD337-NKp30 CD336-NKp44 CD94-NKG2D	FITC PE PerCP APC Alexa Fluor 647 Alexa Fluor 647 APC	SP34 NCAM16.2 3G8 9E2/NKp46 p30-15 p44-8.1 1D11	IgG_3 mouse IgG_{2b}mouse IgG_1 mouse IgG_1 mouse IgG_1 mouse IgG_1 mouse
For Tregs analysis Treg Cocktail BD Becton Dickinson San Jose, CA, USA	CD4 CD25- IL-2Rα CD127- IL-7Ra CD45- LCA	PE Cy7 FITC PerCP	SK3 2 A3 HiL-7R 3G8	IgG_1 mouse IgG_1 mouse IgG_1 mouse IgG_1 mouse

Table 9: **Flow cytometry antibodies for T, NK, T regulatory cells and NK cell activating receptors**

LCA- lymphocyte common antigen; NCAM- natural cell adhesion molecule;

2.6.2. T cell enumeration

T cell analysis was performed by staining with BD Multitest IMK kit for T cells analysis according to the manufacturer's protocol. Briefly, 50µl of anticoagulated whole blood was placed in BD Trucount tubes (Becton Dickinson, Oxford, UK) with 20µl of the monoclonal antibody combination: FITC-CD3/PE-CD8/PerCP-CD45/APC-CD4 (Table 9). After 20 minutes incubation in the dark and at room temperature, 1ml of Quicklysis solution (Cytognos, Salamanca, Spain) was added to each tube prior to a further 15 minute incubation in the dark at room temperature. Quicklysis solution was used to lyse red cells. Cells were acquired and analysed using FACSDiva software version 6.1.3 (BD Biosciences) to determine absolute cell counts of $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$ (T cells) lymphocyte subsets. A minimum of 20,000 $CD45^{+}$ lymphocyte events per sample were collected and analysed (Figure 10 & 11).

2.6.3. NK cell enumeration

NK cells analysis was performed using similar methods as for T cells analysis mentioned above. In brief, 50µl of anti-coagulated whole blood was placed in BD Trucount tubes with 20µl of the following antibody combinations: FITC-CD3/PE-CD56/PerCP-CD45 and 5ul of APC-CD16 (Table 9). Cells were acquired and analysed using FACSDiva software version 6.1.3 to determine absolute NK cell count ($CD3^{-}CD56^{+}CD16^{+}$) and NK cell subsets counts ($CD3^{-}CD56^{bright}$ $CD16^{dim}$ (involved in cytokine mediated cell cytotoxicity) and $CD3^{-}CD56^{dim}CD16^{bright}$ (direct cell death). A minimum of 20,000 $CD45^{+}$ lymphocyte events per sample were collected and analysed (Figure 12 & 13).

2.6.4. T regulatory cell enumeration

T regulatory cell (T regs) analysis was performed by adding 20µl of PerCP-CD45 and T reg antibody cocktail CD4/CD25/CD127 (Table. 9) to 50µl of anticoagulated whole blood placed in BD Trucount tubes. Cell acquisition and analysis was done using BD FACSDiva software version 6.1.3. A minimum of 20,000 $CD45^+$ lymphocyte events per sample were collected and analysed (figure 12.0 & 13.0)(Hardy *et al*, 2013).

2.6.5. Formula for cell count calculation using flow cytometry beads

BD Trucount Tubes were used to determine the absolute counts of various population of cell in the blood as mentioned above. The whole blood with the appropriate monoclonal antibody reagent is placed into the Trucount tubes, the lyophilized pellets in the tube dissolves, releasing a known number of fluorescent beads. The absolute number (cells/µL) of positive cells or cells of interest in the sample can be determined by comparing cellular events to bead events. Using the formula below the absolute cell count can be determined (Nantakomol *et al*, 2010; Hardy *et al*, 2013).

$$\frac{\text{\# of events in region containing cell}}{\text{\# of events in absolute count bead region}} \times \frac{\text{\# of beads per test*}}{\text{test volume}} = \text{absolute count of cell (cells/µl)}$$

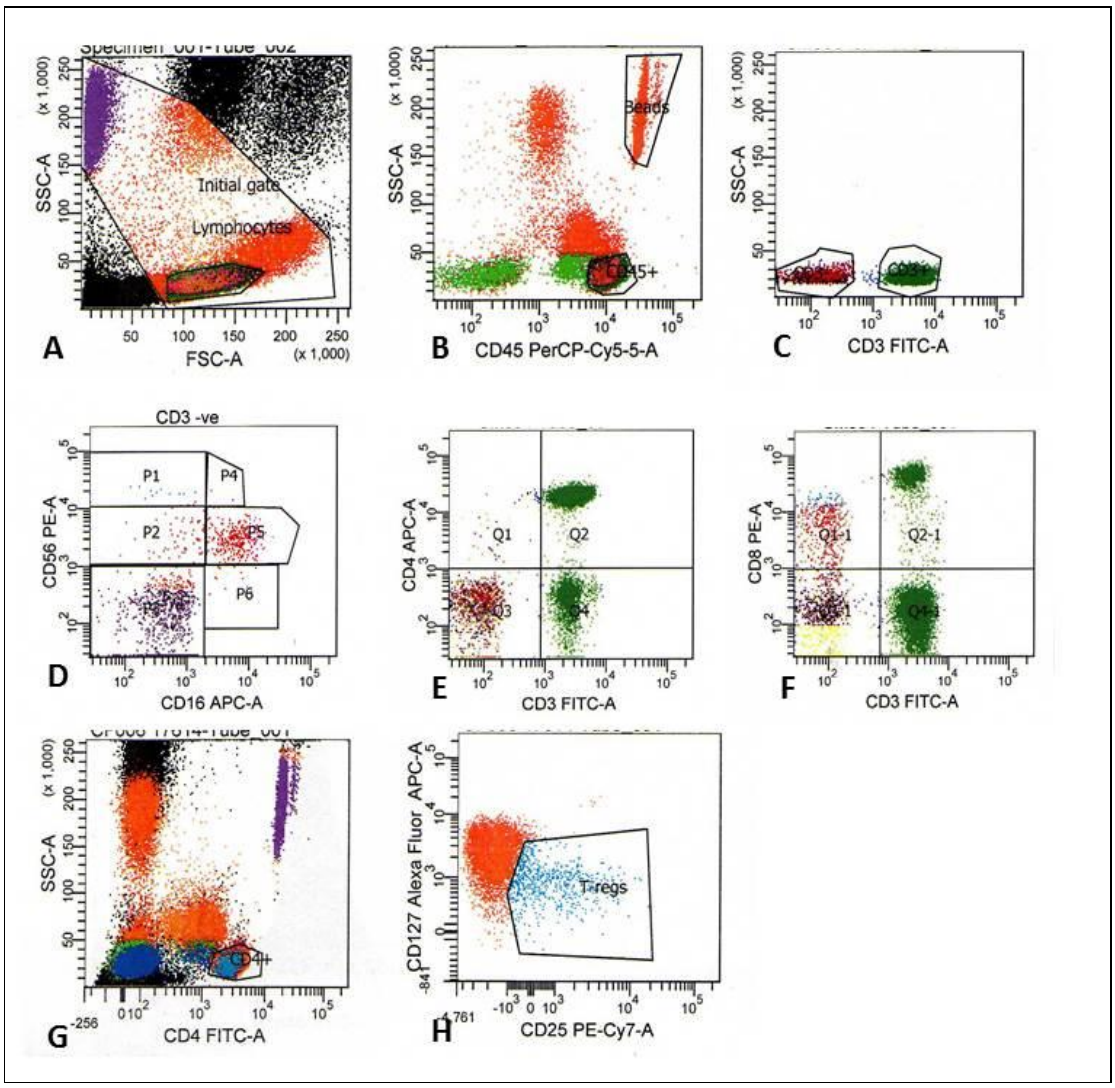


Figure 10: **Flow plot representation of cell acquisition and quantification**

Flow plots cell acquisition and quantification of immune cell subsets in the peripheral blood of subjects on the FACSCanto flow cytometer and analysed by FACSDiva software version 6.1.3.

A. Flow plot represents initial gating on peripheral blood. Forward and side scatter were used to identify the lymphocyte population as outlined.

B. $CD45^+$ lymphocytes were gated to identify the cells of interest including the bead count for quantification.

C. $CD3^+$ and $CD3^-$ cells were identified in the $CD45^+$ populations and gated as shown.

D. Flow plots represent NK cells $CD3^-CD56^+$ cells and subsets of NK cells were identified and counted $CD3^-CD56^{dim}CD16^{bright}$ (P5) and CD3-$CD56^{bright}CD16^{dim}$ (P4).

E & F. Flow plots represent $CD3^+$ T cells and subsets T helper cells ($CD3^+CD4^+$) identified in Q2 and Cytotoxic T cells ($CD3^+CD8^+$) identified in Q2-1.

G & H. Flow plots represent $CD4^+CD25^+CD127^{dim}$ T regulatory cells.

These flow plots are representative for all flow plots analysed in this project.

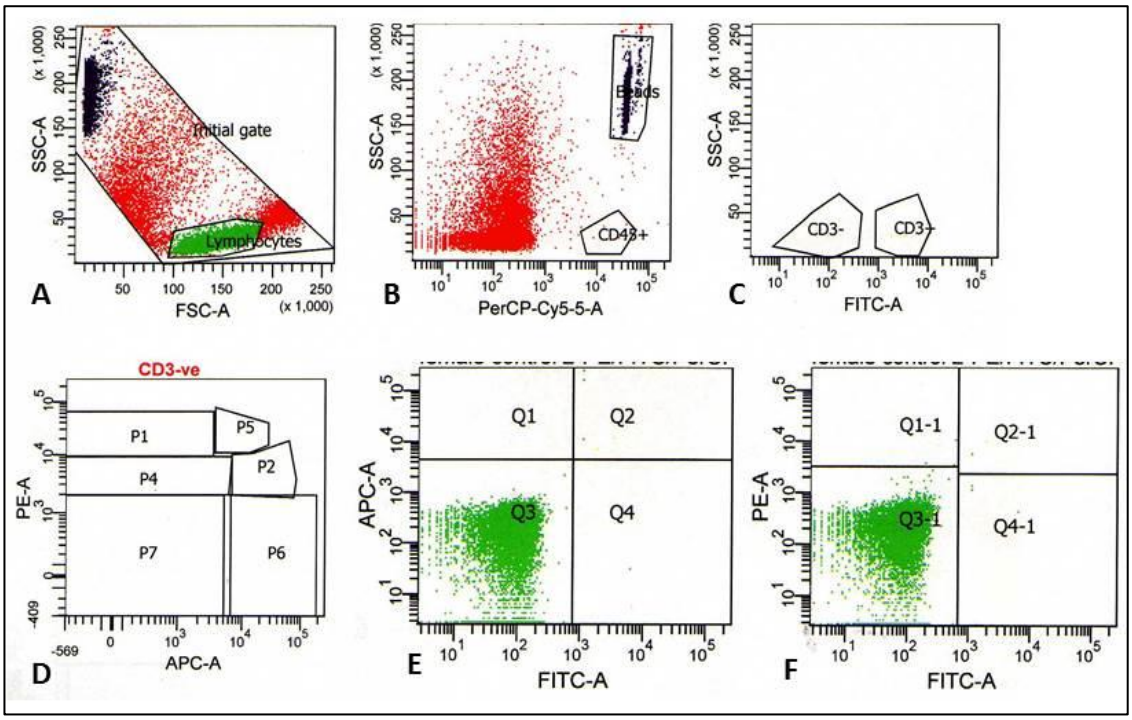


Figure 11: Flow plots representing *isotype controls*

***Isotype controls* for antibodies used in the identification and quantification of immune subsets as shown in figure 10.**

A. Forward and side scatter is used to identify the lymphocytes and are gated for further analysis.

B, C, D, E and F plots represent isotype control demonstrating no fluorescence or slip over of fluorescence. PerpCP-Cy5, FITC-A, APC and PE are all represented respectively.

Flow cytometry was performed on FACSCanto flow cytometer and analysis of the flow plot was on FACSDiva software version 6.1.3.

2.6.6. NK cell activating receptor expression

NK cell activating receptors analysis was perfomed by mixing 20 µl of PerCP-CD45/FITC-CD3/ PE-CD56/APC-NKp46/Alexa Fluor 647-NKp44/APC-NKG2D and 5µl of Alexa Fluor 647-NKp30 (conjugate antibodies) to 50µl of anticoagulated whole venous blood (Table. 8.0). Cell solutions containing the aforementioned antibody combinations were processed using the BD FACSCanto flow cytometer and analysed with the BD FACSDiva software version 6.1.3. In all samples tested, a minimum of 10,000 $CD45^{+}$ lymphocyte

events were collected and analysed (figure 12.0). Results were expressed in mean fluorescence intensity (MFI).

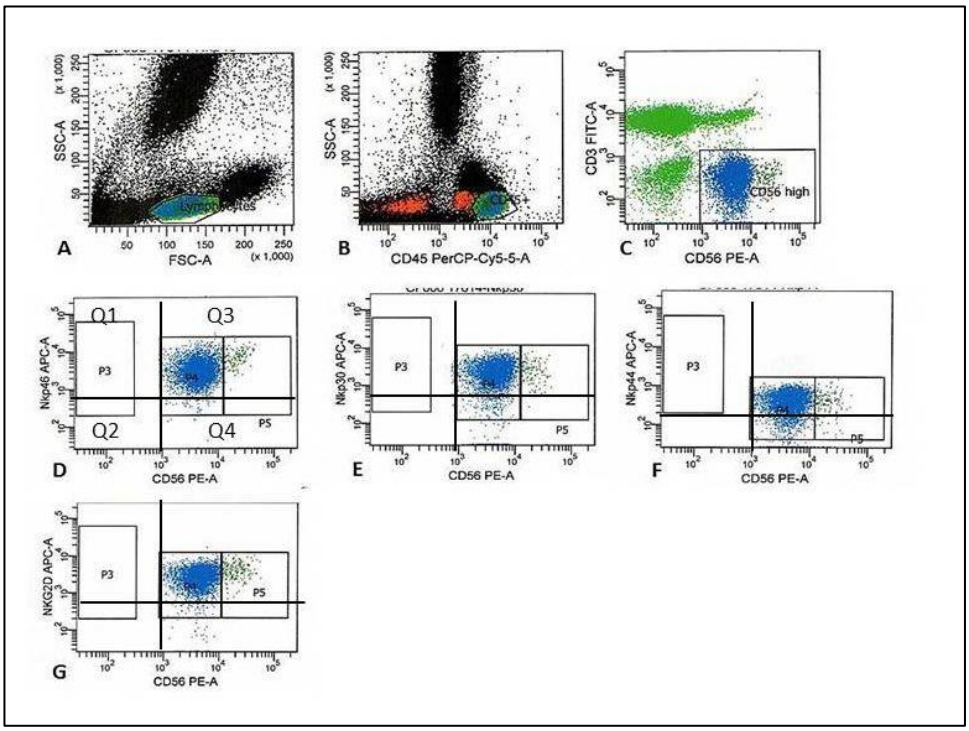


Figure 12.0: **Flow plots to illustrate the analysis of NK cell activating receptors**
NK cell activating receptors; NKp46, NKp30, NKp44 and NKG2D on the NK cells; $CD3^-$ $CD56^+$ in the peripheral blood of the subjects.

Plot A & B. Flow plots represent the identification of lymphocytes by forward and side scatter and $CD45^+$.

Plot C. Flow plot represents the NK cells; $CD3^-CD56^+$, which are gated on as shown.

Plots D, E, F and G. Flow plots represent NKp46, NKp30, NKp44 and NKG2D activating receptors expression on NK cells respectively. They are analysed on both the $CD56^{dim}$ (P4) and $CD56^{bright}$ (P5) expressing NK cells.

Quadrants shown on D exhibit positivity Q3 is the positive quadrant which positive of CD56 and the receptor as shown. Q4 positive for CD56 but not the respective receptor. These flow plots are representative of all flow plots analysed in this study.

Flow cytometry was performed on FACSCanto flow cytometer and analysis was on FACSDiva software version 6.1.3.

2.6.7. Cell viability and cell death analysis by flow cytometry

Principle

Live cells have impermeable membranes to cationic dyes such as Propidium Iodide (PI) and 7-aminoactinomycin D (7-AAD). However, these dyes are capable of entering apoptotic cells due to their plasma membranes being compromised. Referring to the PI dye, upon entry of PI, it labels the cells by binding to the DNA and intercalating between the bases. Once bound, it fluorescence is enhanced by at least 20 fold. Flow cytometry can be used to distinguish live, viable cells (unstained / PI-negative) from dead / apoptotic cells (stained / PI-positive).

Method

Cell lines (Granta 519 and K562), PBMCs and isolated NK cells viability was determined by incubating pre-treated cells with 10µg/ml of PI (Sigma Aldrich, Gillingham, UK) for 10 minutes at 4^0C. Cell viability was then analysed using a BD Accuri C6 flow cytometer and CFlow software (BD Biosciences, Oxford, UK). A minimum of 10,000 events was recorded and the results were presented as a percentage of PI-negative (live / viable) cells. The viability of PBMCs, was assessed after 24 hours incubation in CCM. Isolated NK cell viability was assessed immediately after the completion of NK cell isolation from PBMCs (see section 2.9). For all cell groups used in the experiments described here the viability was >90%, except for NK cell isolation, were by the viability was less than 50% therefore these cells were not used in any experiments. This in turn also resulted in very low number of NK cells to carry out any further viable experiments.

2.6.8. $CD5^+/CD19^+$ quantification of MCL cells in PBMCs from patient samples by flow cytometry

MCL patients can have circulating MCL cells in the peripheral blood and often at diagnosis there are a large numbers of MCL cells circulating. Therefore, effector cell used here from MCL patients had less than 5% dual CD5 (T1,TP67) / CD19 (B4 BCR co-receptor) positive cells. This is to limit the MCL cells affecting the cytotoxicity assay. Typically B cell loose and gain antigens as they develop and mature. In the development of MCL the CD5 and pan B cell antigens such as CD19 and CD20 are present together on the cells to demonstrate the typical immunophenotype of MCL ($CD19^+$, $CD20^+$, $CD5^+$, $CD45^+$,cyclin $D1^+$, $CD23^-$, surface Ig^{bright}). In normal subjects B cells with a dual CD5 and CD19 antigens can range from 3.6 to 20% of B cells (Gupta *et al*, 2004). Thus a 5% cut off was used to ensure that there is minimal impact from native MCL cells and also allow for inclusion of patients with MCL. The quantity of MCL in the isolated PBMC was confirmed by incubating 20µl of BD Oncomark^{TM} (CD5, CD10, CD19)(BD Biosciences, Becton Dickinson, San Jose, CA, USA)(Table 10) antibody with 50µl of isolated PBMCs (approximately 5 x 10^5 cells) in FACS buffer for 20 minutes in the dark at room temperature, prior to analysis by flow cytometry on the BD FACSCanto flow cytometer (as per manufacturer protocol). A minimum of 10,000 lymphocyte events were gated according to their forward and side light scatter properties. Analysis was presented as a percentage of dual staining CD5/CD19 cells using FACSDiva software version 6.1.3 (Figure 13). Results were presented in percentage of cells $CD5^+CD19^+$.

Source	Specificity	Conjugate	Clone	Isotype
Becton Dickinson San Jose, CA, USA	BD Oncomark™ CD5 CD10 CD19	 FITC PE PerCP-Cy5.5	 L17F12 HI10a SJ25C1	 IgG2aKappa IgG_1 Kappa IgG_1 Kappa

Table 10: Flow cytometry antibodies for quantification of MCL cells in PBMCs.

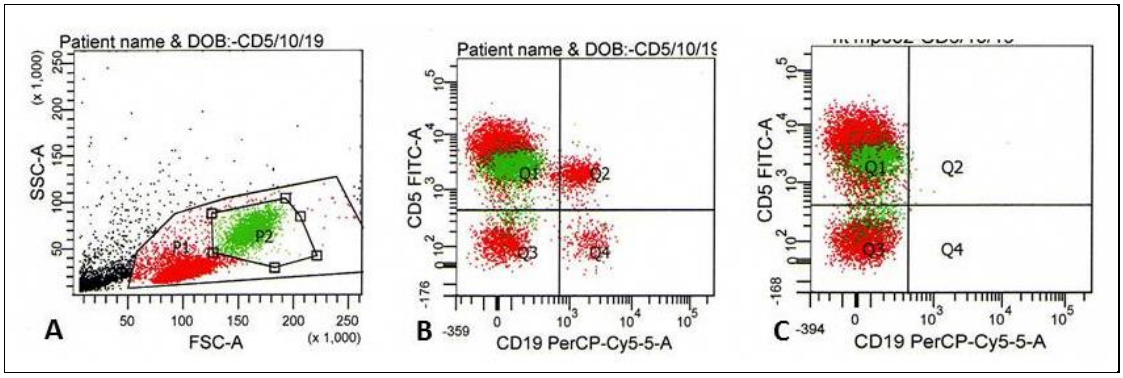


Figure 13: **Flow plots to represent analysis of isolated PBMCs to identify MCL cells**

PBMCS from MCL patients analysed by flow cytometry to identify MCL cells in the PBMC with $CD5^+$ and $CD19^+$ dual fluorescence.

A. Forward and side scatter plot to identify the cells of interest (P1 gating).

B. Example of a flow cytometry plot demonstrating the **presence** of MCL cells in patient PBMC with cells positive for $CD5^+CD19^+$ in Q2 as shown.

C. Example of a flow cytometry plot demonstrating the **absence** of MCL cells in patient PBMC with no cells positive for $CD5^+CD19^+$ cells in Q2 as shown here.

These plots are representative for all the analysis done on patients samples to identify any presence of MCL cells in the PBMC.

2.6a. Statistical Analysis

Results from 5 male and female healthy age matched controls and 3 male and female MCL patients were analysed (unless otherwise stated). All effector (PBMC) and target cells exhibited a >90% viability. Cytotoxicity activity of the effector cells was measured by the percentage cell death of K562 cells and Granta 519 mantle cell lymphoma cells. The analysis was performed on GraphPad version 6 and the results are presented as the percentage mean

difference of cell death +/- standard error of the mean (SEM). Two way ANOVA with sidak post hoc multiple comparison analysis was performed on the cytotoxicity results and one way ANOVA with post hoc Dunns comparison was performed on the results of the enumeration of NK, T , T regulatory cells and NK activating receptors between the groups. The results were presented as the mean total +/- standard error of the mean (SEM). Mann-Whitney U test was performed when comparing mean of two groups.

2.6.9. Quantification of T and NK cells with their subsets, T regulatory cells and expression NK cell activating receptors analysis in PBMC cells post incubation after treatment with oestrone, IL-2 and lenalidomide in various combinations

To determine the proportions of immune subsets and NK cell activating receptors on PBMCs after subjecting isolated PBMCs to various treatments, as described in section 2.7 below, flow cytometry was performed as described above in section 2.6. In brief, a minimum of 5 x 10^5 cells from each treatment arm of the PBMC were taken after they had been washed in PBS and re-suspended in FACS buffer. Approximately 1.25 x 10^5 cells were incubated with the same quantity of antibodies for T cells, NK cells, T regulatory cells and NK activating receptors, as described in section 2.6 (Table 9), for 20 minutes in the dark at room temperature and subsequently analysed. Flow cytometry analysis was on the BD FACSCanto using BD FACSDiva software version 6.1.3. Minimums of 10,000 CD45 positive lymphocyte events were gated according to the side and forward scatter property and CD45+ fluorescence. Results were presented as the mean cell count with +/- standard error of the mean.

2.7. Cell mediated cytotoxicity assay

Principle

The characteristics of cell-mediated cytotoxicity were used to determine the efficiency of cell mediated cytotoxicity between males and females. This involves cytolysis of target cells (e.g. malignant cells) by effector cells (e.g. cytotoxic T cells and/or NK cells). The method of assessing this process was labelling the target cells with a green fluorescent cell staining dye such as carboxyfluorescein succinimidyl ester (CFSE) and then co-culturing the target cells with unlabeled effector cells. After a period of incubation, the dead cells were labelled with a red fluorescent dye such as 7-AAD. Flow cytometry can be used to distinguish live (green signal only cells) from dead (dual green and red signal) target cells (Richardson *et al*, 2010).

Carboxyflourescein diacetate succinimidyl ester (CFDA-SE) is non-fluorescent, is highly cell permeable due to its acetate groups and once within the cell cytoplasm, intercellular esterases remove its acetate groups and coverts the molecule to the fluorescent ester, CFSE (Quah & Parish, 2010). CFSE is retained within the cell by covalent coupling via its succinimidyl group to intracellular molecules, notably lysine residues and other amine sources. Due to the covalent coupling CSFE can be retained in the cell for prolonged periods with no dye transfer to adjacent cells (Quah & Parish, 2010). 7-AAD does not pass through intact cell membranes such as in live / viable cells. Apoptotic cells have compromised cell membranes and 7-AAD can readily enter apoptotic cells to bind to the DNA (Marin *et al*, 2003; Lotze & Thomson, 2005). Its fluorescence once excited is deep red; 440 nm excitation and 650nm emission spectrum therefore it can be utilised with the green fluorescence of CSFE; 494nm excitation and 519nm emission spectrum (Marin *et al*, 2003).

2.7.1. Target cell generation

An aliqout of Granta 519 and K562 cells taken from T75 culture flasks was washed twice in warmed SFM. 10 x 10^6 G519 and K562 cells were labelled with 6µM-9µM of CFDA-SE (Life Technologies, Paisley, UK) and incubated for 15minutes in the dark at 37^0C/5% CO_2. The reaction was stopped by adding equal volumes of CS-HI-FBS for 3 minutes after which the G519 and K562 cells were washed in PBS to remove unincorporated CFDA-SE and re-suspended in CCM.

2.7.2. Effector cell generation

Stored PBMCs from male and female age matched control subjects and MCL patients were removed from the liquid nitrogen dewars and thawed for 1-2 minutes in a water bath (Grant JB2, Grant Instruments, Cambridge, UK) at 37^0C. They were suspended in warmed CCM and incubated at 37^0C / 5% CO_2 for a minimum of one hour. PBMCs were subsequently, washed twice in PBS, counted and re-suspended at 2.5 x 10^6 cells /ml in CCM before being placed into 24-well flat bottom plates (1ml/well). The PBMC cells in the wells were either vehicle control (referred to as control / untreated [DMSO 1ug/ml]) or treated with 40pg/ml oestrone, 100U/ml IL-2, 2µM lenalidomide and a combination of 40pg/ml estrone,100U/ml IL-2 and 2µM lenalidomide. The PBMCs were incubated at 37^0C / 5% CO_2 for 24 hours. Additional PBMCs from control subjects were either untreated controls or treated with DMSO 1µg/ml (vehicle control), 100U IL-2/ml plus 40pg/ml oestrone and 100U IL-2/ml plus 2µM lenalidomide. The concentration of DMSO used throughout the cytotoxicity assay was never more than 0.1%. Due to the limited numbers of PBMCs isolated, the vehicle control was used as the control and any effect of DMSO

being used as a carrier vehicle was evaluated by comparing ten untreated controls with 10 vehicle controls (DMSO1µg/ml).

Following 24hours incubation non-adherent PBMCs were collected, washed in PBS and pelleted by centrifugation. They were re-suspended in CCM prior to counting. A minimum of 1.25 x 10^5 PBMCs cells were analysed for viability by flow cytometry using PI, after 24 hour incubation as described in the above section 2.5.1. 10,000 gated events were recorded and analysed.

2.7.3. Treatment with drugs, hormones and cytokines

Lenalidomide

Lenalidomide was kindly provided by Celgene (San Diego, CA, USA). Primary stock solutions of Lenalidomide (20µM) were prepared by dissolving the compound in DMSO as per manufacturer instruction and stored at -20^{o}C until use.

Oestrone

Oestrone was purchased from Sigma- Aldrich (Dorset, UK). Primary stock solutions of Oestrone were prepared by dissolving the compound in DMSO (as per manufacturer instruction (stock solution 20mg/ml)). Further stock solutions were prepared by diluting the primary stock in CCM to make a working solution of 20ng/ml. This is referred to as the working stock solution herein and stored at 4^0C.

Interleukin-2 (IL-2)

IL-2 was purchased from PeproTech EC.Ltd (London, UK). IL-2 was initially dissolved in 100mM acetic acid and then a working stock of 10,000U/ml in

RPMI with 0.1%BSA was made up and stored at 4^0C. All preparation was done using sterile techniques and solutions.

Fresh working stocks were prepared every 4-6 weeks.

2.7.4. Cell mediated cytotoxicity assay

Following generation of target and effector cells, the target cells were re-suspended at 5 x 10^4 cells/ml and plated into a 96 well flat bottom plate at 5x10^3 cells per well. The effector cells were re-suspended in CCM at 2.5 x 10^6 cells/ml per treatment and plated into the 96 well flat bottom plate containing the target cells at ratios of 1:50, 1:25 and 1:12.5, target to effector cells.

The treated PBMCs were re-suspended at 2.5 x 10^6/ml CCM with 40pg/ml of oestrone. Following four hour incubation at 37^0C / 5% $C0_2$, cell death was determined by staining target cells with 1µg/ml of 7-AAD (Invitrogen, Paisley, UK) and incubating for 10 minutes at 4^oC. Analysis of the mean cell death represented by 7-AAD and CSFE positive cells was performed by flow cytometry. G519 and K562 cells were gated according to forward and side light scatter properties and 1,000 CFSE-labelled events were collected and analysed for each sample (Figure 14). All experiments were performed in triplicate. Results were presented as the mean with standard error mean.

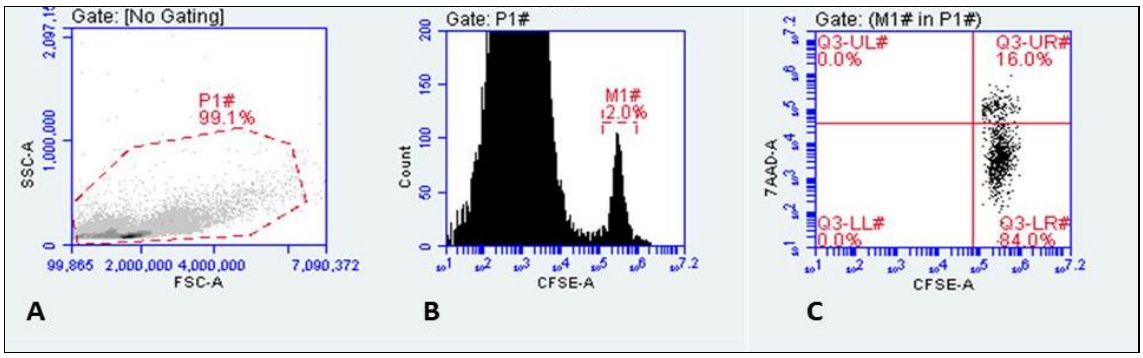


Figure 14: **Flow cytometry plots exhibiting acquisition of cell death data in the cell mediated cytotoxicity experiments**

A. Initial flow plot to illustrate the gating on all cells according to the FSC and SSC (P1) (effector and target cells).

B. CSFE labelled target cells were gated in M1 as shown.

C. Dead target cells with dual CFSE and 7-AAD staining were presented in quadrant 3 –upper right (Q3-UR#).

This graph is representative for all results acquired from the flow cytometry analysis during the cell mediated cytotoxicity experiments.

2.8. Direct cytotoxicity of oestrone and lenalidomide on Granta 519 MCL cell line and K562 cells

To determine the direct effects of oestrone and lenalidomide on MCL, pre-treated Granta 519 cells were transferred from T75 flask and then washed with sterile PBS. The Granta 519 cells were counted by haematocytometer as described in section 2.1 and $2.5x10^6$cells/ml were suspended in CCM. Cells were then placed in 24-well flat bottom plate and left either untreated (CCM alone, control), or treated with DMSO (vehicle control), oestrone (40pg/ml, 80pg/ml or 400pg/ml), 2µM of lenalidomide and a combination of 40pg/ml oestrone with 2µM of lenalidomide. The treated Granta 519 cells were left for 24 and 48 hours at 37^0C / 5% CO_2 prior to analysis.

After the relevant incubation time the treated G519 cells were placed in a 96-well flat bottom plate (5 x 10^5 cells per well) and incubated with 10µg/ml of PI for 10 minutes at 4^0C prior to analysis by BD Accuri C6 flow cytometer and

CFlow software. The results were presented as a percentage of PI-negative (live / viable) cells. All treatment arms were tested in triplicates and the mean cell viability was determined (Results chapter 4, section. 4.3).

K562 target cells were incubated with oestrone as described above and analysed for direct cytotoxic effect of oestrone. K562 target cells were incubated for 0, 24 and 48 hours with the same concentrations of oestrone as described in section 2.6, above with Granta 519 cells (Results chapter 4, section 4.3).

2.8. Immunohistochemistry and Immunofluorescence for identification of immune cell subsets (NK, T and T regulatory cells) and Oestrogen Receptor α (ERα) and β (ERβ)

Principle

Immunohistochemistry (IHC) is a widely used technique that combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. The fundamental concept of IHC is to demonstrate antigens (proteins and carbohydrates complexes within or on the surface of a cell that can bind to a specific antibody) within tissue sections by means of specific antibodies. There are two types of antibodies used in IHC, monoclonal and polyclonal. Polyclonal antibodies have binding affinity to multiple epitopes of target antigens which is prone to cross reaction with non-target antigens. Monoclonal antibodies tend to have an affinity to a single epitope thus producing a more specific and cleaner staining result. All antibodies used here were monoclonal antibodies. The tissue section is exposed to the primary antibody and once the antigen-antibody binding occurs a secondary antibody is introduced which binds onto the primary antibody enhances the staining intensity. To detect the cell or cells of interest following the antigen-antibody

binding of the primary and secondary antibodies, the secondary antibody has an enzyme, Horseradish Peroxidase (HRP) which allows for the chromogenic substance to attach and thus demonstrate a coloured histochemical reaction visible by light microscopy. 3,3'-diaminobenzidine (DAB) is used as a chromogen and it is oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of a brown, alcohol-insoluble precipitate at the site of enzymatic activity. This enables specific identification and visualization of cell or cells and tissue structures that are of interest which have unique antigens that can be targeted by specific antibodies.

Immunofluorescence (IMF) using the same principles as does IHC. It differs by a fluorescently tagged secondary antibody which is visualized on a fluorescence microscope with a near monochromatic light which is filtered to a specific wavelength. The specimen is illuminated with the selected wavelength, which gets absorbed by the fluorochrome, causing it to emit light of a longer wavelength.

Method

All histological samples were from the Department of Haematology, Derriford Hospital, Plymouth, UK and the specimen slides were prepared in the Pathology Department, Derriford Hospital, Plymouth, UK. IHC and immunofluorescence work was performed at the University of Exeter Medical School, Institute of Biomedical and Clinical Science, Exeter, UK with kind permission and guidance from Prof. Noel Morgan and Dr. Sarah Richardson. Some IHC work was also performed with Mr. Phillip Edwards at the Pathology Department, Derriford Hospital, Plymouth.

All histological samples used here were formalin fixed and paraffin embedded (FFPE). Removal of wax was carried out by immersing sample slides in Histo-clear (National Diagnostics, Hessle, UK) for two five minute immersions and subsequently rehydrated with varying concentrations of alcohol (100%, 90%, 70% Methanol each for 1 minute).

Formalin induces molecular modifications of proteins (antigens) which may result in the antibody losing its ability to bind to the antigen. Therefore, antigen retrieval was carried out by Heat-Induced epitope retrieval (HIER) method which utilises heating the specimen to $>100^0$C causing a disruption to the crosslinks between formalin and antigen, thus allowing the retrieval of the antigen. Specimens are heated in various pH retrieval buffers (Citrate pH 6, EDTA pH6; table M4) which further aid in retrieving the antigen by facilitating the breakage of the crosslinks. HIER was carried out by placing rehydrated slides into a pressure cooker with 1 litre of antigen retrieval buffer and heating in a microwave oven for 20 minutes on high power. It was allowed to cool for a further 20 minutes prior to adding the block.

5% normal goat serum (Vector laboratories, Peterborough, UK) was used (incubated for 5 minutes) as a block to prevent non-specific binding of the antibodies. Primary antibodies were subsequently added onto the specimen slides. The primary antibodies used; for MCL cell identification were mouse anti-human CD20 and rabbit anti-human cyclin D1, for NK cells identification were mouse anti-human CD56 and rabbit anti-human CD3, for T regulatory identification were mouse anti-human Foxp3 and rabbit anti- human CD3 and for ERβ identification was the mouse oestrogen receptor antibody. Further details of the antibodies used are described in table 10.0. Oestrogen receptor

beta antibody was incubated overnight in the dark at 4^0C. The remainder of the primary antibodies, were incubated for 1 hour in the dark.

Specimens that were stained to identify NK cells ($CD56^+$, $CD3^-$), T regulatory cells ($CD3^+$,$Foxp3^+$), T cell ($CD3^+$) and MCL cells ($CD20^+$, Cyclin $D1^+$) using the indirect immunofluorescence technique, are described below.

ERα and ERβ identification was done using the indirect IHC technique. Slides were incubated with primary antibody at room temperature in the dark for 1 hour with oestrogen alpha antibody and overnight with oestrogen beta antibody (table 2.5). Following, incubation with the primary antibody, slides were washed three-times in Tris-buffered saline (TBS). They were then immersed for five minutes, in in Peroxidase Block is (DAKO Real peroxidase blocking solution S2023, Dako, Cambridge, UK). The Block was applied to reduce endogenous peroxidase on cells and tissues that can cause non-specific background staining when HRP conjugate antibody is used. Block was washed off by immersion of slides in TBS for five minutes.

Secondary antibody (DakoREAL Envision / HRP, Rabbit/Mouse, DAKO, Cambridge, UK) was then applied and the slides are incubated for 30 minutes in the dark. Secondary antibodies were used to amplify the antigen detected thus allowing for better visualization and greater signal detection. After incubation secondary antibody was washed off by immersion into TBS three times. DAB was added by applying 0.5ml of DAB (Dako REAL DAB) substrate for 10 minutes followed by washing in distilled water.

Haematoxylin stain (Dako, Cambridge, UK) was now applied to provide visual contrast so that the DAB positive cells are easier to visualize. Slides were

further washed in water, dipped in Scott's Tap Water Substitute (STWS) (enhances the blue colour form haematoxylin) and immersed in 2%w/v copper sulphate to enhance the DAB staining. The slides were finally washed in distilled water for 5 minutes and then dehydrated in varying concentrations of alcohol (50%, 70%, 90%, 100% Methanol each for 1 minute).

Slides were then taken and immersed in histo-Clear which pushes out the alcohol and a generous amount of DPX (neutral plastic resin dissolved in xylene) is applied prior to carefully laying on the coverslip. The slides were allowed to dry overnight in a fume cupboard before visualization on the Nikon Eclipse 50i microscope. Images were taken on Nikon digital sight camera with the microscope.

Indirect immunofluorescence method was used to stain for T, NK and T regulator cells as mentioned above. After incubation with the respective primary antibodies, the slides were washed three times, five minutes each in TBS and incubated for 1 hour with secondary antibodies (Table 11). After incubation the secondary antibody was washed off with TBS and Dako fluorescent mounting media S3023 (Dako, Cambridge, UK) was applied over the specimen. A cover slip was gently lowered onto the slide and the mounted slides were kept in the dark over night to dry before visualization on the Leica AF hardware version 2013.1.1 (Leica microscope systems CMS, Germany).

To quantitate the number of NK cells, T cells and T regulatory cells, 13 random images at x200 magnification were taken per slide and positive cells were counted. Results were shown as total numbers and mean in the results chapter 5.

Function	Primary Antibody all monoclonal anti-human	Clone		Primary dilution in antibody diluent	Antibody Diluent	HIER – retrieval buffer
MCL cell identification CD20^{+}, CyclinD1^{+}	Mouse CD20	L26	DAKO, Cambridge, UK	1:1000	DAKO S2022	Citrate pH 6
	Rabbit CyclinD1	SP4	Abcam, Cambridge, Uk	1:100	DAKO S2022	Citrate pH 6
T cell identified CD3^{+}	Rabbit CD3	SP7	Abcam, Cambridge, UK	1:100	DAKO S2022	EDTA pH 8
NK cell identification CD56^{+}, CD3^{-}	Mouse CD56	NCL-L CD56	Novocastra Lieca microsystem, Milton Keynes, UK	1:50	DAKO S2022	EDTA pH 8
	Rabbit CD3	SP7	Abcam, Cambridge, UK	1:100	DAKO S2022	EDTA pH 8
T regulatory cell identification CD3^{+}, FoxP3^{+}	Mouse FoxP3	236A/E7	Abcam, cambridge, UK	1:100	DAKO S2022	EDTA pH 8
	Rabbit CD3	SP7	Abcam, Cambridge, UK	1:100	DAKO S2022	EDTA pH 8
Oestrogen Beta receptor identification	Mouse oestrogen Beta	14C8	Abcam, Cambridge, UK	1:200	DAKO S2022	Citrate pH 6
Oestrogen Alpha Receptor identification	Rabbit oestrogen Alpha	EP1	DAKO, Cambridge, UK	1:200:		
Secondary antibodies						
Immunoflourescence	Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)			Life Technologies, Paisley, UK		
Immunoflourescence	Alexa Fluor® 546 Goat Anti-Rabbit IgG (H+L)			Life Technologies, Paisley, UK		
Immunoflourescence Nuclear staining	DAPI (4′,6-diamidino-2-phenylindole)			Life Technologies, Paisley, UK		
For oestrogen IHC receptor antibodies	DAKO REAL Envision HRP, anti-mouse/ anti - rabbit			DAKO, Cambridge, UK		

Table 11: Primary and secondary antibodies used in Immunohistochemistry and Immunofluorescence

2.9 NK cells Isolation

Principle

Negative selection of NK cells was undertaken, resulting in untouched NK cells in the final solution. Non-target cells consisting of T cells, B cells, dendritic cells, monocytes, granulocytes and erythroid cells are indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies against lineage specific antigens and a cocktail of MicroBeads. Isolation of the untouched NK cells is achieved by passing the solution through a column in a magnetic field thus depleting the magnetically labelled cells (non-NK cells) form the solution which passes through the column.

Method

Buffer solution was prepared by adding 0.5% CS-HI-FBS with 2mM EDTA to sterile PBS. After isolation of PBMCs, the total cell number was determined. PBMC cells were resuspended in 40µl of buffer per 10^7 total cells, followed by the addition of 10µl of NK cell Biotin-Antibody Cocktail per 10^7 total cells and incubated for 5 minutes at 4^0C. After incubation, 30µl of buffer per 10^7 total cells was added with 20µl of NK cell MicroBead Cocktail per 10^7 total cells. This was further incubated at 4^0C for ten minutes after which the volume was adjusted to a minimum of 500µl of buffer. Magnetic separation was done with miniMACS^{TM} separator and MACS column (Miltenyi Biotec, Surrey, UK). The columns were primed with buffer in the magnetic field of the MACS separator and cells were applied gently to the top of the column. The effluent consisting of negatively selected NK cells were collected in a 5ml FACS tube. The cells were resuspended in CCM and counted.

2.9.1. NK cell mediated cytotoxicity

Target Cells

K562 cells were used here as they have been widely accepted as lacking in MHC-1 antigen making them sensitive to NK mediated cell death. Target cells were generated using the same technique described in section 2.7.

Effector cells and cytotoxicity assay

Isolated NK cells were counted and were planned to be incubated with target cells at a ratio of 1:25, target to effector cell, or lower ratios, depending on the final number of NK cells isolated. Once the target and effector cells were plated in a 96 flat bottom well plate, they were incubated for 4 hours prior to analysis by flow cytometry using the same method as in section 2.7.

Of note in reference to section 2.6.7, viability of NK cells after this isolation technique was poor <50%, and in addition the number of NK cells were insufficient to conduct any further viable cytotoxicity experiments. Due to the limited patient material the NK isolation and cytotoxicity assay was not performed.

Chapter 3

Results

Enumeration and characterisation of immune cell subsets; NK cells, T cells, T regulatory cells and NK activating receptors on NK cells in the peripheral blood of untreated male and female MCL patients

3.1. Background

The gender differences in the incidence of cancer is well documented, with the overall incidence of all cancers in males being 1.4 times higher than in females (Cook *et al*, 2011). The incidence of MCL in males is up to three times higher than in females (McKay *et al*, 2012; Chandran *et al*, 2012). There are a number of factors, such as the influence of hormones, environment and genetics, that have been implicated to influence this difference (Pennell *et al*, 2012; Libert *et al*, 2010; Dorak & Karpuzoglu, 2012). One of these factors is the difference between the innate and adaptive immune systems of males and females (Dorak & Karpuzoglu, 2012; Oertelt-Prigione, 2012; Fairweather *et al*, 2008; Chang *et al*, 2014). Females mount a better immune response than males (Klein, 2000; Hewagama *et al*, 2009; Pal & Hurria, 2010) and survive sepsis at higher rates (Dorak & Karpuzoglu, 2012). The difference between the cancer incidence and infection rates in male and females may be accounted in part by the immune system and its role in infection / pathogen surveillance and elimination. This mechanism is similar to cancer immunosurveillance by the immune cells (Gajewski *et al*, 2013; Swann & Smyth, 2007).

Cancer immunosurveillance is a process by which the immune system identifies cancerous and / or precancerous cells and eliminates them before they can cause any harm (Swann & Smyth, 2007). This forms a part of the immunoediting concept which provides a more complete explanation of the mechanism by which the immune system acts on tumours (Swann & Smyth, 2007). The concept of immunoediting is divided into three phases; elimination, equilibrium and escape as described in further details in chapter 1 section 1.1.7. The NK cells have been implicated in the process of immunosurveillance (Imai *et al*, 2000; Vitale *et al*, 2014) and one of the mechanisms by which NK cells

eliminate tumour or unhealthy cells is through the activation of NK activating receptors (Koch *et al*, 2013). A group of NK activating receptors, NCRs, NKp46, NKp30 and NKp44 have been described to deliver potent signals through ITAMS to stimulate the NK cells directly to lyse cancerous cells (Hudspeth *et al*, 2013) . This occurs after engagement with their ligands on tumour cells without the need for prior antigen activation thus allowing for rapid clearance of the cancerous cells (Hudspeth *et al*, 2013). Additionally, the activating receptor NKG2D on the surface NK cells, has also been described to be crucial for immunosurveillance of epithelial and lymphoid malignancies (Iannello & Raulet, 2013; Vivier *et al*, 2012b) by inducing direct cytotoxic activity of NK cells. NKG2D activation by its ligands; the MHC class I chain related protein A (MICA) and B (MICB) family and the UL16- binding proteins (ULBPs) family (López-Soto *et al*, 2014), results in the phosphorylation of the signal adaptor protein DNAX-activating protein and recruitment of phosphatidylinositol-3-kinase (PI3K). Activated PI3K in turn activates mitogen activated protein kinase (MEK) for stimulation of extracellular signal-regulated kinase (ERK). This ultimately leads to actin polymerisation and the granules (perforin and granzymes) become polarised towards the target. Exocytosis occurs, which results in target cell apoptosis. Thus activation of this pathway enables NK cells to secrete cytokines (IFN-γ) and induces exocytosis of cytotoxic granules to facilitate killing of the target cells (López-Soto *et al*, 2014; Nandagopal *et al*, 2014; Anel *et al*, 2012) (for further details on immunosurveillance and immunoediting please refer to chapter 1 section 1.1.7).

The concept of immunosurveillance has been supported by the increased incidence of cancer in conditions where the immune system is suppressed (Yanik *et al*, 2013). Individuals with primary or acquired immunodeficiency, for

example, as in post-organ transplantation patients who are immunosuppressed by drugs, have been observed to have an increased incidence of secondary cancers such as lymphoma (Ponce *et al*, 2014). The risk of B cell lymphoma is the highest in the first year post transplantation. This has been attributed to the greater immunosuppression during this period, which causes either reduced numbers of immune cells and activating receptors or reduced function of the immune cells during this period to reduce graft rejection (Opelz & Döhler, 2004).

Furthermore, studies in various subtypes of lymphoma have shown circulating numbers of T cells or NK cells to be lower and in turn act as surrogates for prognosis depending on the enumeration at diagnosis. A study in follicular lymphoma has demonstrated a lower number of absolute NK cells (<150/µl) correlating to an inferior outcome, p=0.02 (Shafer *et al*, 2013). Another study has demonstrated a lower absolute lymphocyte count (ALC) results in a poor outcome (ALC ≥ 1.0 x 10^9/L versus ALC ≤ 1.0^9/L, overall survival of 175 months vs 73 months, respectively, p<0.04) in patients with follicular lymphoma (Siddiqui *et al*, 2006). Variations in the immune cell subset counts have been demonstrated to influence the outcome in other subtypes of lymphomas like Diffuse Large B-cell Lymphoma (DLBCL) and lymphoproliferative disorders like chronic lymphocytic lymphoma (Palmer *et al*, 2008; Mittal *et al*, 2008). It has also been demonstrated that females exhibit a better response to lenalidomide, an immunomodulatory drug, which provides further support of the immune system's role in immunosurveillance (Eve *et al*, 2012). In other haematological malignancies such as chronic myeloid leukaemia, low number of NK activating receptors such as the NKG2D have also been reported (Boissel *et al*, 2006). There is a lack of data with regards to inhibitory NK receptors an MCL and further studies are needed.

To date, there has been no published data showing the difference in the immune cell subsets between males and females with MCL. On the basis of the above data and discussion, it was undertaken to enumerate the circulating immune cells (T cells, NK cells and T regulatory cells) and measure the NK activating receptor (NKp46, NKp30, NKp44 and NKG2D) expression on NK cells.

The hypothesis proposed was that women with MCL have a higher number of circulating immune cells and higher expression of NK activating receptors on NK cells.

The aims of this chapter were;

1) To determine if there is a difference in the number of circulating NK cells, T cells and T regulatory cells in the peripheral blood between untreated male and female patients with MCL.
2) To determine if there is a difference in the NK activating receptors (NKp46, NKp44, NKp30 and NKG2D) on NK cells in the peripheral blood of male and female with MCL.

Enumeration of NK cells, T cells, T regulatory cells and the expression of NK activating receptors was performed by flow cytometry on BD FACSCanto flow cytometer and analysis performed using the BD FACSDiva software. For further details of the materials and methods please refer to chapter 2 section 2.2.

3.2. Statistical testing

The results are presented as the mean of each group (male and female patients and controls) and +/- standard error of the means (SEM). Statistical analysis was performed using Kruskal–Wallis test for non-parametric data when

comparing more than two groups a post-hoc Dunn's multiple comparison test was applied to identify a significant difference between any of the 4 groups. The non-parametric Mann-Whitney U test was used to compare two groups, that is, between male and female patients. Statistically significant results were taken as a p value of less than 0.05. Confidence interval was at 95%. Statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad Software, San Diego, USA).

3.3. Enumeration and characterisation of immune subsets; T / NK / T regulatory cells in the peripheral blood

Enumeration of immune cell subsets was performed on peripheral venous blood from the participants. All patient samples were taken prior to the individual having had any form of therapy for MCL.

3.3.1. Enumeration of total NK cell and NK cell subset numbers

The total number of NK cells and NK subset cells were assessed in males and females from control and untreated MCL groups. The NK cell subsets included in the analysis were; CD3-$CD56^{bright}CD16^{dim}$ (involved with cytokine release in their cytolytic response) and CD3-$CD56^{dim}CD16^{bright}$ (involved with direct cytotoxic response).

There was no significant difference observed in the quantification of the total number of NK cells in the peripheral blood from male and female patients and healthy control (figure 17.0). However, there was a trend towards greater number of NK cells in male MCL patients compared to female MCL patients (figure 17.0). In addition, in only one male MCL patient out of 6 and one female MCL patient out of 6, the NK cell quantification was below the normal reference

range adopted by Derriford Hospital Combined Laboratories; NK cells 67-1134 cell/µl.

Further analysis of the subsets of NK cells did not exhibit a significant difference in the mean number of NK $CD56^{dim}$ $CD16^{bright}$ cells and NK $CD56^{bright}$ $CD16^{dim}$ cells between male and female patients nor was there any significant difference between healthy controls and MCL patients cells (Figure 15)(P=0.713 and P=0.588, $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$, respectively).

However, there was a trend towards the NK $CD56^{dim}$ $CD16^{bright}$ cells in the male MCL patients demonstrating a higher mean count (34% higher) than mean of the female MCL patients (Figure 15 B).

Conversely, the mean number of the NK $CD56^{bright}$ $CD16^{dim}$ cells was greater in the female patient group, but on examining the individual female results it was noted that one of the female patient subjects had an unexpectedly high number of these cells (Figure 15 C).

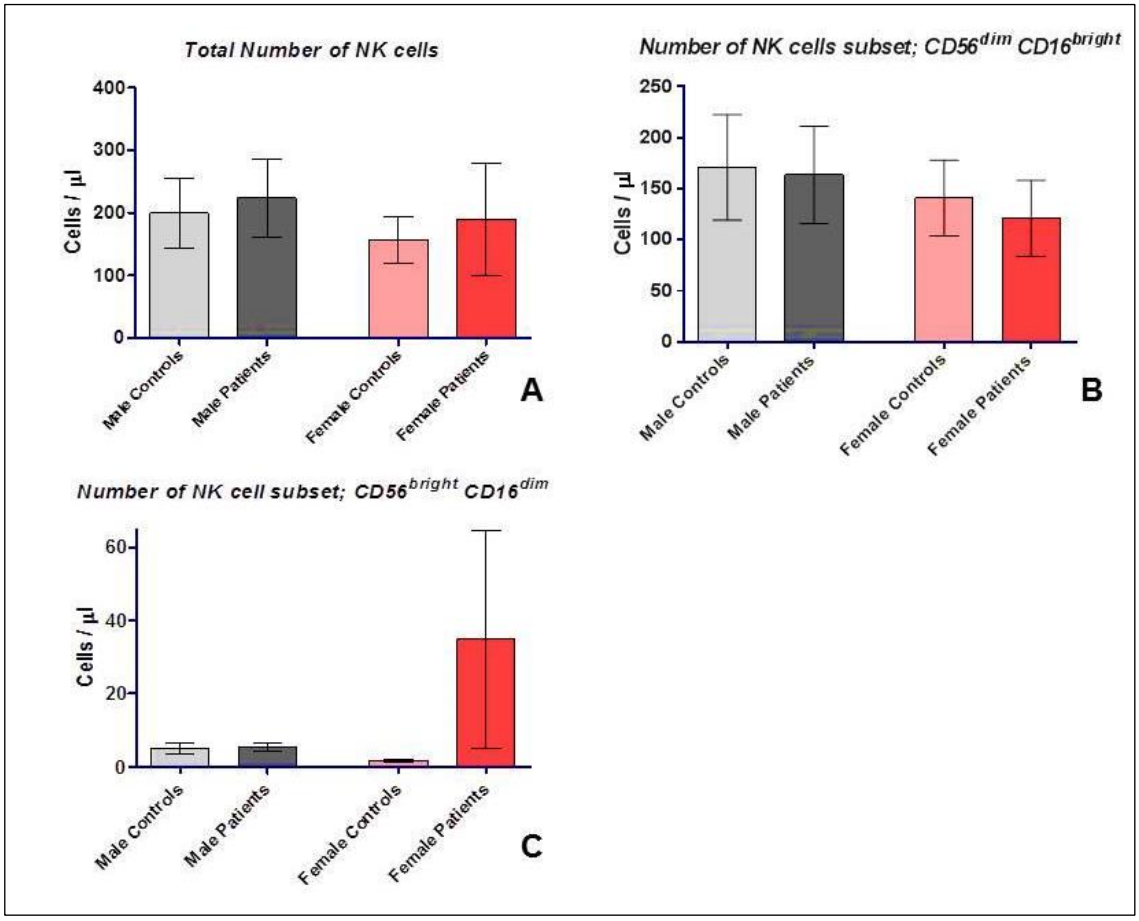


Figure 15: **NK cells and NK cell subsets numbers in peripheral blood of healthy controls and MCL patients**

Panel A: total number of NK cells determined in the peripheral blood of MCL patients and healthy control subjects, no significant difference was observed between the male and female MCL patients (p=0.91). There was a trend toward higher number in the patient group compared to the healthy controls.

Panel B: number of $CD56^{dim}$ and $CD16^{bright}$ positive NK subset cells, no significant difference was observed between the genders p=0.86, however there was trend towards male MCL patients exhibiting greater number of these cells compared to the females MCL patients, p=0.50.

Panel C: number of $CD56^{bright}$ and $CD16^{dim}$ positive NK cells. No statistical difference was observed between the female and males in either group p=0.08, however one female patient exhibited an unexpectedly high number of these cells which account for this result.

n= 5 healthy male controls, n=6 male MCL patients, n= 6 healthy female controls, n=5 MCL females MCL patients.

Interestingly, there was a trend toward patients with clinically indolent MCL exhibiting higher number of NK cells compared to the clinically aggressive patients. Therefore, further analysis of the NK cell numbers in respect to clinically indolent and aggressive MCL patients was evaluated. Patients with clinically indolent MCL were defined as asymptomatic and not requiring treatment for up to a minimum of 1 year. Clinically aggressive patients were symptomatic and requiring immediate therapy. Patients with clinically indolent MCL exhibited a higher mean number of NK cells compared to the patients with clinically aggressive MCL (Table 12). Not all patients could be included as some did not meet the criteria that defined clinically indolent MCL or clinically aggressive.

MCL patient gender	Clinically Indolent Total NK cells (cells/µl)	Clinically aggressive Total NK cells (cells/µl)	MCL patient gender
Male	344	101	Male
Male	412	94	Male
Female	114	98	Female
		176	Female
MEAN	**290 (+/- 90.2)**	**117 (+/- 19.6)**	

Table 12: **Indolent versus aggressive MCL in relation to NK cells**
Indolent MCL patients in general tended to exhibit higher number of NK cells in the peripheral blood compared to patients with clinically aggressive MCL, p=0.11. A direct comparison between male and female MCL patients in either the clinically indolent or aggressive group could not be assessed due to the limited number of subjects.

Subsequently NK $CD3^{-}CD56^{dim}$ $CD16^{bright}$ subset numbers were evaluated in patients with clinically indolent MCL and aggressive MCL. The results exhibited greater number of NK $CD3^{-}CD56^{dim}$ $CD16^{bright}$ cells in the indolent patients compared to the clinically aggressive patients (p=0.06) (Table 13). The difference between males and females with clinically indolent and aggressive

MCL was not feasible to evaluate, due to the small numbers and unequal distribution of the clinical characteristics between the genders.

MCL patient gender	Clinically Indolent NK subset: $CD56^{dim}$ $CD16^{bright}$ (cells/µl)	MCL patient gender	Clinically aggressive NK subset: $CD56^{dim}$ $CD16^{bright}$ (cells/µl)
Male	254	Male	37
Male	213	Male	45
Female	148	Female	84
		Female	68
MEAN	**205 (+/- 10.7)**		**59 (+/- 30.9)**

Table 13: Clinically indolent versus aggressive MCL patients in relation to $CD3^{+}CD56^{dim}CD16^{bright}$ NK cell subset

Enumeration of the $CD3^{+}CD56^{dim}CD16^{bright}$ NK cell subsets in relation to the clinical presentation of MCL in patients. The data presented here is groups according to the clinical presentation; aggressive vs indolent MCL. Direct comparison between male and female MCL patients in each clinical group could not be analysed due to the limited number of subjects.

3.3.2. Enumeration of T cells; T cells $CD3^{+}$, $CD3^{+}$ $CD4^{+}$ (T-helper cells) and $CD3^{+}$ $CD8^{+}$ (cytotoxic T cells) between male and female patients with MCL and age matched healthy controls.

The enumeration of T helper and cytotoxic T cells was performed due to their central role in cancer immunosurveillance and to determine if there was any gender bias in the untreated MCL patients (Kallies, 2014; Swann & Smyth, 2007; Gajewski *et al*, 2013).

There was no significant difference found in the mean numbers of the total T helper or cytotoxic T cells between male and female MCL patients (Figure 16) and additionally, no statistical difference was observed when all 4 groups were compared (P>0.5).

The male patients exhibited a trend towards greater number of both the T helper and cytotoxic T cells in the peripheral circulation compared to the male controls and female patients. Therefore, all the T cells ($CD3^+$) were enumerated in all the groups to determine if there was a gender bias. The male patient group exhibited significantly greater number of T cells in the peripheral blood compared to both the male control group and the female patient group (Figure 16).

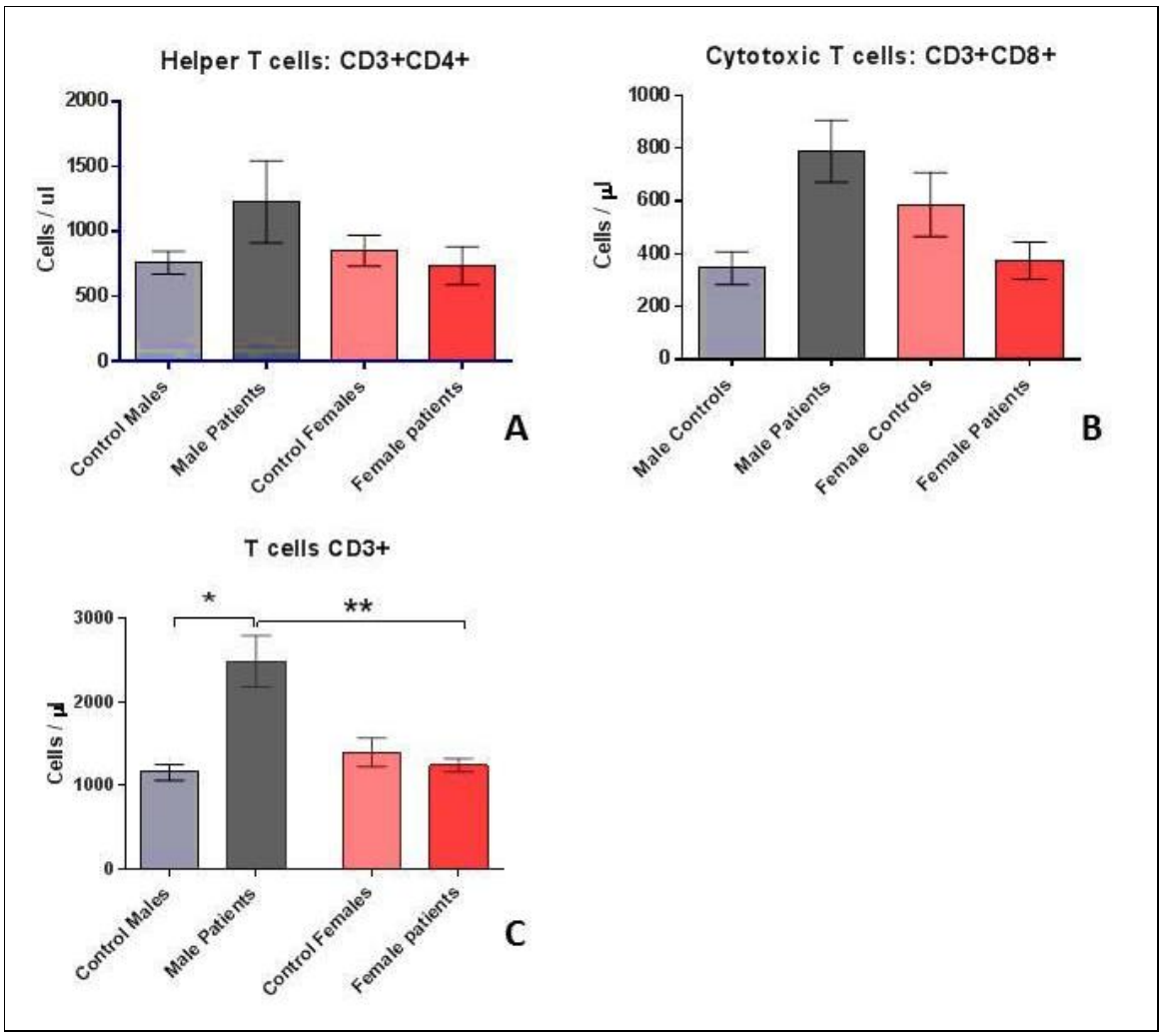


Figure 16: Enumeration of T cells ($CD3^+$), T helper cells ($CD3^+CD4^+$) and Cytotoxic T cells ($CD3^+CD8^+$)

T cell and T cell subset numbers in peripheral blood of age matched healthy controls and MCL patients

Panel A: total number of T helper cells ($CD3^+CD4^+$) in MCL patients and age matched healthy control subjects, no statistical difference was observed between the genders in either MCL patients or healthy controls patients (P=0.240). There was a trend towards male MCL patients (1229 cells/µl) exhibiting greater number of T helper cells compared to the female MCL patients (738 cells/µl).

Panel B: Number of Cytotoxic T cells ($CD3^+CD8^+$) in MCL patients and age matched healthy controls, no significant difference was observed between the genders of either MCL patients or healthy control (P=0.082) , however as with T helper cells the male MCL patients (789 cell/µl) exhibited a greater number of cytotoxic T cells compared to the female MCL patients (375 cell/µl).

Panel C: Total number of T cells $CD3^+$, male patients demonstrate a significantly greater number of circulating T cells compared to age matched male controls, *p=0.006 and female patients, **p=0.03.

n= 5 healthy male controls, n=6 male MCL patients, n= 6 healthy female controls, n=5 MCL females MCL patients.

As with the NK cells additional evaluation was performed on the T cells ($CD3^+$) numbers in clinically aggressive male (n=3) and female MCL (n=3) patients to determine whether the clinical characteristics of MCL would influence the immune cell numbers. There was no significant difference between the genders (p=0.10) (Table 14). However, there was a trend towards the male MCL patients exhibiting greater number of T cells in the peripheral circulation compared to the female MCL in this cohort of subjects which are age matched and all have stage IV disease. The number of T cells in clinically indolent MCL had similar results as those of clinically aggressive MCL however direct comparison was not feasible due to limited numbers (n=2 males and n=1 females). This could perhaps be reflective to the difference seen in the tumour microenvironment between the male and female MCL patients. It is possible that female patients could exhibit higher number of T cells within the tumour microenvironment thus resulting in fewer T cells in the peripheral blood as the T cells egress towards the affected nodes. It is not possible to comment on the activation of the T cells between the genders with these results as they primarily reflect the numbers of T cells in the peripheral circulation.

	T cells ($CD3^+$) (cells/µl)	
	Male clinically aggressive MCL	**Female clinically aggressive MCL**
	1473	1456
	2224	1355
	2537	1268
Mean	**2078 (+/- 316)**	**1360 (+/- 54.3)**

Table 14: T cells numbers in the peripheral blood of 3 male and 3 female MCL age matched

(median age for the MCL patients; males 70 years and females 68 years) patients presenting with clinically aggressive MCL. All the subjects in both male and female MCL groups presented here has stage IV disease (p=0.10).

3.3.3. Enumeration of T regulatory cells; $CD4^{+}CD25^{+}CD127^{dim}$ in male and female patients and age matched healthy controls

Enumeration of T regulatory cells was performed due to their vital role in cancer immunosurveillance as discussed above. T regulatory cells are primarily function to suppress the immune response. They are involved in the suppression of T cells which in turn indirectly inhibits the T cells (T helper cells) activity against cancerous cells as described in chapter 1 section 1.1.4.

No significant difference was found in the mean numbers of T regulatory cells between male and female patients or when all 4 groups were compared (p=0.476)(Figure 17).

In both the male and female MCL patient groups there was a trend exhibiting greater number of T regulatory cells when compared to their respective controls groups (Figure 17).

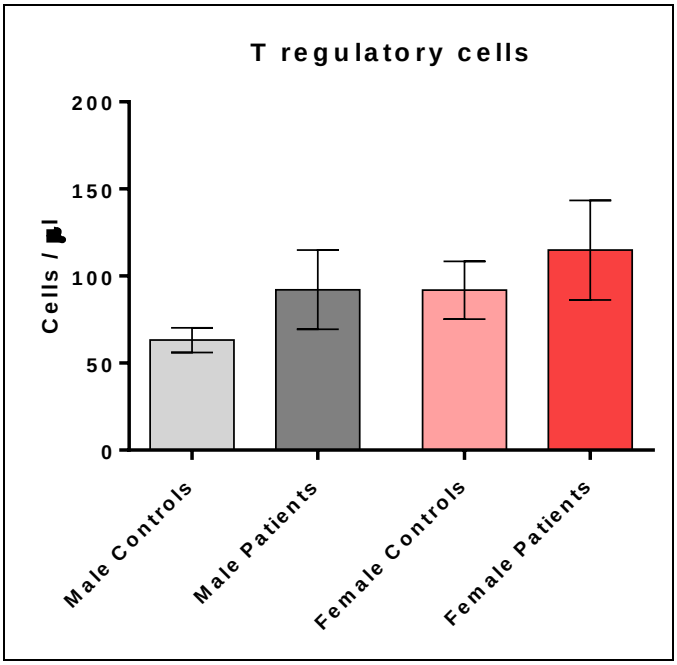


Figure 17: Enumeration of T regulatory cell ($CD4^{+}CD25^{+}CD127^{dim}$) in peripheral blood of all 4 groups. Age matched subjects; n=5 healthy males, n=6 MCL males, n= 6 healthy females, n=5 MCL females measured by FACS analysis (p=0.59).

Additional analysis was performed to determine if there was a gender difference in relation to the numbers of T regulatory cells between clinically indolent and aggressive MCL patients. There was no significant difference between the male and female MCL patients when comparing the clinically aggressive MCL. However there was overall trend toward higher numbers of T regs in female MCL patients (Table 15). Due to the limited number of clinically indolent MCL patients direct comparison was not possible.

One female patients had a lower T regulatory cell count compared to the rest of the female MCL subjects shown here (Table 15). This difference was could not fully be accounted for by the clinical features of the patient as this was consistent with the rest of the group. It is thought to reflect individual variation in T regulatory cell numbers. It is not possible to conclude further on this 'outlier' result with the one female aggressive MCL, however greater number of subjects would be warranted to fully describe this result.

T regulatory cells (cells/µl)	
Male clinically aggressive MCL patients	**Female clinically aggressive MCL patients**
54	143
99	48
64	102
Mean	
72 (+/- 10.7)	97 (+/- 27.5)

Table 15: Quantification of T regulatory cells in the peripheral blood of clinically aggressive MCL male and female MCL patients
All subjects had stage IV disease at diagnosis. Both male and female MCL subjects presented here were age matched (median age 70 years male MCL and 68 years female MCL patients) (p=0.40).

The results demonstrating the enumeration of the T regulatory cells in the peripheral blood of age matched male and female MCL patients exhibit no significant difference between the genders. However there is a trend towards female MCL patients in this cohort exhibiting a greater number of T regulatory cells. When compared to age matched controls the patient groups exhibit a trend towards greater number of T regulatory cells in the peripheral circulation, however this did not achieve statistical significance.

3.4. Examining the expression of the NK cell activating receptors; NKp46, NKp30, NKp44 and NKG2D in male and female patients and age matched controls

NK activating receptors; NKp46, NKp30, NKp44 or NKG2D on the two subsets $CD3^{-}CD56^{bright}$ and $CD3^{-}CD56^{dim}$ where examined to determine whether there was any evidence of gender difference. NK activating receptors have an important role in NK-mediated elimination of cancerous cells (Koch *et al*, 2013; López-Soto *et al*, 2014).

3.4.1. Evaluating the expression of the NK activating receptor; NKp46, NKp30, NKp44 and NKG2D on $CD3^{-}CD56^{bright}$ NK cells

The $CD3^{-}CD56^{bright}$ NK cells (cytokine subset of NK cells) exhibited high levels of NKp46, NKp30 and NKG2D and a moderate level of NKp44 activating receptor in both the control and patient groups when compared to the isotype controls for each activating receptor (Figure 18).

None of the NK activating receptors; NKp46 ($p=0.75$), NKp30 ($p=0.69$), NKp44 ($p=0.34$) or NKG2D ($p=0.50$) on the $CD3^{-}CD56^{bright}$ NK cells exhibited a statistically significant difference between the males and females in the control and patient groups. Furthermore, there was no significant difference between

the male control group and the male patient group or the female control group and the female patient group (p=0.50) (Figure 18).

Although there were small variations amongst the groups of the expression of the NK activating receptors, there were no notable trends in the groups.

Therefore, in conclusion there was no significant difference in the expression on the NK activating receptors between the male and female MCL patients on the $CD3^{-}CD56^{bright}$ NK cells (cytokine subset of NK cells).

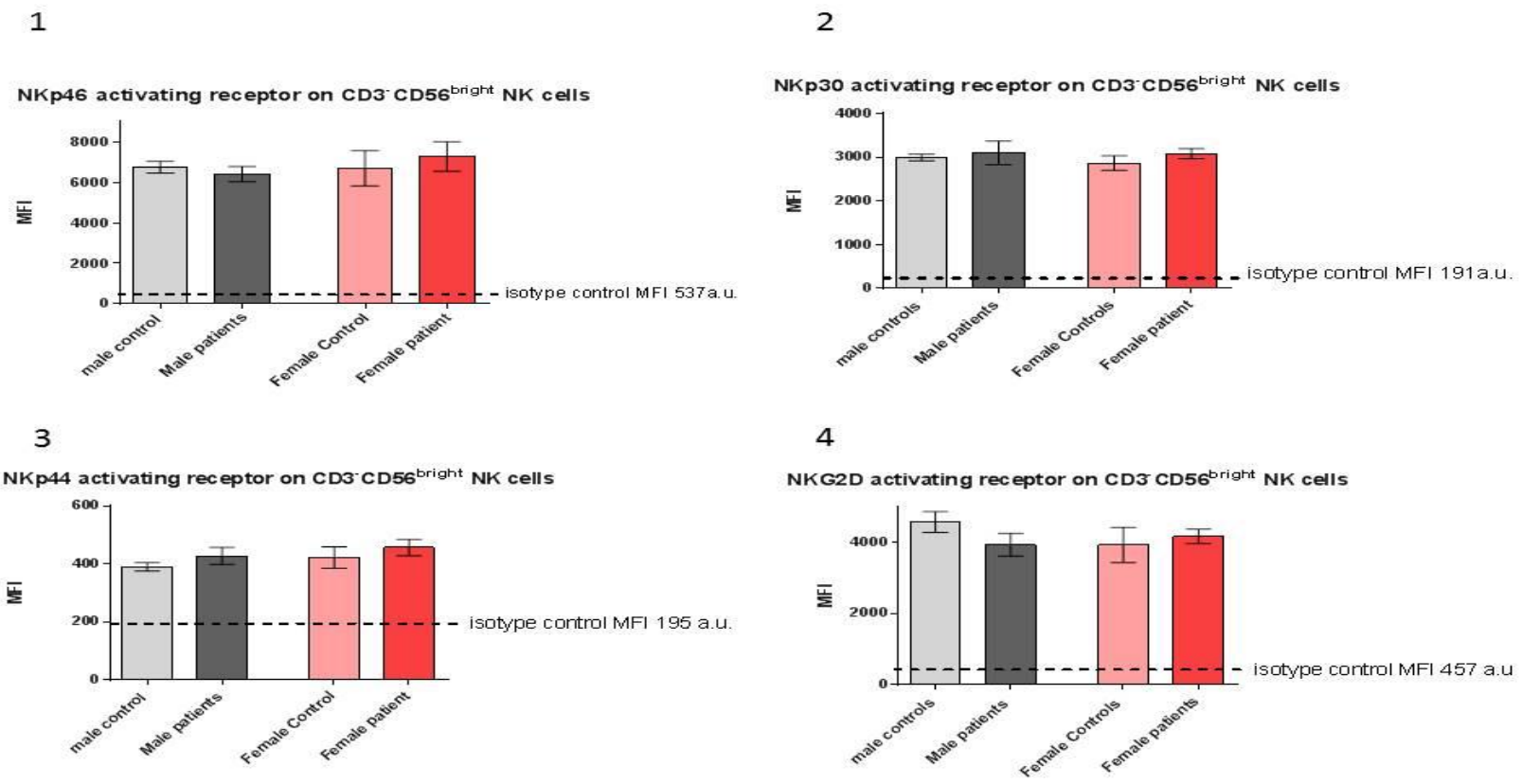


Figure 18: Expression of NK cell activation receptors on NK cytokine subset CD3$^-$CD56^{bright} cells in the peripheral blood of 5 male and 6 female age matched healthy controls and 6 male and female MCL patients (p >0.05, Kruskal-Wallis). All NK activating receptors had greater expression than isotype controls. 1. NKp46 expression ; 2. NKp30 expression; 3. NKp44 expression; 4. NKG2D expression on NK cytokine subset CD3$^-$CD56^{bright} cells.

3.4.2. Comparison of the expression of NK activating receptors on NK cells; $CD3^{-}CD56^{dim}$ between male and female MCL patients and controls

3.4.2.1 Evaluating expression of NK activating receptor NKp46, NKp30, Nkp44 and NKG2D on $CD3^{-}CD56^{dim}$ NK cells

The $CD3^{-}CD56^{dim}$ NK cells (cytotoxic NK cells) exhibited high levels of NKp46, NKp30 and NKG2D and a moderate level of NKp44 activating receptor in both the control and patient groups when compared to the isotype controls for each of activating receptor. There were no significance difference in the expression of all the four NK activating receptors when compared between all the groups (NKp46 p=0.68; NKp30 p=0.40; NKp44 p=0.66; NKG2D p=0.34; Kruskal-Wallis test)(figure 21.0) and specifically there was no significant difference exhibited between male and female MCL patients (Figure 19). However, there was a trend towards a greater expression of NK activating receptor NKG2D in the male and female controls when compared to the respective patient groups (Figure 19).

In conclusion there was no significant difference between the male and female MCL patients with respect to the activating receptors on the cytotoxic NK cells $CD3^{-}CD56^{dim}$.

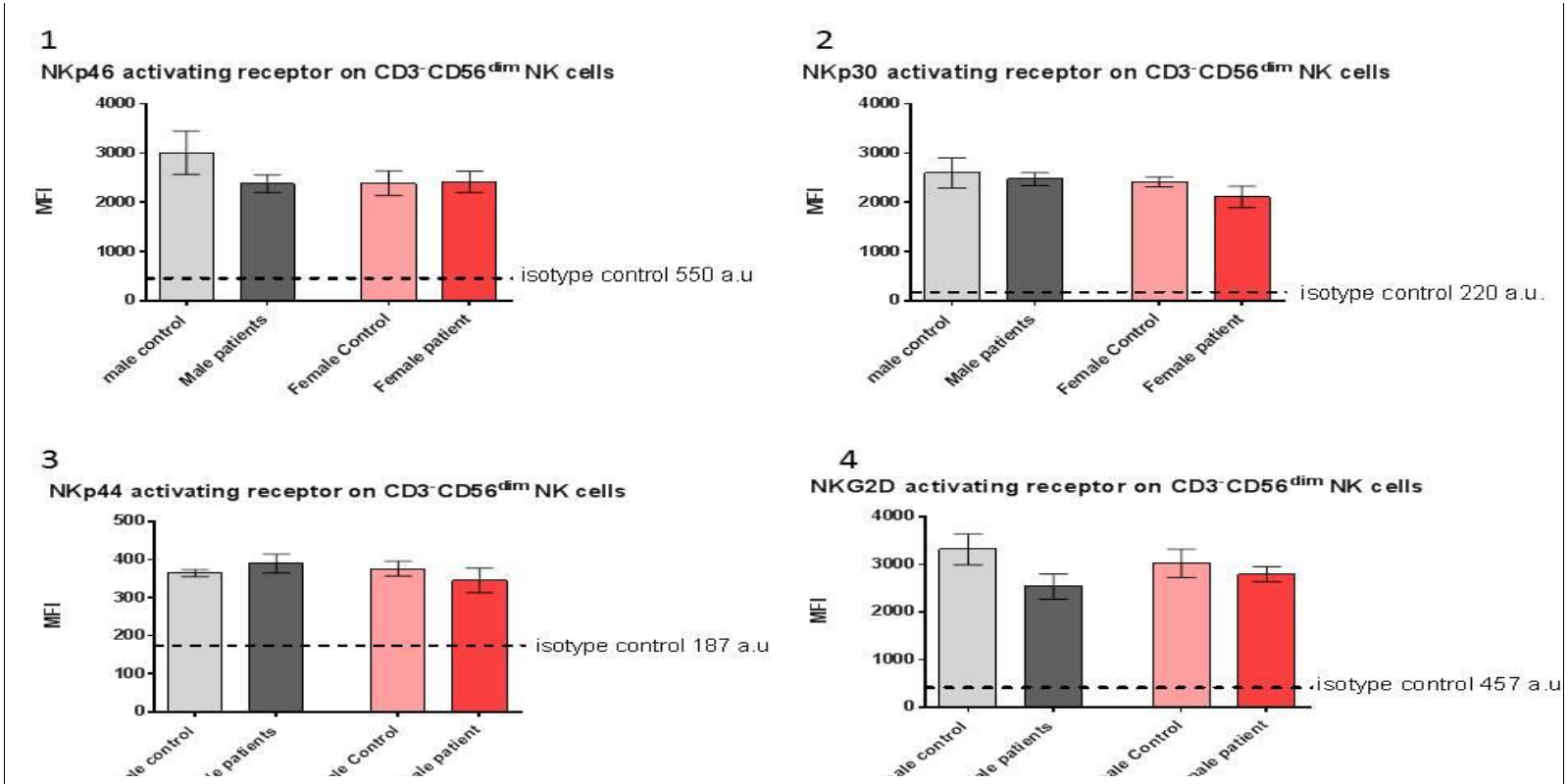


Figure 19: Expression of NK cell activation receptors on cytotoxic NK cells CD3-CD56^{dim} cells in the peripheral blood of 5 male and 6 female healthy age matched controls; and 6 male and female MCL patients(p>0.05, Kruskal-Wallis). Expression of the NK activating receptors were analysed by flow cytometry. All receptors had greater expression than isotype controls. **1.** NKp46 activating receptor; **2**. NKp30 activating receptor; **3.** NKp44 activating receptor; **4.** NKG2D activating receptor expression on cytotoxic NK cells.

3.5.2. T cells $CD3^+$, $CD3^+CD4^+$ (T- helper cells) and $CD3^+C8^+$ (Cytotoxic T cells)

There was a significant increase in the number of circulating T cells ($CD3^+$) in male MCL patients when compared to female MCL patients, p=0.04.

There was no significant difference exhibited when comparing the male and females MCL patients with regards to the T cell subsets; T helper and cytotoxic cells. However, in both these subsets, there was a trend towards greater numbers in male MCL patients compared to the female MCL patients.

3.5.3. T regulatory cells

The T regulatory cells did not exhibit a significance difference between male and female MCL patients or healthy controls.

However there was a trend toward female MCL patients exhibiting greater number of T regulatory cells compared to the male MCL patients. It is important to note that the enumeration of the T regulatory cells is in the peripheral blood. Therefore, as T regulatory cells have been implicated in regulation and suppression of the T cells (Josefowicz *et al*, 2012), it is probable that the number of T regulatory cell in the tumour microenvironment of female MCL patients could be significantly lower than compared to the male MCL patients, thus enabling females to mount a greater immune response to the MCL cells.

3.5.4. Expression of NK activating receptors on NK cell subset $CD3^-$ $CD56^{bright}CD16^{dim}$

There was no significant difference observed between the expression of the NK activating receptors between the male MCL patients and female MCL patients.

3.5.5. Expression of NK activating receptors on NK cell subset: $CD3^{-}$ $CD56^{dim}CD16^{bright}$

There was no significant difference between the expression of the NK activating receptors between the male MCL patients and female MCL patients.

There was a trend exhibiting greater expression of the NKG2D receptor on control groups compared to the patient groups.

As previously described in chapter 1, NCRs receptors (NKp30, NKp44 and NKp46) belong to the immunoglobulin superfamily. These receptors comprise of an extracellular ligand-binding domain, transmembrane domain and a short cytosolic domain. They involve the ITAM-dependent signalling molecules to activate several intercellular pathways and in addition it has been described that they can activate each other through phosphorylation of the adapter molecules thus increasing their efficiency (Koch *et al*, 2013). Individually they are activated by ligands which attach onto the extracellular domains. NKp30 receptor has been shown to be activated by the tumour antigen B7-H6 which are tumour specific as this ligand is not found on healthy cells (Brandt *et al*, 2009; Koch *et al*, 2013). Interestingly the expression of NKp44 is increased only after activation and also correlates to the efficiency of the NK cell activity and killing (Fauriat *et al*, 2007). However the activating ligand for NKp44 is yet to be described as with the NKp46 receptor's ligand which again remains unknown. Nonetheless the activating NCRs can crosstalk between each other and is thought to enhance their efficiency to stimulate the NK cells to either release cytokines or activate cytotoxicity (Augugliaro *et al*, 2003; Koch *et al*, 2013). These NCRs are found on both subsets of NK cells and although they can potentially be stimulated individually they also complement each other for activation. It has been described that lower expression of NCRs lead to reduced

activity (Fauriat *et al*, 2007) and the results do not show any significant difference between the expression of the NCRs between the genders suggesting that these activating receptors may not play a significant role in the immune response mounted by either the male or female MCL patients.

NKG2D is a c- type lectin like receptor and it is not able to respond if stimulated alone but needs another 'co-activation' signal from other activating receptors such as NKp46. It has two MICA and MICB ligands that activate the receptor. It is interesting that the expression of NKG2D has a higher trend in the control group compared to the MCL patient. This could relate to lower activation of the NK cells in MCL patients possibly due to inhibitory ligands expressed by MCL cells. There is no difference between the genders which may negate the role of the NK cells in between the genders in terms of the activating receptors investigated here.

3.6.0 Discussion

Despite the results showing very few statistically significant differences between the male and female immune cell subsets and NK activating receptors, some interesting trends emerged that are further discussed below:

3.6.1. NK cells and NK cell subsets

NK cells, cytokine NK cells $CD3^{-}CD56^{bright}$ $CD16^{dim}$ and cytotoxic NK cells $CD3^{-}CD56^{dim}$ $CD16^{bright}$

In summary, the quantification of NK cells and the subsets demonstrated no significant difference between male and female MCL patients. There was a trend towards greater mean number of total NK cells in MCL patients compared to healthy controls. In addition there was a trend towards greater number of NK cells as a whole in male MCL patients compared to the female MCL patients.

To date there is no published data that has examined whether there is a difference in NK cell numbers between male and female patients with MCL or B cell lymphoproliferative disorders.

The trend exhibited by the total number of NK cells described above may be explained by the differing distribution of clinically indolent MCL in male and female patients. In this cohort of patients there were 50% of male patients and 15% of the female patients with clinically indolent MCL patients (table 6.0, chapter 2). Patients with a clinically indolent subtype of MCL (asymptomatic and not requiring therapy for > 1 year) tend to present a more favourable outcome compared to the more aggressive subtype (symptomatic and needing therapy immediately) (Hsi & Martin, 2014; Shah & Rule, 2014). The results also showed that clinically indolent patients exhibited a higher mean of NK cells (290 cells/µl +/- 90.2) compared to patients with clinically aggressive MCL (117 cells/µl +/- 19.6)(table 11.0). Patients with clinically indolent MCL have better OS compared to clinically aggressive MCL as discussed in chapter 1 (Hsi & Martin, 2014). The NK cell numbers observed between clinically indolent and aggressive disease correlate in part to other studies which demonstrated an association between patients exhibiting a greater number of NK cells and improved overall survival (Palmer *et al*, 2008; Shafer *et al*, 2013; Siddiqui *et al*, 2006; Sanchez *et al*, 2011). Another study reported by Shafer, *et al*, demonstrated a low number of peripheral NK cells was associated with an inferior outcome in patients with follicular lymphoma (5% versus 2% had died p=0.02, low NK numbers versus high NK numbers respectively) (Shafer *et al*, 2013). This has also been demonstrated in patients with high grade diffuse large B cell lymphoma with low NK cells numbers in peripheral circulation resulted in an inferior survival (p=0.02) (Plonquet *et al*, 2007).

In addition there was a trend towards greater mean number of NK cells in the peripheral circulation of MCL patients compared to the healthy controls. This possibly reflects the response of the immune system against the cancerous cells. The trend observed here correlates to a study which demonstrated higher number of circulating NK cells in CLL patient compared to controls ($p<0.05$) (Costello *et al*, 2012). They accounted for the increase in absolute NK cell numbers to greater numbers of $CD3^{-}CD56^{dim}$ NK cells subset which are involved in direct cytotoxicity, suggesting the increase in NK cells was due to the immune system's response to eliminate CLL cells (Costello *et al*, 2012). Majority of the subjects (91%) described in the study by Costello *et al*, exhibited Binet stage A disease (low stage, indolent) disease, the results here with clinically indolent MCL patients (table 12.0) also correlate to their findings (Costello *et al*, 2012) suggesting that a similar mechanism may exist with NK cells and MCL.

NK Cell subsets: Cytokine NK cells $CD3^{-}CD56^{bright}CD16^{dim}$

The numbers of the NK cell subset; $CD3^{-}CD56^{bright}CD16^{dim}$ was found to be higher in female patients compared to male patients. This was accounted for by one female patient exhibiting an unexpectedly high number of this NK subset which notably skewed the result (Chapter 2.0; Table 6.0; patient FP004). Interestingly, the cytokine NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$ makes up about 10% of the NK cells in the circulation and 95% of the NK cells in lymphoid tissue (Caligiuri, 2008; Campbell & Hasegawa, 2013; Ferlazzo *et al*, 2004). Cytokine NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$ bridge the innate and adaptive immune system by producing cytokines to induce T cells (Caligiuri, 2008). IFN-γ is considered to be the prototypic NK-cell cytokine produced by NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$. This NK subset needs two signals to produce IFN-γ.

One always includes IL-12 and the second can be either IL-1, IL-2, IL-15, or IL-18, or engagement of an NK-activating receptor (Cooper *et al*, 2001). The release of cytokines IFN-γ, TNF-α and MIP-1α occurs rapidly and the release of IFN-γ by the NK cells can activate the APCs to upregulate MHC class 1 and increase APC cytokine secretion which help to bridge and activate the adaptive immune system (Fehniger *et al*, 2003). In addition the Cytokine NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$ expresses the high-affinity IL-2 receptor complex (HA IL-2αRβγ and IA IL-2Rβγ) and competes for picomolar concentrations of IL-2 that are released by the T cells in the lymph nodes. This co-stimulation with IL-2 derived from T cells and IL-12 derived monocytes or DC cells results in large amounts of IFN-γ production. Thus, consolidating the process by which the NK cells link the innate and adaptive immune system.

The cytokine NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$ expresses high levels of L-selectin and CCR7, both of which are involved in trafficking of immune cells to the lymph nodes. Therefore it is probable that these NK cells could traffic to the site of developing adaptive immune responses and have dynamic interactions with the T cells and APCs. This could also explain the excess number of NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$ present in the peripheral blood of this one female patient, where these cells may be migrating to the affected lymph nodes. A possible method to determine whether these cells are migratory is to analyse the expression of the CCR7. CCR7 is expressed on the NK cell subset $CD3^{-}CD56^{bright}CD16^{dim}$ and not usually present on the NK cell subset $CD3^{-}CD56^{dim}CD16^{bright}$ .High expression of CCR7 is related to NK cells homing to affected nodes. The expression of CCR7 could be used as a surrogate marker to compare their expression to control NK cells to determine if these cells are

migratory (Somanchi *et al*, 2012). Discounting this result from the data set no overall difference was observed between male and female MCL patients.

NK subset: Cytotoxic NK cells $CD3^{-}CD56^{dim}CD16^{bright}$

Interestingly, Cytotoxic NK cell subset; $CD3^{-}CD56^{dim}CD16^{bright}$ which is involved in direct killing of tumour cells was found to exhibit a trend towards lower numbers in female MCL patients compared to male MCL patients. This subset is deemed to be the more mature of the NK cell subset and represents 90% of the circulating NK cell population (Sanchez *et al*, 2011; Caligiuri, 2008). It could be that these cells remain and continue to mature in the affected lymph nodes in order to kill the MCL cells. This may explain the lower numbers present in the circulation.

Further analysis of the NK cell subset $CD3^{-}CD56^{dim}CD16^{bright}$ between clinically aggressive and indolent MCL patients found that the patients with a clinically aggressive MCL had lower numbers than patients with clinically indolent MCL (table 3.0b). The lower numbers of NK cell subset $CD3^{-}CD56^{dim}CD16^{bright}$ in female patients could be accounted for by the higher numbers of female patients with aggressive disease in this cohort.

It is probable that lower number of NK cells observed in the peripheral circulation may reflect higher numbers in the affected lymph node or that aggressive MCL could be inhibiting the bone marrow and lymph nodes' capacity to produce these cells. A study with mouse models has demonstrated increased number of NK cells in lymph nodes affected with lymphoma in λ-myc transgenic mice which are prone to develop spontaneous lymphomas compared to wild type mice, thus supporting the possibility of NK cells migrating to affected nodes (Brenner *et al*, 2010).

One of the limitations of the enumeration of NK cells and NK cell subsets has been the exclusion of $CD3^{-}CD56^{+}CD16^{-}$ cells. Although the overall result may be unaltered it would be interesting to explore this. Currently, there are no pan-antibody markers for NK cells and some studies have used NKp46 as a marker for all NK cells types (Caligiuri, 2008; Tomasello *et al*, 2012) but this has also been shown to be expressed on T cells, thus limiting its specificity for NK cells .It would have been interesting to explore this by flow cytometry where the CD3 negative, CD4 negative cells, CD56 positive and NKp46 positive cells are identified and evaluated to determine whether this encompasses all NK cells.

Although there was no significant difference observed with the number of NK cells and the subsets between male and female MCL patients it would be interesting to determine whether there is a difference in the base line activation of the NK cells between the genders in MCL. One possible method is to measure the surface expression of the lysosomal-associated membrane protein–1 (CD107a) by flow cytometry. As the expression of CD107a correlates to NK cell activation (Penack *et al*, 2005) this would potentially provide a rapid quantification of activated cytotoxic NK cells between the genders.

3.6.2. T cells $CD3^{+}$, $CD3^{+}CD4^{+}$ (T Helper cells) and $CD3^{+}CD8^{+}$ (cytotoxic T cells)

The results exhibited a significantly greater numbers of $CD3^{+}$ T cells in male MCL patients compared to female MCL patients. Although the numbers of the T cell subsets $CD3^{+}$ $CD4^{+}$ and $CD3^{+}$ $CD8^{+}$ showed no statistical difference between the genders there was a trend towards male MCL patients exhibiting higher numbers in the peripheral blood.

The findings above may be explained by the migration of T cells to the tumour microenvironment. It is possible that the female patients may have higher

number of T cells surrounding the MCL cells within the affected lymph nodes and therefore lower numbers in the peripheral circulation. As mentioned previously the burden of MCL in the lymph nodes in clinically aggressive MCL may inhibit the proliferation or migration of the T cells resulting in fewer T cells in the circulation. As this cohort of patients had more females with clinically aggressive MCL patients this may be another possible explanation for male patients exhibiting a higher mean number of T cells in the peripheral circulation compared to female patients. It would be interesting to analyse the chemokine receptors on T cells that are involved with the migration of T cells. For example CCR7 chemokine receptor is associated with migration of T cells into lymph nodes (Von Andrian & Mackay, 2000). Analysing the expression of this receptor by flow cytometry on T cells of female and male MCL patients may aid to explain the difference in in the number of T cells in the peripheral blood (Von Andrian & Mackay, 2000; Pham *et al*, 2008).

The analysis of the enumeration of T cells in 6 clinically aggressive male (n=3) and female (n=3) patients with MCL found no statistically significant difference (p=0.1). However there was a trend towards the male MCL patients exhibiting greater number of T cells.

Another T cell subset that would be of further interest to explore is the Natural Killer like T cells (NKT cells). These cells are T cells with a T cell receptor, but unlike conventional T cells that detect peptide antigens presented by the conventional MHC molecules, NKT cells recognize lipid antigens presented by CD1d, a non-classical MHC molecule (Rossjohn *et al*, 2012; Vivier *et al*, 2012b). They bridge the innate and adaptive immune system by producing large and rapid quantities of cytokines that can stimulate T cells and NK cells

(Terabe & Berzofsky, 2008). NKT cells are represented by two subsets; type I NKT cells (also known as invariant NKT or iNKT cells) expressing the invariant Vα24Jα18 TCR and Type II NKT cells expressing the more diverse set of α chains in their TCR, however both are CD1d restricted (Godfrey *et al*, 2004). NKT cells are primary class of T cells that can provide the immune system with a mechanism of specific recognition of lipid antigens as CD1d is found to be present in lipids or glycolipids rather than peptides (Godfrey *et al*, 2010).

Type I NKT cells are activated by α-GalCer or endogenous lipids (may be tumour derived) presented by CD1d on immature dendritic cells. They produce interferon-γ which induces maturation of the dendritic cells which promotes the dendritic cells to produce IL-12, which augments the IL-2 and IFN-γ production by type I NKT cells. Production of IL-2, IL-12 and IFN-γ activates NK cells, $CD8^+$ T cells and macrophages (Terabe & Berzofsky, 2008). Additionally activation of NK cells ($CD56^{bright}$ subset) would potentially lead to increase in IFN-γ production resulting in a positive feedback loop to enhance the T cells and NK cells activity.

Type II NKT cells tend to suppress tumour immunity. These cells are activated by tumour derived glycolipids presented by CD1d to produce IL-13. Together with TNF-α induces expression of TGF-β which suppresses activation of cytotoxic $CD8^+$ T cells. In some setting the IL-13 may induce M2 macrophages that also suppress $CD8^+$ T cells (Terabe & Berzofsky, 2008; Rossjohn *et al*, 2012).

Interestingly it has been reported that NKT cell have anti-tumour effect on MCL cells by the production of IFN-γ. This has been demonstrated in vitro by human NKT cells producing higher levels of IFN-γ when exposed to B cells derived

from MCL patients compared to healthy donors which suggest mechanisms by which NKT cells recognize and respond to malignancy (Li *et al*, 2014). It would therefore be interesting to enumerate these cells in MCL patients to identify if there is any difference between the genders. As reduced numbers of iNKT cells could potentially related to poorer immunity against MCL.

3.6.3. T regulatory cells

T regulatory cells are an immunosuppressive T cell subset that functions to prevent autoimmunity and regulate immune response. These cells are important in the immunosurveillance process and data has suggested that their role is context-dependent. In some solid tumours such as ovarian, breast and prostate cancers higher number of infiltrating T regulatory cells is associated with a poor outcome (Ebelt *et al*, 2009; Cureil *et al*, 2004). However, in lymphoid malignancies a number of studies have shown a better outcome with higher number of T regulatory cells, especially in follicular lymphoma and diffuse large B cell lymphoma (Głowala-Kosińska *et al*, 2013; Kelley & Parker, 2010). These studies have proposed that the better outcome could be due to the possible direct cytotoxic effect of T regulatory cells on the lymphoma cells or by interfering with T-cell help. One of the mechanisms proposed in suppressing malignant B cells is related to the suppressive effects of the T regulatory cells on T cells. T regulatory cells release IL-10, TGF-β and IL-35 which are largely inhibitory cytokines. IL-10 induces anergy in both $CD4^+$ and $CD8^+$ T cells and downs regulates the expression of co-stimulatory molecules, adhesion molecules and MHC-class II on antigen presenting cells (Steinbrink *et al*, 2002). TGF-β inhibits IL-2 production and in turn can inhibits T cell proliferation. IL-35 can also inhibit T cell proliferation and conversely stimulate T regulatory cell proliferation. Malignant B cells require the microenvironment to develop and this

relates to close contact with T helper cells. It is likely that T regulatory cells which inhibit T helpers cells can disrupt the microenvironment of the malignant B cells to inhibit its proliferation by indirectly inhibiting the T helper cells (Lindqvist & Loskog, 2012). A recent study has demonstrated direct interaction of T regulatory cells with malignant B cells. When T regulatory cells from healthy controls and from patients with CLL were exposed to autologous malignant B cells (CLL) they exhibited lysis of the malignant B cells and killing was confirmed by granzyme ELISAs (Lindqvist *et al*, 2011). This study suggested that the T regulatory cell's natural function as a suppressor of autoimmunity is related to direct cytotoxicity of the malignant B cells. It has been proposed that CLL is driven by autoantigens and CLL cells were shown to produce autoantibodies. In addition CLL has been connected to several different autoimmune conditions (Ansell *et al*, 2011). T regulatory cells controlling B cells express the B cells in an antigen specific manner (T cell receptor – MHC II- restricted) because malignant B cells express MHC-II and killing via death receptor ligands or granzyme release is commonly regulated via T cell antigen recognition (Lindqvist *et al*, 2011; Lindqvist & Loskog, 2012).

However there are studies as mentioned previously that correlate high number of T regulatory cells to poor outcomes. These discrepancies may be due to the methods used to detect T regulatory cells, an example being the use of PCH101 antibody can mistakenly also stain activated T cells. Thus what are thought to be T regulatory cells could represent activated T cells. Currently, The role of T regulatory cell in mantle cell lymphoma has not been fully investigated and require further studies.

There was no significant difference found between male and female MCL patients or controls with the quantification of T regulatory cells. Interestingly, the patient group tended to have a higher mean compared to the controls. This may suggest that the patients possess a more activated immune system in order to deal with the cancerous cells or alternatively due to the lymph node infiltration with MCL there is an egress of T regulatory cells into the peripheral blood. This trend also conforms to T regulatory findings in other studies with lymphoma, where the numbers have been higher in patients compared to controls (Mittal *et al*, 2008; Kelley & Parker, 2010). One such study reported by Mittal S, *et al*, demonstrated T regulatory cells increased in patients with B cell non-Hodgkin's disease compared to healthy controls (20.4% versus 3.2% patients vs. controls respectively, p=0.001)(Mittal *et al*, 2008). Furthermore, the results show a trend of higher T regulatory cell numbers in female patients. This concurs with the finding reported by Eve et al (Eve *et al*, 2012).

Therefore, the trend towards female MCL patients in this study having a higher number of T regulatory cells compared to male MCL patients could provide a reason for the gender difference observed in MCL. However this trend observed with T regulatory cells needs to be further correlated in studies with larger number of MCL participants. As mentioned above, the exact role of T regulatory cells in MCL needs to be understood.

3.6.4. NK activating receptors; NKp46, NKp30, NKp44 and NKG2D on $CD3^-$ $CD56^{bright}$ NK cell subset and $CD3^-CD56^{dim}$ NK cell subset

The high expression of NKp46 activating receptor has been shown to correlate with higher NK cell cytotoxicity activity and is also associated with improved outcomes in haematological malignancies such as acute myeloid leukaemia (Fauriat *et al*, 2007; Epling-Burnette *et al*, 2007; Sivori *et al*, 1999). Interestingly

majority of the evidence in relation to NK cells role in tumour clearance in leukemia has come from killer cell inhibitory receptor (KIR) mismatch in allogenic stem cell transplantation in the treatment of acute myeloid leukemia (Moretta *et al*, 2014). Study by Fauriat et al, demonstrated reduction in NCR expression in patients with AML. The expression of the NCRs improved after therapy when patients achieved a complete remission. AML patients with low expression of NCRs on the NK cells played a role in the patients' outcome suggesting a possible surveillance role of the NK cells against leukemia cells (Fauriat *et al*, 2007).

However, there was no significant difference observed in the expression of NKp46 activating receptor on either of the NK cell subsets between male and female MCL patients or when compared to the control groups. NKp46 is constitutively expressed on NK cells as is NKp30 (Hudspeth *et al*, 2013), which also demonstrated no significant difference between the male and female patients or the controls. It is possible that circulating NK cells are not activated or stimulated and thus the expression of the NKp46 and NKp30 is not altered significantly between the genders or controls. It is also possible that these circulating NK cells remain dormant until they reach the tumour site where they are stimulated by the antigen on the tumour itself, dendritic cells or T cells and their production of cytokines within the microenvironment (Moretta, 2002). Both the NK 56^{dim} and NK 56^{bright} cells expression cytokine receptors which influence their functions. The following cytokine receptors are expression on both subsets of NK cells; IL-12R (IL-12Rb1 and IL-12Rb2), IL-15R (IL-15Rα and IL-2/15Rβγ_c), IL-18R (IL-18Rα/R1 and lL18Rβ/RAP), IL-21R (IL-21Rγ_c) and IL-2R (IL-2Rαβγ; $CD56^{bright}$ and IL-2Rβγ; $CD56^{bright}$ and $CD56^{dim}$) (Romee *et al*, 2014). Cytokines produced by dendritic cells, T cells and macrophages in the

microenvironment activate the NK cells and encourage further cytokine production, proliferation, survival and cytotoxicity of the NK cells (Romee *et al*, 2014).

IL-2 and IL-15 have been the most studied cytokine activators of NK cells. The signalling through the IL-2/IL15 receptor induces proliferation, and enhances cytotoxic effects. IL-2 and IL-15 share the IL-2/IL-15Rβ and γ_c as the primary signalling subunits. Both $CD56^{bright}$ and $CD56^{dim}$ cells have high affinity IL-2 receptors that respond to picomolar concentrations of IL-2 (Nandagopal *et al*, 2014). IL-15 is produced by APCs and can activate the IL-2/IL-15 receptor. It has been suggested that picomolar concentrations of IL-2 only activates the IL-2Rαβγ receptor and high concentrations are needed to activate both the IL-2/IL-15Rβγc and therefore IL-15 could be a more useful cytokine to activate NK cells as it will act through both the IL-2 and IL-15 receptor (Vitale *et al*, 2014). Interestingly, IL-2 also activates T regulatory cells which can limit the function of the NK cells thus inducing an inhibitory effect (Romee *et al*, 2014).

In resting NK cells the NKp44 expression is induced by the presence of IL-2 (Moretta *et al*, 2001) . The examination of the NKp44 activating receptor demonstrated a moderate expression on both subsets of NK cells compared to the isotype control. It is possible that the NK cells in the peripheral circulation of the male and female MCL patients are unstimulated and it may be this that results in the lack of significant expression of the NKp44 receptor. It would be interesting to examine the expression of the NKp44 on NK cells from affected lymph nodes to correlate if there is a higher expression due to the activated microenvironment of the tumour. This could be achieved by analysing cells

obtained by fine needle aspiration from the affected lymph node and using flow cytometry to measure the expression of the NKp44 activating receptor.

The expression of NKG2D activating receptor exhibited no significant difference between male and female MCL patients. There was a trend which demonstrated lower expression of the NKG2D receptor on the patient NK cells subset $CD3^{-}CD56^{dim}$ compared to the control group. This suggests that the NK cells subset $CD3^{-}CD56^{dim}$ is less activated in MCL patients. It is possible that MCL itself could inhibit the NK cells by producing inhibitory ligands thus resulting in lower expression of this receptor compared to the healthy controls (Sanchez *et al*, 2011; López-Soto *et al*, 2014). Evaluation of certain activating ligand such as B7-H6, MICA or MICB in the primary MCL patients may shed further light on certain aspects of the NK cell activating receptor expression. Currently inhibitory ligands to NK cells in mantle cell lymphoma have not been identified. However the inhibitory ligand B7-H1 has been shown to be expressed by mantle cell lymphoma cells which inhibit T cells function, which was recently reported by Wang et al (Wang *et al*, 2013a).

Larger studies are required to confirm the observed trend and further research is needed to determine if MCL exhibits any inhibitory ligands to NK receptors.

In this study only four activating receptors that have been implicated in haematological malignancies were evaluated (Costello *et al*, 2012; Epling-Burnette *et al*, 2007; Sanchez *et al*, 2011). However, one of the future plans is to evaluate the NK inhibitory receptors such as KIR and CD92 to determine whether the expression of these inhibitory receptors is different between the genders.

One of the major limitations of this study was the low number of participants. Given that MCL is a rare disease recruitment of patients that were either newly diagnosed or untreated was challenging. Therefore, in order to research these trends and results further, a larger cohort of patients is required. This would also allow for more robust statistical testing as individual variations could be better accounted for.

3.7. Conclusion

The major finding in this chapter was that the male MCL patients exhibited a significantly higher number of T cells in the peripheral circulation compared to the female MCL patients. Additionally, there was a trend towards greater number of T regulatory cells in the female MCL patients compared to the male MCL patients. These results suggest that there is possibly an egress of the immune cells from the lymph node. It is possible that higher number of T cells would be present in the affected lymph nodes of female patients compared the males thus inversely correlating to the number of T cell in the circulation. As discussed earlier T regulatory cells have an inhibitory effect on T cells and thus if this observation remains consistent then fewer T regulatory cells in the microenvironment could reduce their inhibitory effects in female patients. Hence an assumption could be made that, female patients with MCL are potentially able to mount a stronger immune response within the microenvironment of the MCL. The exact role of T regulatory cells and the other immune cells in the context of MCL needs additional studies and importantly these results illustrate the cell numbers in the peripheral blood of the patients, which could be significantly different to the numbers and function in the microenvironment of MCL. Nonetheless, larger studies are warranted to explore further these results and trends observed.

Chapter 4

Results

The cytotoxic analysis of peripheral blood immune cells in male and female MCL patients and healthy controls. Analysis of the effects of oestrone and IL-2 on immune cell numbers and cytotoxic action.

4.0. Background

Immunosurveillance and immunoediting is a process by which the innate and adaptive immune system eliminates cancerous cells (Swann & Smyth, 2007; Ponce *et al*, 2014; Plonquet *et al*, 2007). The evidence for this process occurring in humans is provided in part by the increased cancer incidence in immunosuppressed transplant patients compared to age matched healthy controls (Opelz & Döhler, 2004). In addition, patients with human immunodeficiency virus (HIV) have an increased incidence of lymphoma rates especially when the CD4 counts are low (Yanik *et al*, 2013). T cell have been implicated in immunosurveillance and a recent study has demonstrated their importance in B cell lymphoma proliferation, where by removal of T cells accelerates the progression of lymphoma (Afshar-sterle *et al*, 2014; Kallies, 2014). NK cell are well recognised to play a pivotal role in the first defence against cancerous cells by inducing direct cell mediated death on defective cells (Vivier *et al*, 2011). Furthermore, quantification of T and NK cells in B cell malignancies have been associated with prognostication implying their importance in immunosurveillance (Głowala-Kosińska *et al*, 2013; Shafer *et al*, 2013; Plonquet *et al*, 2007). A recent study has demonstrated that the presence of T cells within the tumour environment can be a positive prognostic indicator in MCL (Nygren et al. 2014). Therefore, cell mediated cytotoxicity by immune cells is an important first line defence and whether females mount a better response in MCL is warranted for further study.

The role of IL-2

Functional enhancement of T, T regulatory and NK cells has been demonstrated by IL-2 (Boyman & Sprent, 2012; Bordignon *et al*, 1999; Liao *et al*, 2013). IL-2 stimulates the production of lymphokine activated killer cells

(LAK) which are stimulated NK and cytotoxic T cells. LAK cells have been proven to be more effective in killing tumour cells (Bordignon *et al*, 1999; Grimm *et al*, 1982). Furthermore, by utilising and enhancing the hosts immune cells with IL-2, it has been demonstrated that certain malignancies such as melanoma and renal cell carcinoma have been treated with some success (Rosenberg, 2014). In addition IL-2 has been recognized to have beneficial effects in the treatment of acute myeloid leukaemia (AML) by stimulating the host's immune system to eliminate any remaining leukemic cells following initial induction chemotherapy (Romero *et al*, 2009). Therefore, stimulation of PBMCs by IL-2 increases the immune cell cytotoxic function and it would be interesting to determine whether female patients with MCL have a superior response to IL-2. There is no data on the effects of oestrone on IL-2 receptors or its effect in the context of B cell lymphomas and this may warrant further studies.

Oestrone

As mentioned in chapter 1, the incidence of MCL has a male to female prevalence of approximately 3:1 and a study has demonstrated that females with MCL respond better to immunomodulatory therapy such as lenalidomide (Eve *et al*, 2012). Although the exact mechanism of this gender difference is not fully understood, there appear to be several factors that may influence it. These include the ability of the female immune system to mount a greater immune response, better response to activating cytokines such as IL-2 and possibly the influence of oestrogens (Klein, 2012).

The median age of the female patients participating in this study was 62 years and oestrone is the most abundant female hormone in postmenopausal women of the three female hormones; oestriol, oestrodiol and oestrone (Gruber *et al*,

2002). Studies have also demonstrated oestrogen receptor α and β presence on immune cells; T cells and NK cells (Phiel *et al*, 2005; Henderson *et al*, 2003; Yakimchuk *et al*, 2013) and in turn oestrone has an affinity to both receptors (Gruber *et al*, 2002) with a capability of activating them both. Furthermore, oestrogen receptors have been identified on B lymphocytes and some B cell lymphomas (Römer & Pfreundschuh, 2014)(chapter 1 section 1.3). Therefore investigating the effects of oestrone on the immune cell mediated cytotoxicity on target cell would be interesting to determine and determine whether addition of oestrone would enhance the cell mediated cytotoxicity.

Lenalidomide

A recent study has demonstrated better response rates with lenalidomide in female patients with relapsed / refractory MCL thus suggesting a possible influence due the gender (Eve *et al*, 2012). Due to the small size of the study the researchers could not fully address this observation. However, it could be possible that the female immune cells or hormones may have influenced this result. Lenalidomide, a derivative of thalidomide, is an immunomodulatory drug which has been shown to enhance T cell and NK cell cytotoxic activity against lymphoma cells (McDaniel *et al*, 2012; Richardson *et al*, 2010)(chapter 1 section 1.2.2d). Therefore the cytotoxic function of these cells was evaluated following incubation with lenalidomide to determine if there was a difference between the male and female immune cells in patients with mantle cell lymphoma.

On the basis of the above data the aim of this chapter was to;

1) Determine whether effector cells (PBMCs) from female MCL patients have a greater cell mediated cytotoxicity compared to males.

2) Determine whether incubation with IL-2, oestrone and lenalidomide exerts an effect which is different between the cell mediated cytotoxicity of female MCL patients' effector cells compared to male MCL patients' effector cells.

3) Determine whether incubation with IL-2, oestrone and lenalidomide influences the quantity of immune cells compared to untreated control cells.

4) Determine the effects of IL-2, oestrone and lenalidomide on the NK activating receptors; NKp46, NKp44, NKp30 and NKG2D.

It must be taken into consideration that due to the problems with isolation of NK cells on viability, effector cells were cultured for 24 hours in media containing various treatments (chapter 2, section 2.7). Unless otherwise stated, all the effector cells utilised in this chapter were non-adherent cells which were either untreated (control) or treated with IL-2, oestrone, lenalidomide, lenalidomide combined with oestrone and lenalidomide combined with oestrone and IL-2. The effector cells contained T cells, NK cells, NK-T cells and neutrophils. In all the experiments presented in this chapter the effector cells were from clinically unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

'Clinically unselected' is defined; that there was no pre-selection of patient in relation to their clinical characteristics but due to the limited number of material and patients available, any suitable patient sample was utilised as described above throughout this chapter (chapter 2).

4.1. Direct cytotoxicity effect of oestrone and lenalidomide on Granta 519 (G519) mantle cell lymphoma cells and K562 cells

The direct effect of oestrone and lenalidomide was evaluated on both cell lines to determine whether there was an effect on the target cells (G519 and K562), prior to investigating the cell mediated cytotoxicity of PBMCs (effector cells) on target cells when incubated with these compounds. There was no significant direct cytotoxic effect of oestrone or lenalidomide on G519 cells at various concentrations when incubated for up to 48 hours (Figure 20). Similarly, there was no significant direct cytotoxicity exhibited on K562 cells when incubated with oestrone and lenalidomide at similar concentrations (data not shown).

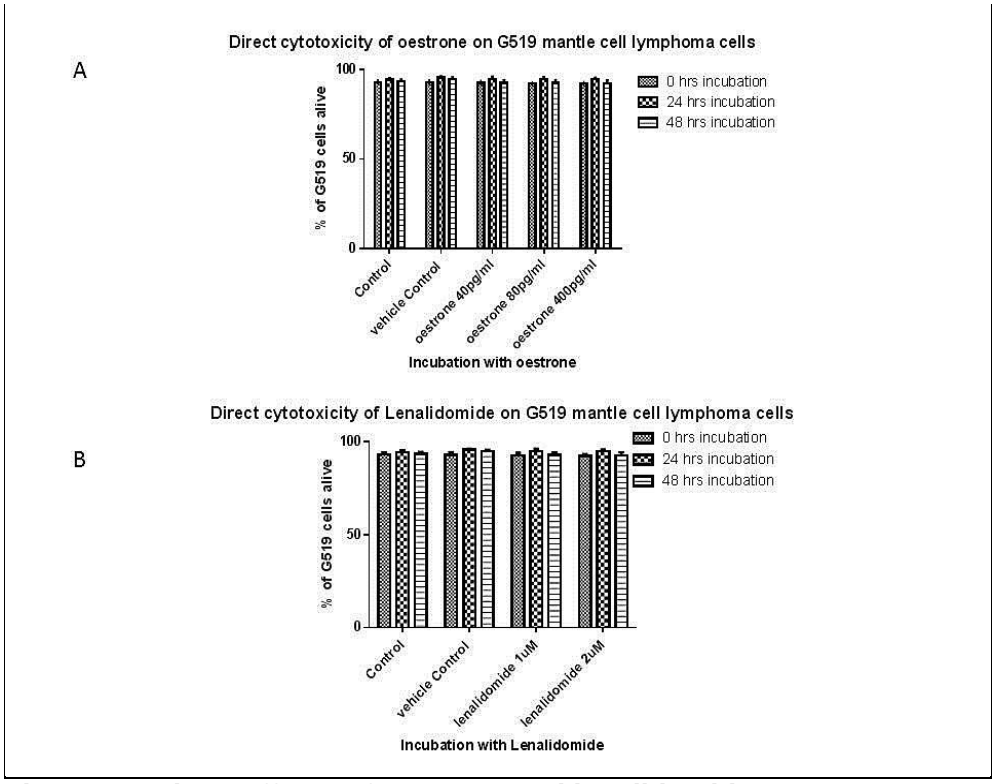


Figure 20: **Direct effects of oestrone and lenalidomide on G519 target cells**
A. G519 cells were incubated with varying concentrations of oestrone as shown over a period of 48 hours incubation to assess direct cytotoxic effect of oestrone on G519 cells.
B. Similarly G519 cells were incubated with lenalidomide at 1µM and 2µM concentration over a period of 48 hours incubation.
(similar results were observed when oestrone and lenalidomide were used in combination as well as oestrone, lenalidomide and IL-2 (100IU) at the above stated concentrations, data not shown).

4.2. Cell mediated cytotoxicity of control (untreated) effector cells from male and female healthy controls and MCL patients on K562 target cells

The K562 cells lack the MHC-class 1 complex and thus exhibit good sensitivity to NK cytotoxicity. The activating receptors on NK cells sense the absence of MHC-class 1 and target these cells for destruction. Therefore, K562 cells are a good model in which to assess the cytotoxic ability of PBMC population due to their extreme sensitivity to NK cytotoxicity. The cell mediated cytotoxicity of K562 target cells by effectors cells were analysed by flow cytometry as described in the methods chapter 2, section 2.7.

The control effector cells which were incubated with vehicle control DMSO (1µl/ml, <0.001%) will be referred to as untreated throughout this chapter. There was no difference observed between effector cells with or without DMSO (data not presented).

K562 target cells were exposed to untreated effector cells from male and female healthy controls and unselected MCL patients (consisting patients exhibiting both clinically indolent and aggressive disease) (Figure 21). There was a significant increase in cell mediated cytotoxicity on the K562 cells by effector cells from female controls compared to male controls, $p=0.04$ at E:T ratio of 50:1 (Figure 21 B). No significant difference was observed in the cell mediated cytotoxicity of effector cells from male and female MCL patients, $p=0.9$ (Figure 21 C). Interestingly the base line activity of MCL patients was similar to the male controls.

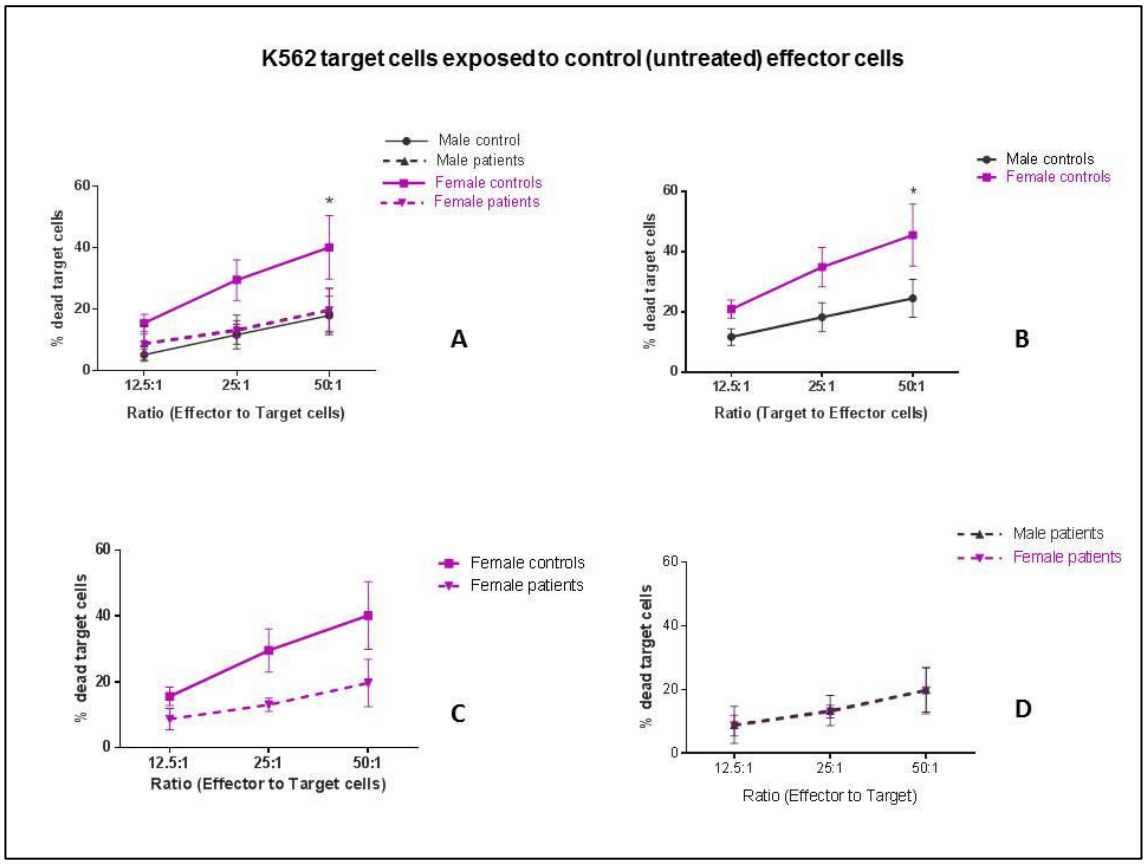


Figure 21: Cell mediated cytotoxicity of effector from both MCL patients and healthy control groups on K562 target cells

Effector cells from unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used. Plots above demonstrate cell mediated cytotoxicity by mean (+/- SE) percentage cell death of target (K562) cells.

Plot **A** exhibits cell mediated cytotoxicity of effector cells from male and female healthy controls and unselected male and female MCL patients.

Plot **B** exhibits cell mediated cytotoxicity of effector cells from male and female controls.

Plot **C** exhibits cell mediated cytotoxicity of effector cells from female MCL patients and female controls.

Plot **D** exhibits cell mediated cytotoxicity of effector cells form unselected male and female MCL patients.

4.3. Cell mediated cytotoxicity of control effector cells (untreated) from male and female healthy controls and MCL patients on Granta 519 target cells

The baseline cell mediated cytotoxicity of control (untreated) effector cells from male and female controls and MCL patients on Granta 519 target cells was analysed. The cell mediated cytotoxicity was analysed by the flow cytometry as described in the methods chapter 2, section 2.7.

The Granta 519 target cells were exposed to the untreated effector cells from the male and female healthy controls and unselected MCL patients. There was no significant difference in cell mediated cytotoxicity between the female and male control group, p=0.9 (Figure 22 A & B) and there was no significant difference observed in the cell mediated cytotoxicity between the male and female MCL patients, p=0.8 (Figure 22 C).

The healthy control groups demonstrated an overall greater cell mediated cytotoxicity compared to the MCL patients (Figure 22). However, this only reached significance in the female MCL patients versus female healthy controls, p=0.03 at E:T ratio 50:1 (Figure 22 D).

The cell mediated cytotoxicity exhibited when using G519 target cells differed compared to K562 target cells (figure 24.0) largely due to the K562 cells being more sensitive to NK cell mediated cell death. G519 cells have expression of MHC- class 1 and therefore are not killed as effectively as K562 cells by NK cells as mentioned in section 2.4.2.

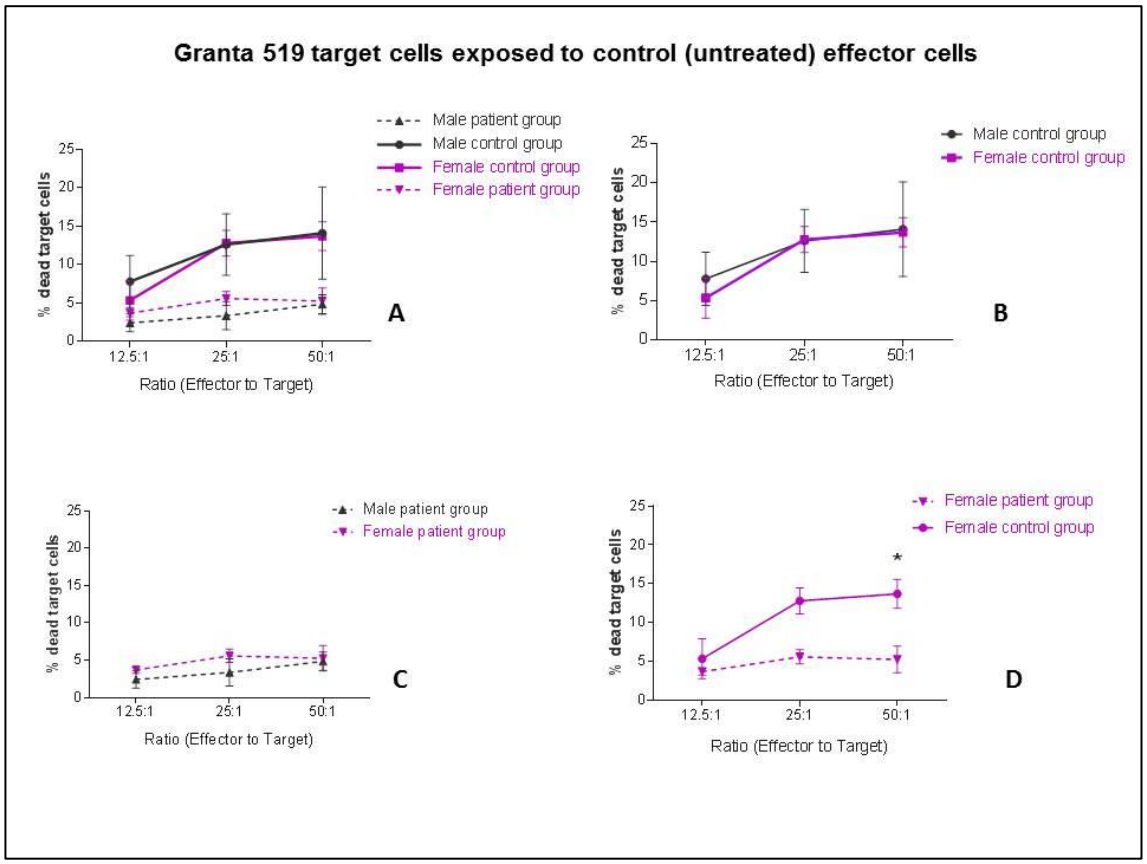


Figure 22: Cell mediated cytotoxic activity of male and female untreated effector cells from unselected MCL patients and healthy control groups on Granta 519 target cells

Cell mediated cytotoxicity is presented by mean percentage (+/- SE) of target cell (G519) death. Effector cells from unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Plot **A** exhibits the cell mediated cytotoxicity of the effector cells from healthy male and female controls compared to the unselected male and female MCL patients.

Plot **B** exhibits cell mediated cytotoxicity of the effector cells from healthy female and male controls.

Plot **C** exhibits cell mediated cytotoxicity of effector cells from the unselected male and female MCL patients.

Plot **D** exhibits cell mediated cytotoxicity of effector cells from female controls compared to female MCL patients.

4.4. Cell mediated cytotoxicity function of IL-2 treated effector cells from male and female healthy controls and MCL patients on K562 and Granta 519 target cells

PBMCs (effector cells) from the subjects were incubated with IL-2 100IU for 24 hours as described in the methods chapter 2, section 2.7. Incubation with IL-2 produces LAKs as described earlier.

K562 target cells

K562 target cells were exposed to LAK cells from both healthy controls and MCL patients. Incubation with IL-2 resulted in an overall increase in cell mediated cytotoxicity in all groups when compared to untreated effector cells ($^{\dagger+\dagger\dagger}$p=0.02, Figure 23, Table 16). The LAK cells from the female controls exhibited a significant increase in cell mediated cytotoxicity when compared to the untreated effector cells from the same group (*p=0.04, **p=0.003 and ***p=0.04, Figure 23, Table 16).

The LAKs cells from the female controls demonstrated a greater increase in cell mediated cytotoxicity compared to the male controls (Figure 23, Table 16) and they also exhibited the greatest overall cytotoxicity activity (Figure 23). Interestingly, the cell death observed with LAKs cells from clinically unselected MCL patients was similar to male controls. LAKs cells from the female controls exhibited a significantly greater cell death compared to the male controls, *p=0.009, at ratio 25:1 E:T cells (Figure 23B). Additionally, LAK cells from female controls exhibited a significantly greater cytotoxicity compared to the clinically unselected female MCL patients, *p=0.02 (Figure 23 D).

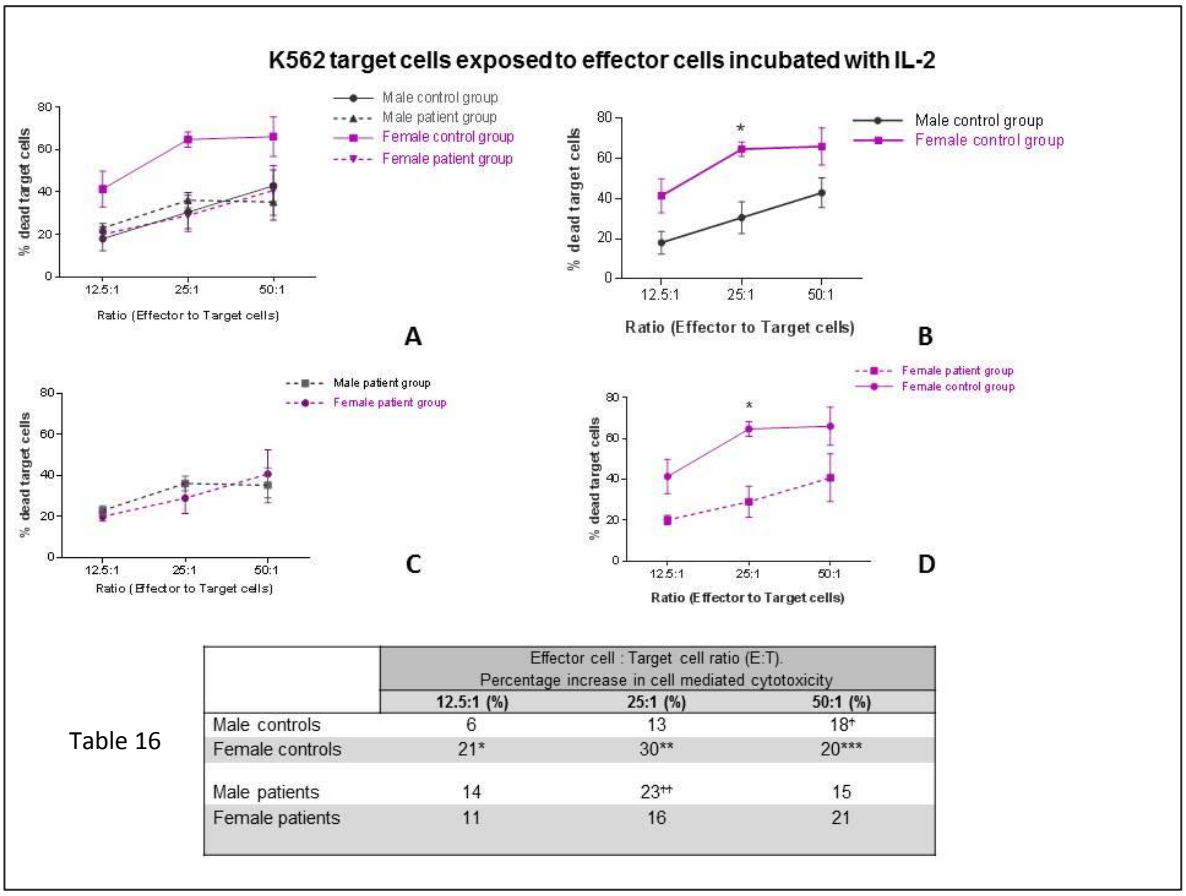


Table 16

	Effector cell : Target cell ratio (E:T). Percentage increase in cell mediated cytotoxicity		
	12.5:1 (%)	25:1 (%)	50:1 (%)
Male controls	6	13	18†
Female controls	21*	30**	20***
Male patients	14	23††	15
Female patients	11	16	21

Figure 23: Cell mediated cytotoxic activity of effector cells treated with IL-2 on K562 target cells

Cell mediated cytotoxicity is presented by mean percentage (+/- SE) of target cell (K562 cells) death. Effector cells from unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Plot **A** compares cell mediated cytotoxicity activity of LAK cells on K562 target cells in all 4 groups.
Plot **B** demonstrates cell mediated cytotoxicity of LAK cells from female controls and male controls.
Plot **C** demonstrates cell mediated cytotoxicity of LAK cells from clinically unselected male and female MCL patients.
Plot D demonstrates cell mediated cytotoxicity of LAK cells from female controls and MCL female patients.
Table 16: Percentage increase in cell mediated cytotoxicity of LAK cells on K562 target cells when compared with untreated effector cells.

Granta 519 Target Cells

Granta 519 target cells were exposed to effector cells treated with IL-2 (to from LAK cells) from all four groups. The LAK cells exhibited an overall greater increase of cell mediated cytotoxicity activity when compared to the cell mediated cytotoxicity of the untreated effector cells (Table 17, *p=0.03, **p=0.005 and ***p=0.03). As with the K562's target cells, the control groups exhibited greater overall increase when compared to the patient groups. Interestingly, the female MCL patients did not exhibit an increase in the cell mediated cytotoxicity after the effector cells were incubated with IL-2 (Table 17).

When comparing the cytotoxic activity between the groups of the LAK effector cells on Granta 519 target cells there was a trend towards male controls exhibiting greater cell mediated cytotoxicity compared to the rest of the groups and interestingly when compared to the female control group (Figure 24 A & B). However this did not reach statistical significance, p=0.9 (Figure 24). Additionally the male MCL patient group exhibit a significantly greater cell mediated cytotoxicity compared to the female MCL group (E:T – 50:1, *p=0.04, figure 28 C). The female and male control group demonstrated a greater cell mediated activity compared to the respective patient groups (E:T ratio 25:1 and 50:1, *p=0.03 and **p=0.002 respectively, Figure 24 D).

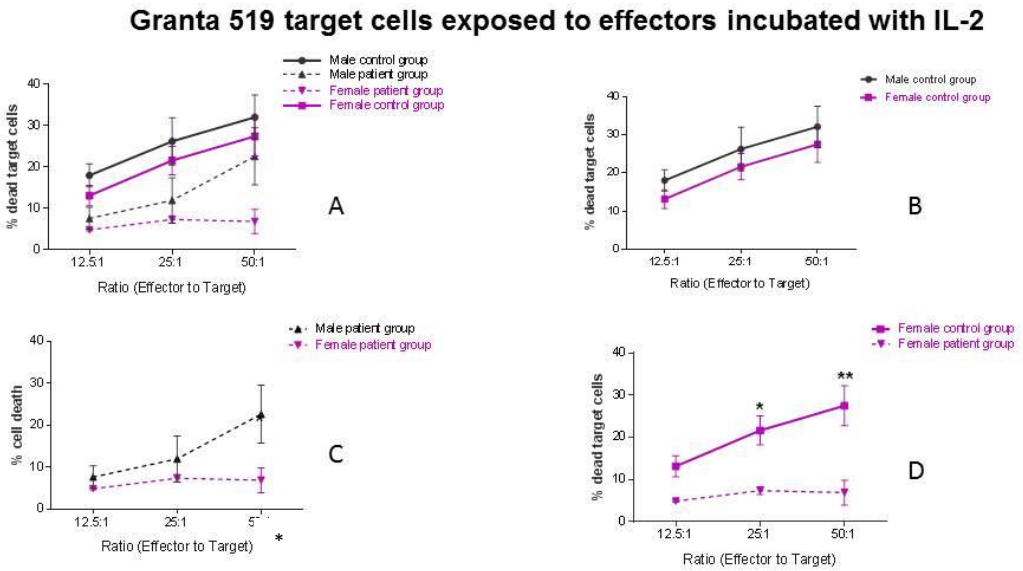


Table 17

	Effector cell : Target cell ratio (E:T). Percentage fold increase in cell mediated cytotoxicity		
	12.5:1 (%)	**25:1 (%)**	**50:1 (%)**
Male controls	10	13	18*
Female controls	7	11	13**
Male patients	5	9	17***
Female patients	1	1	2

Figure 24: Cell mediated cytotoxicity of effector cells treated with IL-2 (LAKS) on Granta 519 target cells

Cell mediated cytotoxicity by mean percentage (+/- SE) of target cell (G519) death. Effector cells from unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used**.**

Plot **A** compares cell mediated cytotoxicity of LAK cells from male and female controls and MCL patients.

Plot **B;** cell mediated cytotoxicity of LAK cells from female and male control groups.

Plot **C;** cell mediated cytotoxicity of LAK cells from male and female patient groups.

Plot **D;** cell mediated cytotoxicity of effector cells compared between female patients and female controls.

Table 17: Percentage increase in cell mediated cytotoxicity of effector cells on G519 cells from the subjects incubated with IL-2 compared to untreated effector cells.

4.5. Cell mediated cytotoxicity of effector cells incubated with oestrone from male and female healthy controls and MCL patients on K562 and Granta 519 target cells

The median age at which MCL presents is 65 years (McKay *et al*, 2012). The male and female patients in this study had a median age of 64 and 62 years respectively. All the healthy control subjects were over the age of 50 years (median age 54 years). The physiological level of oestrone in post-menopausal women is 40pg/ml, which was used throughout the experiments to determine the effects of oestrone on the cytotoxic activity of effector cells (Gruber *et al*, 2002). The effector cells were incubated with oestrone for 24 hours and subsequently used to analyse the cell mediated cytotoxicity on the target cells by flow cytometry. Oestrone was included in the media in which the cell mediated cytotoxicity was carried out.

K562 and Granta 519 target cells were exposed to effectors cells incubated with oestrone from both healthy controls and MCL patients. No significant change was observed in the cell mediated cytotoxicity when compared to the cell mediated cytotoxicity activity of the untreated effector cell (Table 18 and 19). There was a trend towards a marginally greater cell mediated cytotoxicity activity in the male MCL patients compared to the female MCL patients. This is probably due to the marginally greater number of NK cells and T cells in the male MCL patients compared to the female MCL patients rather than an effect of oestrone (Figure 25 and 26)

K562 and Granta 519 target cells were exposed to effector cells incubated with oestrone from male and female healthy controls and MCL patients. There was no significant difference observed between the male and female healthy controls and MCL patients.

The cell mediated cytotoxicity on K562 target cells exhibited a trend towards greater activity in female controls compared to male controls and MCL patients, p=0.9 (Figure 26, B & D). There was no significant difference exhibited of the cell mediated cytotoxicity on K562 target cells between the male and female MCL patients, p=0.5 (Figure 26 C).

There was no significant difference in the cell mediated cytotoxicity between male and female healthy controls on G519 target cells, p=0.9 (Figure 26 A & B). Similarly there was no significant difference was exhibited in the cell mediated cytotoxicity between male and female MCL patients, p=0.7 (Figure 26 C). However at the E:T ratio of 50:1 there was a trend towards greater activity in the male MCL patients compared to female patients, p=0.3 (Figure 26 A & C). This result is possibly a reflection of the variation in numbers of effector cells between the two groups (Figure 25 & 26). Notably, the percentage of target cell death was less in the G519 cells compared to the K562 cells. This is probably accounted for by K562 cells being more sensitive to NK cell mediated death due to the lack of MHC-class 1. The G519 cells exhibit reduced MHC-class 1 expression and are therefore less sensitive to NK cell mediated cell death.

In relation to both the K562 and Granta 519 target cells the results observed are comparable to the cell mediated cytotoxicity exhibited by the untreated effector cells (Figure 25 & 26).

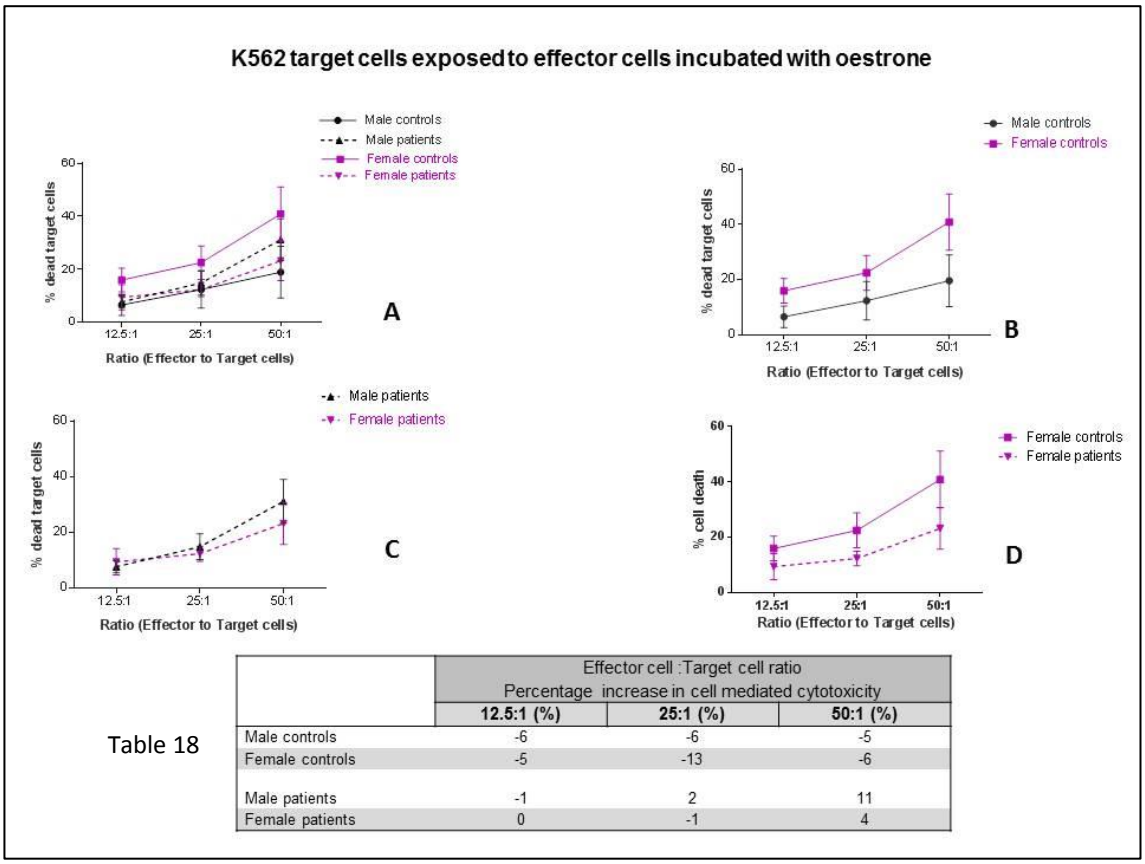


Table 18

	Effector cell :Target cell ratio Percentage increase in cell mediated cytotoxicity		
	12.5:1 (%)	**25:1 (%)**	**50:1 (%)**
Male controls	-6	-6	-5
Female controls	-5	-13	-6
Male patients	-1	2	11
Female patients	0	-1	4

Figure 25: Cell mediated cytotoxicity of effector cells incubated with oestrone on K562 target cells

Cell mediated cytotoxicity presented by mean percentage (+/- SE) of target cell (K562 cells) death. Effector cells from unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Graph **A:** Cell mediated cytotoxicity activity comparing all 4 groups.

Graph **B:** Cell mediated cytotoxicity activity of male and female healthy controls.

Graph **C:** Cell mediated cytotoxicity activity of male and female MCL patient groups.

Graph **D:** Cell mediated cytotoxicity of healthy female controls and female MCL patients

Table 18: Percentage increase in cell mediated cytotoxicity on K562 target cells after effector cells from the subjects were incubated with oestrone.

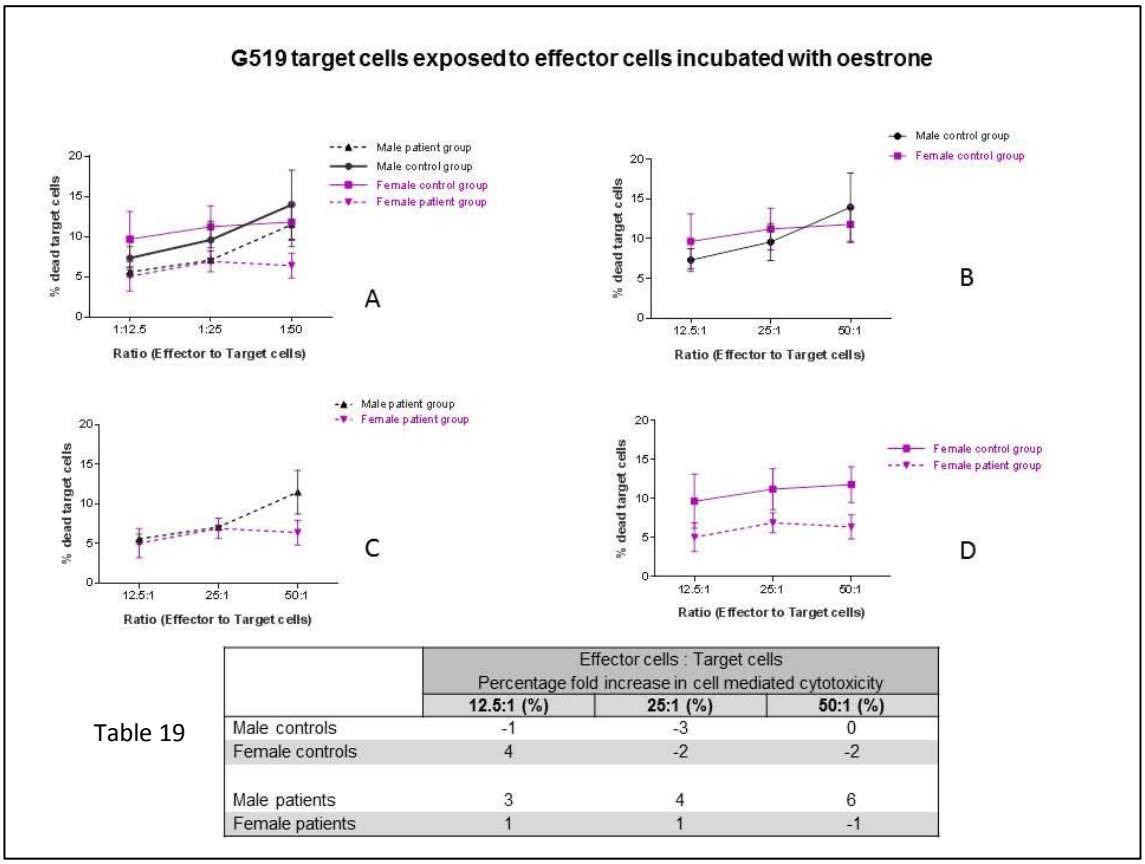


Table 19

	Effector cells : Target cells Percentage fold increase in cell mediated cytotoxicity		
	12.5:1 (%)	**25:1 (%)**	**50:1 (%)**
Male controls	-1	-3	0
Female controls	4	-2	-2
Male patients	3	4	6
Female patients	1	1	-1

Figure 26: Cell mediated cytotoxicity of effector cells incubated with oestrone on Granta 519 target cells

Cell mediated cytotoxicity presented by mean percentage (+/- SE) of target cell (G519 cells) death. Effector cells from unselected male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Graph **A**: Cell mediated cytotoxicity of effectors cells from male and female controls and patients.

Graph **B**: Cell mediated cytotoxicity of effector cells from female and male control groups.

Graph **C**: Cell mediated cytotoxicity of effector cells from male and female patient group.

Graph D: Cell mediated cytotoxicity of effector cells from female controls and female MCL patients

Table 19: The percentage increase in cell mediated cytotoxicity of effector cells incubated with oestrone compared to untreated (control) effector cells.

4.6. Cell mediated cytotoxicity of effector cells incubated with lenalidomide and a combination of lenalidomide and oestrone from male and female healthy controls and MCL patients on Granta 519 target cells

Lenalidomide has been described to modulate the immune cells and female patients with MCL have demonstrated greater response rates (Eve *et al*, 2012). Therefore, in addition to lenalidomide, a combination of lenalidomide and oestrone was also used to determine whether simulating a postmenopausal 'female' environment with oestrone would demonstrate greater cell mediated cytotoxicity by effector cells. The cell mediated cytotoxicity on Granta 519 cells of effector cells from healthy controls and MCL patients that had been incubated with lenalidomide 2µM and a combination of lenalidomide 2µM and oestrone for 24 hours was analysed. The cell mediated cytotoxicity was analysed by flow cytometry as described in chapter 2, section 2.7. Due to the limitation on the quantity of effector cells available for each subject, the cell mediated cytotoxicity was only evaluated on G519 cells and not on K562 cells. This was done primarily to evaluate the effects on MCL cells.

Granta 519 target cells exposed to effector cells incubated with lenalidomide and a combination of lenalidomide and oestrone from healthy controls and MCL patients demonstrated no significant increase in cell mediated cytotoxicity when compared to untreated effector cells from the same groups (Table 20 and 21).

Effector cells incubated with only lenalidomide demonstrated no significant difference in the cell mediated cytotoxicity between the male and female healthy control groups, p=0.5 (Figure 27 A). However, the healthy male and female control groups exhibited greater cell mediated cytotoxicity on G519 target cells compared to the MCL male and female patient groups (p=0.04*; E:T 50:1 Figure 31 D; male healthy controls compared to male MCL patients). There was

no significant difference in the cell mediated cytotoxicity between the genders in either the healthy controls, p=0.5 or MCL patient, p=0.4 (Figure 27 B & C).

Effector cells incubated with lenalidomide and oestrone in combination exhibited similar results to effector cells incubated with only lenalidomide as described above. There was no significant difference observed between the genders from healthy controls or MCL patients, p=0.45 (Figure 28 A & B). However, there was a significantly greater cell mediated activity in the healthy controls compared to the MCL patients (p=0.02* and p=0.04*; Figure 28 C & D, respectively).

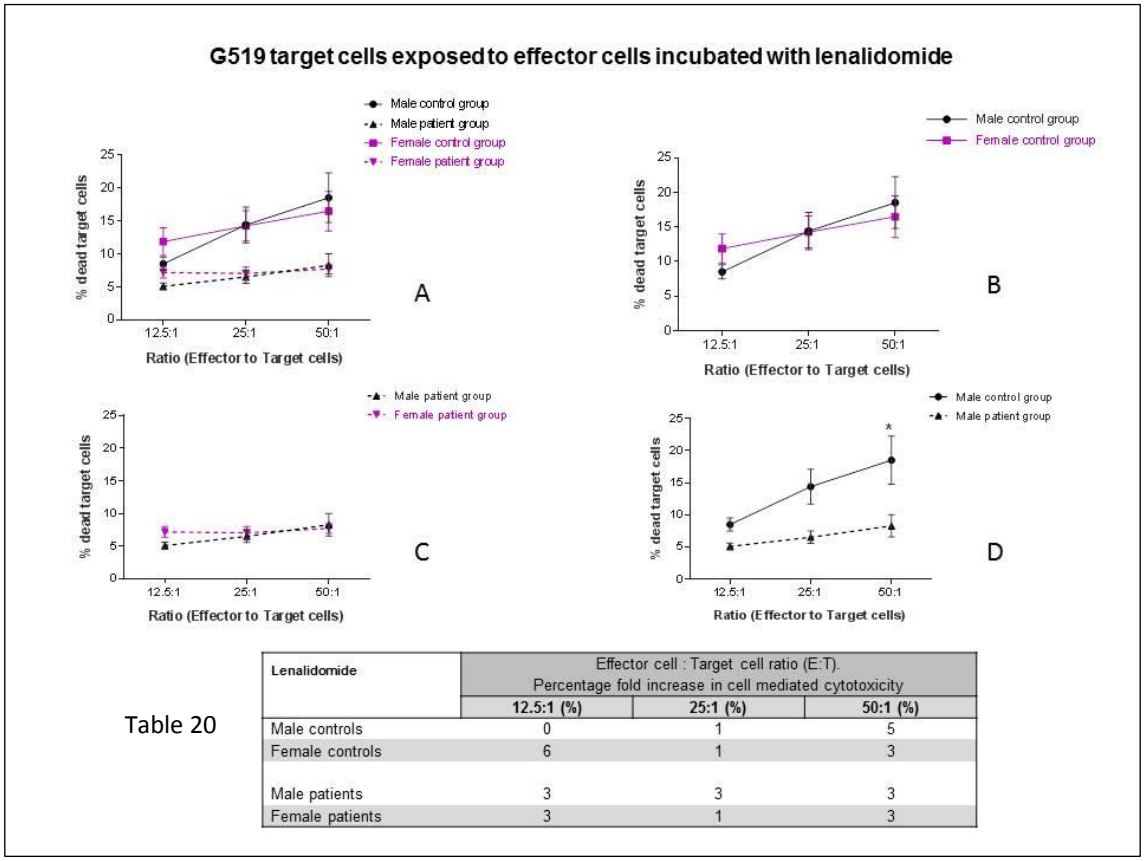


Table 20

Lenalidomide	Effector cell : Target cell ratio (E:T). Percentage fold increase in cell mediated cytotoxicity		
	12.5:1 (%)	25:1 (%)	50:1 (%)
Male controls	0	1	5
Female controls	6	1	3
Male patients	3	3	3
Female patients	3	1	3

Figure 27: Cell mediated cytotoxicity of effector cells incubated with lenalidomide on Granta 519 target cells

Cell mediated cytotoxicity presented by mean percentage (+/- SE) of target cell (G519 cells) death. Effector cells from male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Graph **A**: Cell mediated cytotoxicity of effectors cells from male and female controls and MCL patients.

Graph **B**: Cell mediated cytotoxicity of effector cells from female and male healthy control.

Graph **C**: Cell mediated cytotoxicity of effector cells from male and female MCL patient.

Graph **D**: Cell mediated cytotoxicity of effector cells from male patients and male healthy controls.

Table 20: **Percentage increase of cell mediated cytotoxicity of effector cells incubated with lenalidomide compared with untreated (control) effector cells on Granta 519 target cells.**

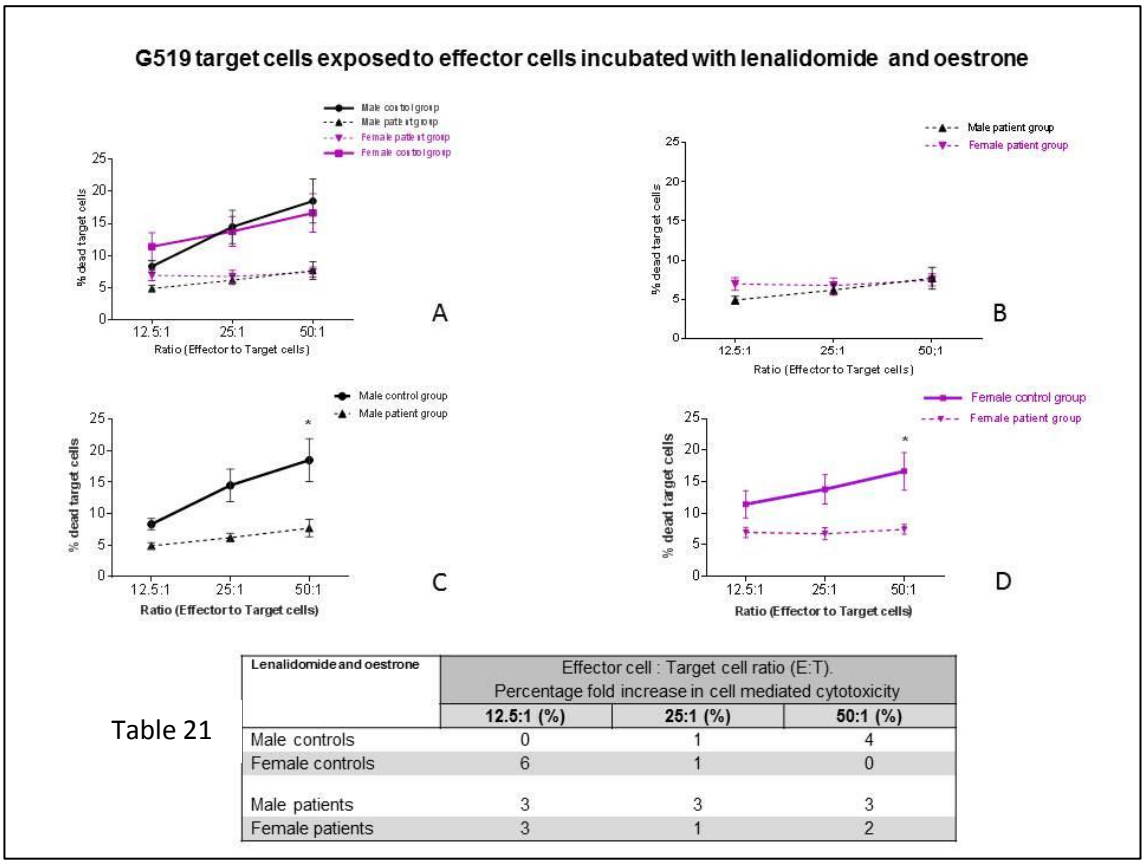


Table 21

Lenalidomide and oestrone	Effector cell : Target cell ratio (E:T). Percentage fold increase in cell mediated cytotoxicity		
	12.5:1 (%)	**25:1 (%)**	**50:1 (%)**
Male controls	0	1	4
Female controls	6	1	0
Male patients	3	3	3
Female patients	3	1	2

Figure 28: Cell mediated cytotoxicity of effector cells incubated with lenalidomide and oestrone in combination

Cell mediated cytotoxicity presented by mean percentage (+/- SE) of target cell (G519 cells) death. Effector cells from male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Graph **A**: Cell mediated cytotoxicity of effectors cells from male and female controls and MCL patients.

Graph **B**: Cell mediated cytotoxicity of effector cells from female and male patient groups.

Graph **C**: Cell mediated cytotoxicity of effector cells from male healthy control.

Graph **D**: Cell mediated cytotoxicity of effector cells from female healthy controls and female MCL patients.

Table 21: Percentage increase in cell mediated cytotoxicity of effector cell incubated with a combination of lenalidomide and oestrone compared with untreated (control) effector cells on Granta 519 target cells.

4.7. Cell mediated cytotoxicity of effector cells from male and female patients and healthy controls incubated with a combination of lenalidomide, IL-2 and oestrone on Granta 519 target cells

The effector cells were treated with a combination of lenalidomide 2µM, IL-2 100IU and oestrone 40pg/ml for 24 hours. This was to determine if LAKs mounted a greater response in the presence of lenalidomide and oestrone. The combination of IL-2, oestrone and lenalidomide was used to ascertain whether there was an enhanced response to IL-2 in the presence of oestrone and lenalidomide and in turn if the 'female environment' enhanced the cell mediated cytotoxicity. The cell mediated cytotoxicity was performed in media containing lenalidomide, IL-2 and oestrone. The analysis was performed by flow cytometry as described in chapter 2 section 2.7. Cell mediated cyctotoxicity on K562 target cell was not analysed to limitations in the availability of adequate number of effector cells from the subjects.

Granta 519 target cells exposed to effector cells incubated with a combination of IL-2, oestrone and lenalidomide from male and female healthy controls and MCL patients exhibited a percentage increase in the cell mediated cytotoxicity when compared to untreated effector cells (Figure 29, Table 22). There was a significant increase in the cell death observed in healthy male and female controls at the T:E ratios of 1:50, *p=0.001, ***p=0.001 (Table 22). There was no statistically significant increase observed in the female MCL patients, however a trend toward greater increase in cell mediated cytotoxicity was observed in male MCL patients compared to the female MCL patients (Table 23).

There was no significant difference in the cell mediated cytotoxicity of the effector cells from male and female controls, p=0.8 (Figure 29 A & B). However there was a trend towards male controls exhibiting greater cell mediated cytotoxicity compared to the female controls. Additionally the control groups exhibited a trend towards greater cell mediated cytotoxicity compared to the MCL patients. Female controls exhibited a significantly greater cell mediated cytotoxicity compared to the female MCL patients (*p=0.02, **p=0.001, Figure 29D). Interestingly, the male MCL patients exhibited significantly greater cell mediated cytotoxicity when compared to the female patients (*p=0.01, Figure 29 C). This observed result is largely related to the greater number of T and NK cells in male MCL patients compared to female MCL patients (Figure 29)

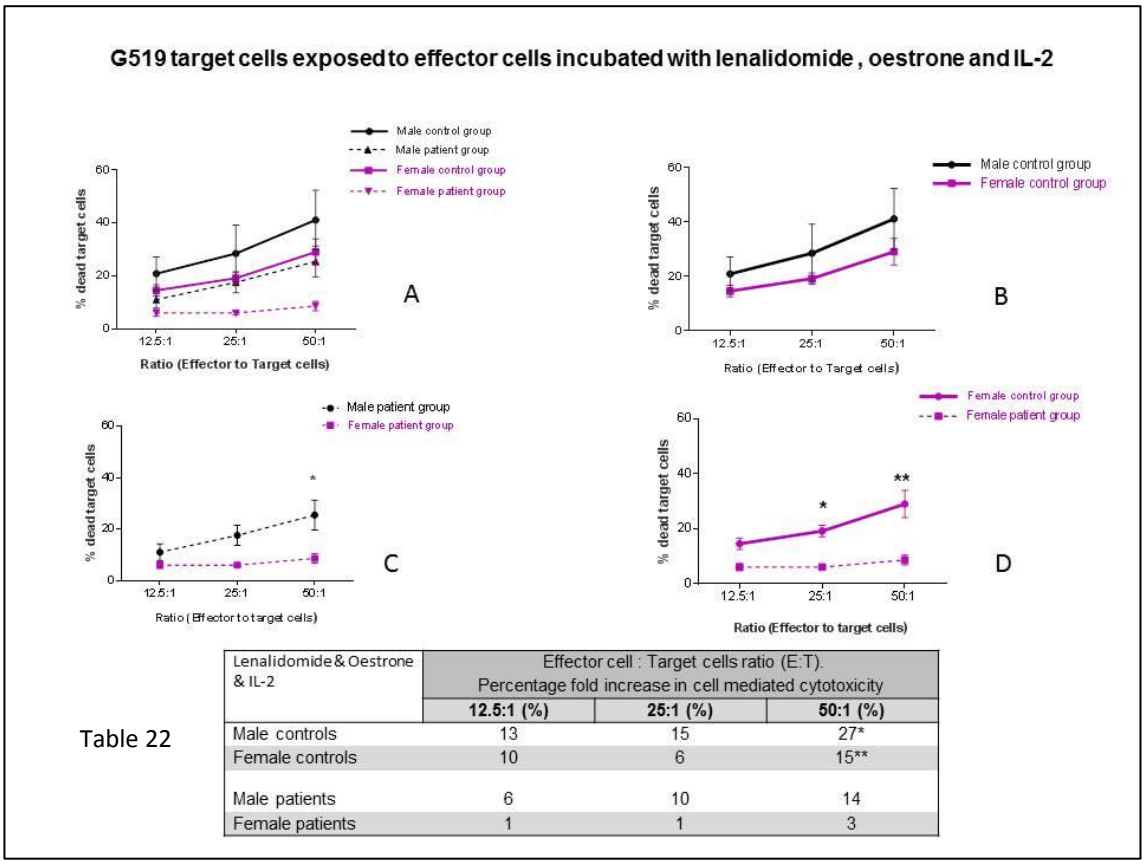


Table 22

Lenalidomide & Oestrone & IL-2	Effector cell : Target cells ratio (E:T). Percentage fold increase in cell mediated cytotoxicity		
	12.5:1 (%)	**25:1 (%)**	**50:1 (%)**
Male controls	13	15	27*
Female controls	10	6	15**
Male patients	6	10	14
Female patients	1	1	3

Figure 29: Cell mediated cytotoxicity of effector cells incubated with a combination of lenalidomide, IL-2 and oestrone

Cell mediated cytotoxicity presented by mean percentage (+/- SE) of target cell (G519 cells) death. Effector cells from male and female MCL patients consisting of 3 male patients; 2 clinically aggressive and 1 clinically indolent and 3 female patients; 1 clinically aggressive and 2 clinically indolent were used. Effector cells from 5 healthy age matched male and female controls were used.

Graph **A**: Cell mediated cytotoxicity of effectors cells from male and female controls and patients.

Graph **B**: Cell mediated cytotoxicity of effector cells from female and male control groups.

Graph **C**: Cell mediated cytotoxicity of effector cells from male and female patient group.

Graph **D**: Cell mediated cytotoxicity of female healthy controls and female MCL patients.

Table 22: Percentage increase in cell mediated cytotoxicity after effector cells incubated with combination of IL-2, oestrone and lenalidomide compared with untreated effector cells on Granta 519 target cells.

4.8. The increase in cell mediated cytotoxicity was due to an IL-2 effect

Following the results of the cell mediated cytotoxicity analysis, the most notable increase was observed in effector cells incubated with IL-2 or IL-2 in combination with lenalidomide and oestrone. Further cell mediated cytotoxicity experiments were carried out with effector cells from 3 male and female healthy controls incubated with oestrone 40 pg/ml, IL-2 100IU, lenalidomide 1µM, IL-2 100IU and oestrone 40pg/ml, IL-2 100IU and lenalidomide 2µM, IL-2 100IU, lenalidomide 2µM and oestrone 40pg/ml. These combinations were utilised to evaluate whether the increased cell mediated cytotoxicity was largely due to IL-2 or whether the other treatments also has an influence.

The results demonstrated a notable increase in cell mediated cytotoxicity with effector cells incubated with IL-2 or with IL-2 combined with lenalidomide and or oestrone. This increase in cell mediated cytotoxicity was concluded to be a result of the effects of IL-2 rather than lenalidomide or oestrone (Figure 30). Notably, in both male and female groups, there was no significant difference in the cell mediated cytotoxicity between IL-2 incubated effector cells compared to effector cells incubated with IL-2 and lenalidomide or IL-2, lenalidomide and oestrone or IL-2 and oestrone, $p>0.05$ (Figure 30).

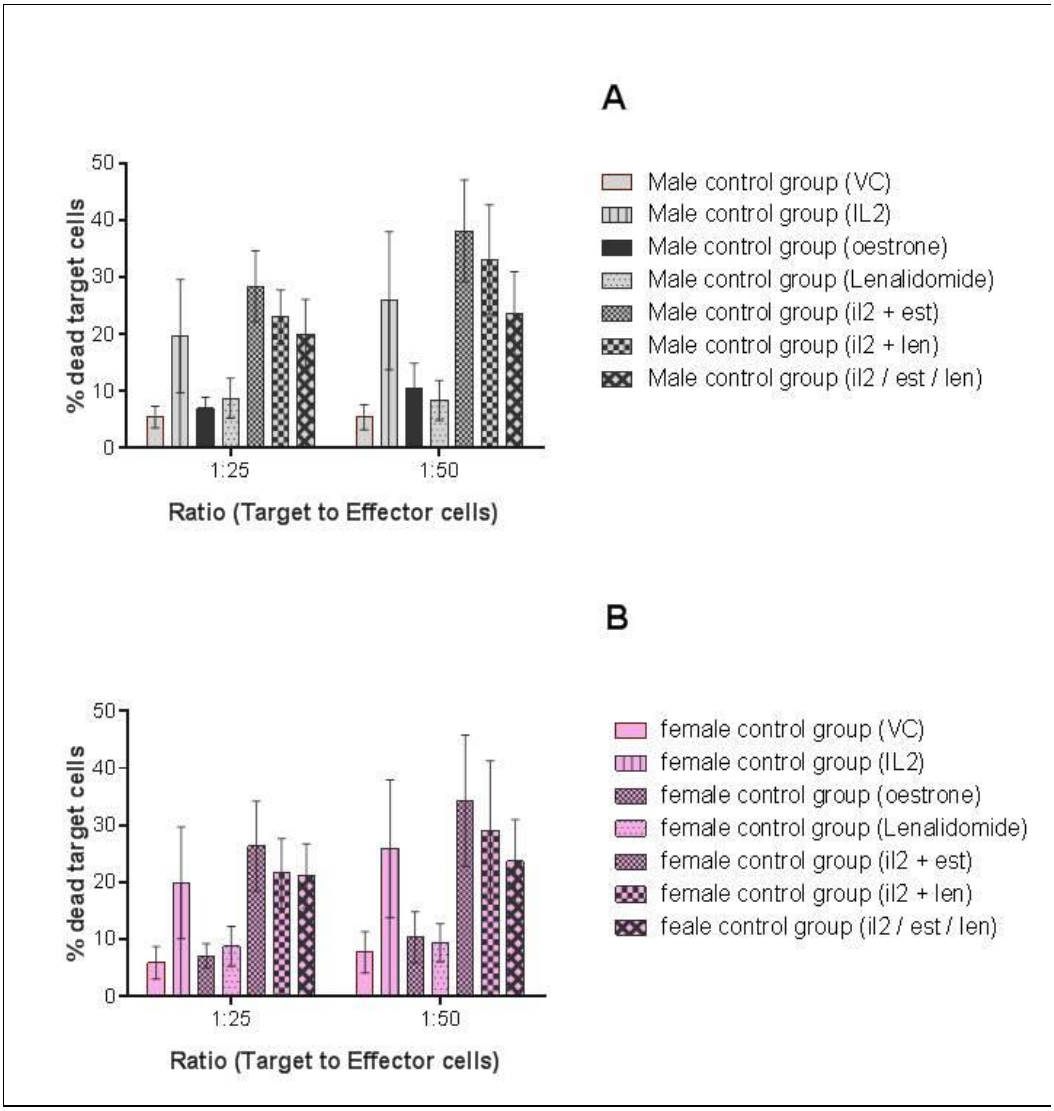


Figure 30: Cell mediated cytotoxicity of effector cells treated with IL-2 with and without lenalidomide, oestrone and combination of the three on G519 target cells

Actvity of cell mediated cytotoxicity was measured as percentage of dead target cells (+/- SE). Effector cells from 3 healthy age matched male and female controls were used in these analysis.

Graph **A:** Cell mediated cytotoxicity of effector cells from male control subjects

Graph **B:** Cell mediated cytotoxicity of effector cells from female control subjects.

VC-vehicle control DMSO; IL2- interleukin-2; IL2 +est - IL-2 with oestrone; il2+len- IL-2 with lenalidomide; il2/est/len- IL-2 + oestrone + lenalidomide.

4.9. Incubation with either lenalidomide, oestrone or IL-2 had no quantitative effects on NK cells, T cells or T regulatory cells

Quantification of the NK cells, T cells and T regulatory cells was performed on the PBMCs from the male (n=4) and female (n=4) controls and male (n=3) and

female (n=3) patient groups after 24 hours incubation with control media (DMSO), IL-2 (100iu), oestrone (40pg/ml), lenalidomide (2µM) and a combination of lenalidomide (1µM) and oestrone (40pg/ml) plus a further combination of IL-2, oestrone and lenalidomide. These PBMCs were taken from the same solution used in the cell mediated cytotoxicity experiments described earlier (Chapter 2 and chapter 4). Due to the limited available numbers of PBMCs, only the treatments mentioned above were analysed. There were no additional PBMCs available for this analysis with other combination of treatments. Analysis was performed to determine whether there was proliferation of the immune cells after incubation with the above agents compared to untreated effector cells (Figures 31, 32, 33 & 34). Quantification of the cells was performed by flow cytometry as described in the methods chapter 2.

There was no significant change in the numbers of NK cells ($CD3^-CD56^{bright}CD16^{dim}$ and $CD3^-CD56^{dim}CD16^{bright}$), T cells; $CD3^+CD4^+$ (T helper cells), $CD3^+CD8^+$(cytotoxic T cells) and T regulatory cells ($CD4^+CD25^+CD127^{dim}$) after incubation compared to the untreated effector cells (Figures 31 & 32).

Notably the overall mean number of NK cells in the female MCL patients was lower than the male MCL patients in the PBMCs, although this difference was not significant, p=0.6 (Figures 31). The numbers in the NK cell subset $CD56^{bright}$ $CD16^{dim}$ were small and did not shown significant differences, p>0.05.

The T cells: T cell subsets ($CD3^+CD4^+$ and $CD3^+CD8^+$) numbers overall were observed to be greater in the female controls compared to the male controls

and in the male patients compared to the female patients, however the difference was no significant ($p>0.05$) (Figure 32 and 34).

The mean number of T regulatory cells were similar in all groups with marginally greater numbers observed in the female groups compared to the male groups ($p>0.05$) (Figure 36). Interestingly, there was a trend towards an increase in numbers of the T regulatory cells when incubated with IL-2 or IL-2 in combibation with lenalidomide and oestrone in the female controls and male and female MCL patients. The male control group did not exhibit a notable trend, which could be related to the lower number of T regulatory cells resulting in no demonstrable change in numbers (Figure 32). In additional there was a trend toward greater increase in numbers of T regulatory cells demonstrated in the female controls after treatment with IL-2 only compared to IL-2 combined with lenalidomide and oestrone. No firm conclusions could be drawn from this due to the limited numbers and there was no significant difference in the PBMC numbers between the two treatments, $p>0.05$ (Figure 32). Nonetheless, it would have been expected that there would be a greater increase in T regulatory numbers after incubation with IL-2 in combination with lenalidomide and oestrone as both lenalidomide and oestrone enhances the production of IL-2 from T cells thus in turn simulating the increase in T regulatory cell numbers directly and indirectly (Ku *et al*, 2009; Kotla *et al*, 2009).

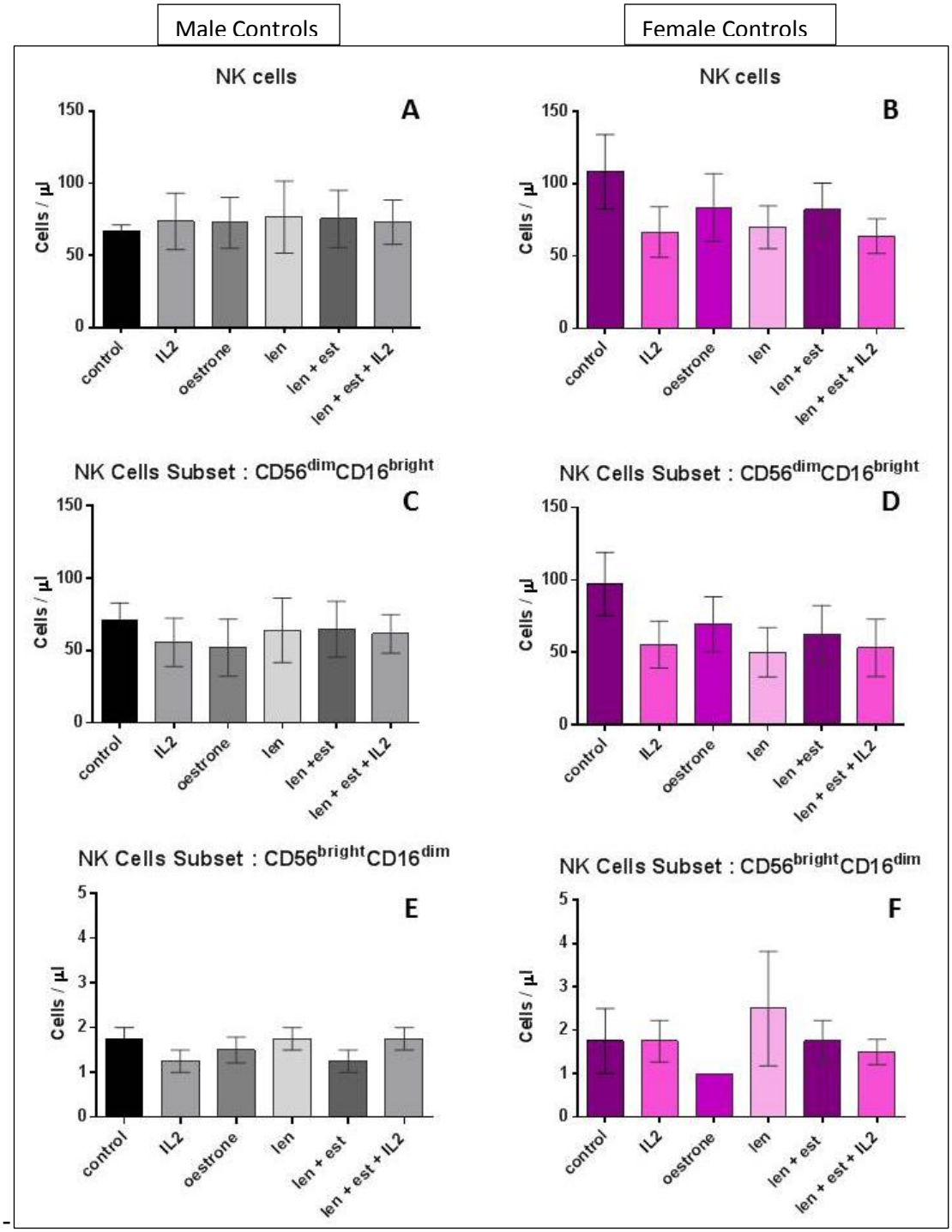


Figure 31: Healthy controls: $CD3^{-}CD56^{+}$ NK cells, $CD3^{-}CD56^{bright}CD16^{dim}$ and $CD3^{-}CD56^{dim}CD16^{bright}$NK cell subsets numbers in PBMCs.

PBMCs from 4 male (grey bars - A, C & E) and female (pink bars – B, D & F) healthy controls post 24 hours incubation with various treatments as shown. Mean number of cells/microliter (+/- SE) are presented.

(Len – lenalidomide; len + est - lenalidomide and oestrone; len + est + IL2 – combination of lenalidomide, oestrone and IL-2).

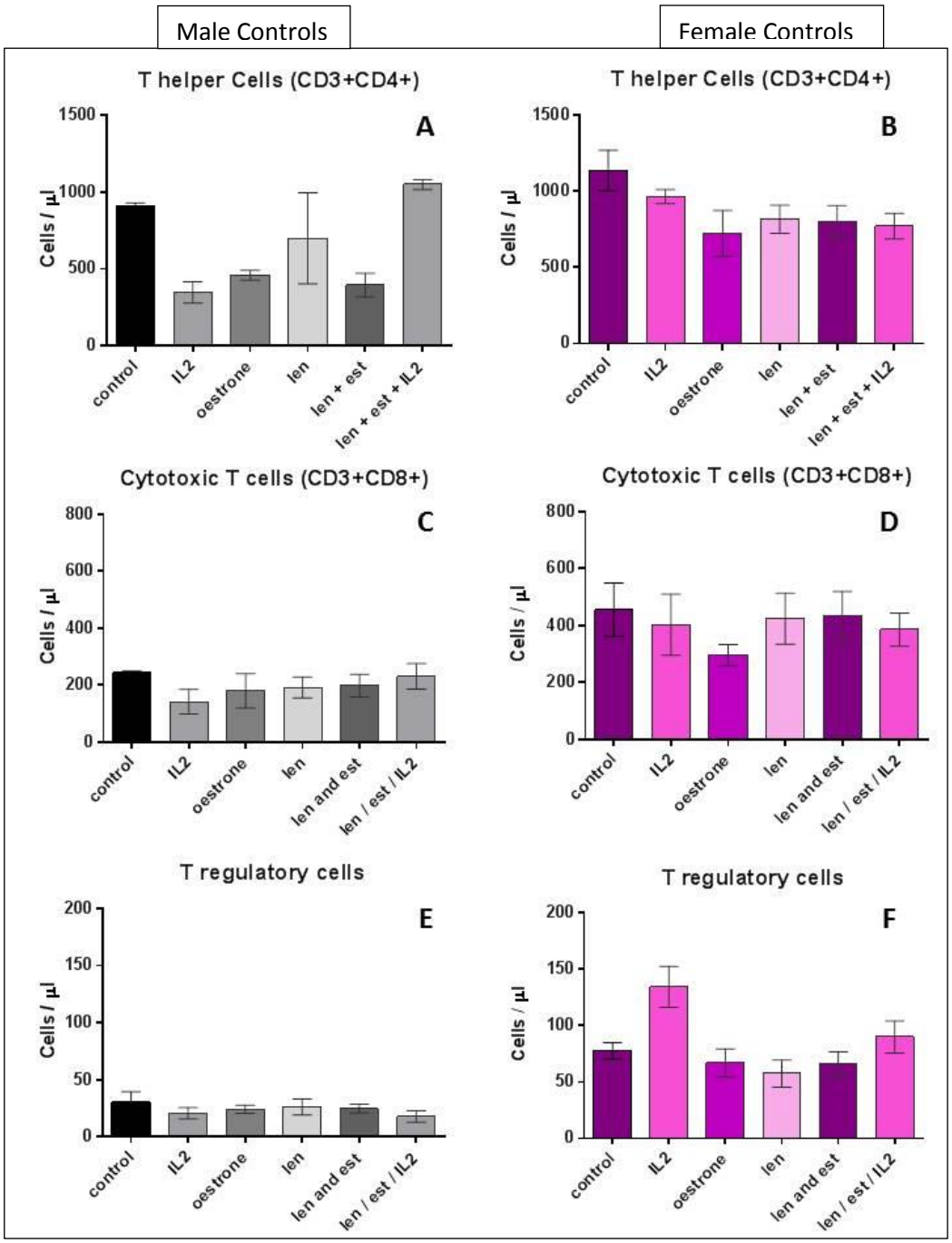


Figure 32: Healthy controls: T cells subsets and T regulatory cells quantification in PBMCs.

PBMCs from 4 male (grey bars - A, C & E) and female (pink bars – B, D & F) healthy controls post 24 hour incubation with various treatments used in the cell mediated cytotoxicity experiments. Mean number of cells/microliter (+/- SE) are presented.

(Len – lenalidomide: len + est = lenalidomide and oestrone; len + est + IL2 – combination of lenalidomide, oestrone and IL-2).

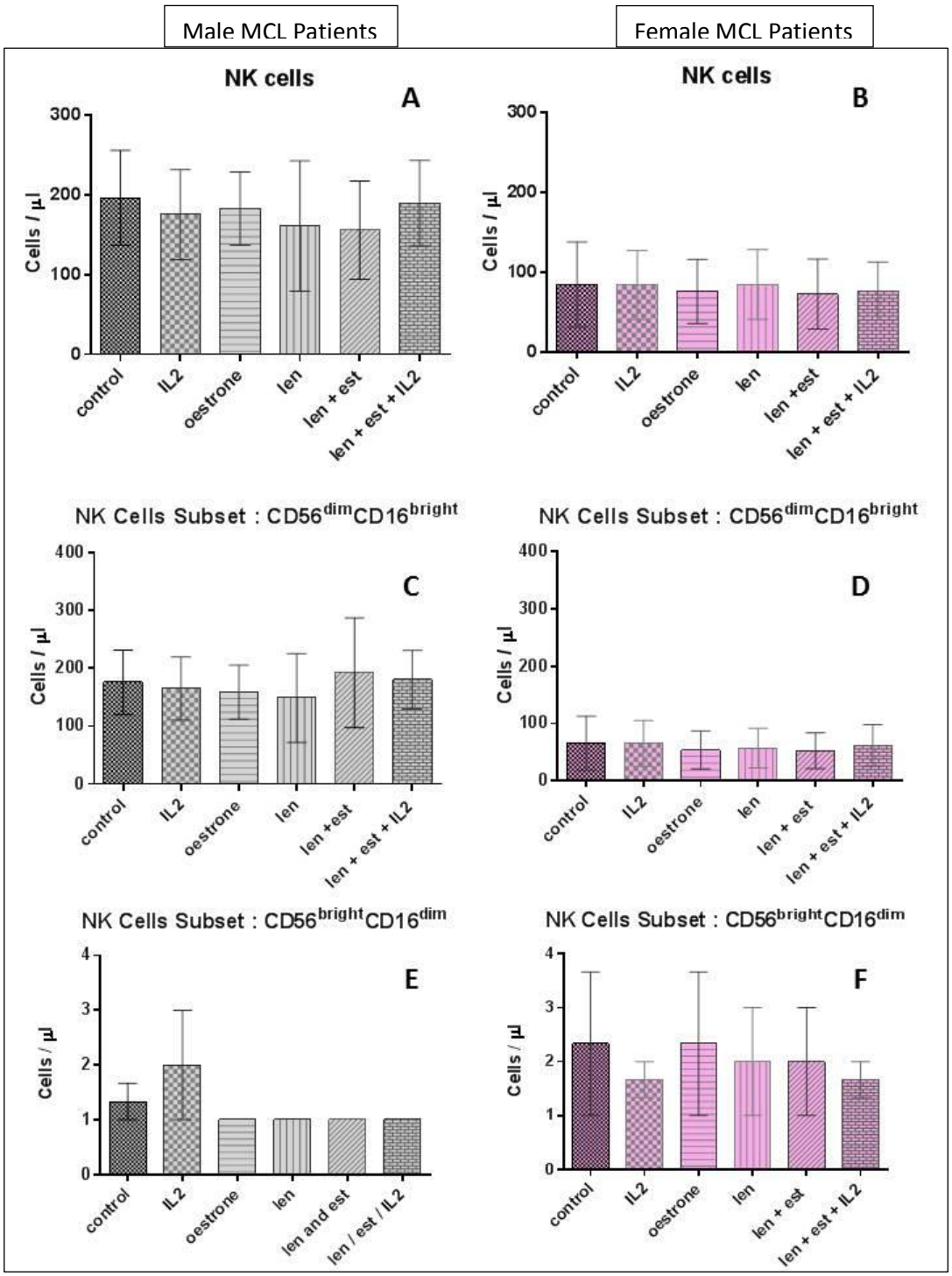


Figure 33: NK and NK cell subsets quantification in PBMCs of MCL patients after incubation.

PBMCs from 3 male (grey patterned bars - A, C & E) and female (pink patterned bars – B, D & F) MCL patients post 24 hour incubation with various treatments as shown. Mean number of cells/microliter (+/- SE) are presented.

(Len – lenalidomide: len + est = lenalidomide and oestrone; len + est + IL2 – combination of lenalidomide, oestrone and IL-2)

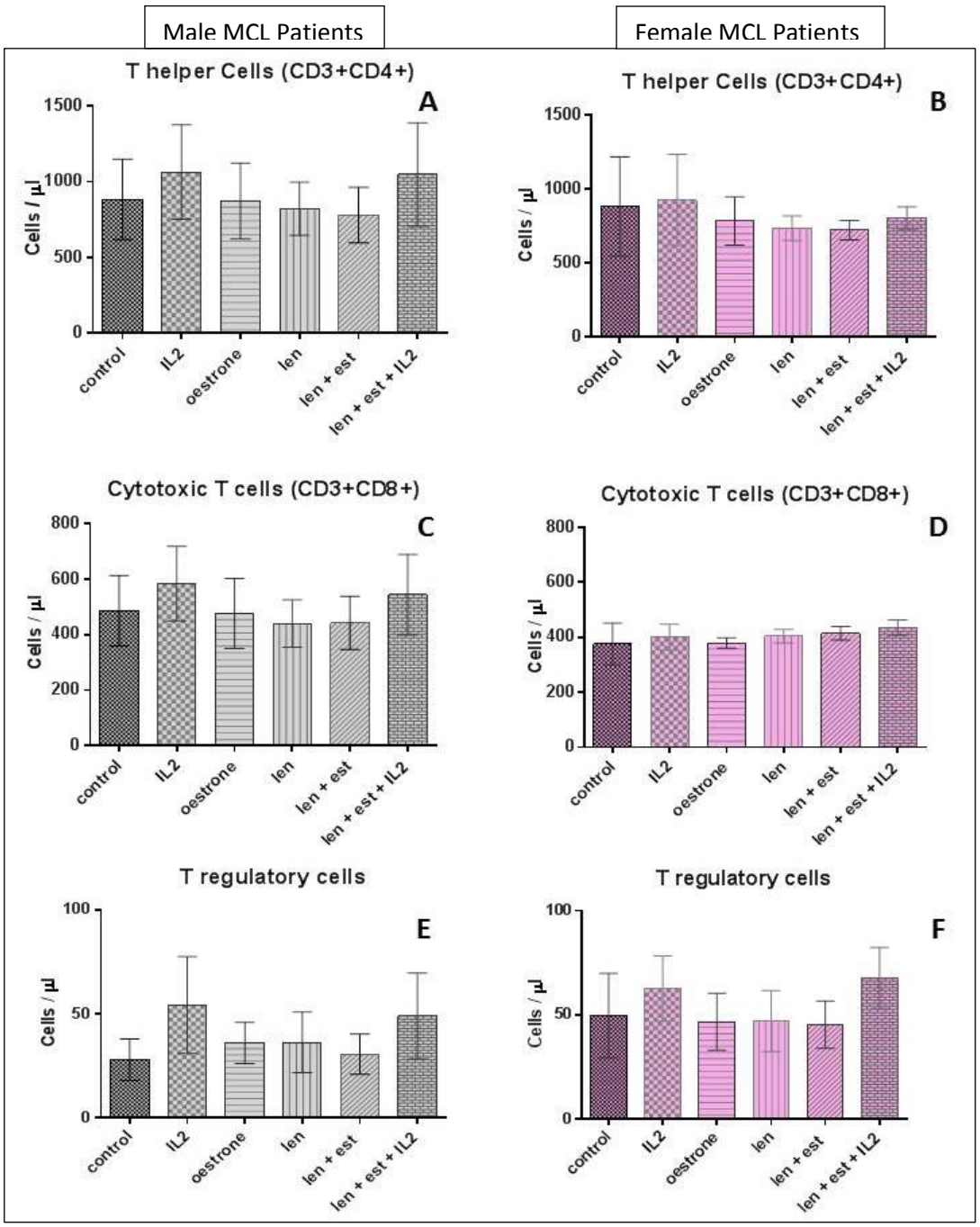


Figure 34 : T cell subsets and T regulatory cell quantification in PBMCs from MCL patients post incubation

PBMCs from 3 male (grey patterned bars - A, C & E) and female (pink patterned bars – B, D & F) MCL patients post 24hour incubation with various treatments. Mean number of cells/microliter (+/- SE) are presented.

(Len – lenalidomide; len + est = lenalidomide and oestrone; len + est + IL2 – combination of lenalidomide, oestrone and IL-2).

4.10. Effects of IL-2, oestrone and lenalidomide on NK activating receptors; NKp46, NKp30, NKp44 and NKG2D on NK cells subsets; CD3-CD56^{bright} and CD3-CD56^{dim} subsets.

The effector cells from male (n=4) and female (n=4) healthy controls and male (n=3) and female (n=3) MCL patients were analysed by flow cytometry to assess the expression of NK activating receptors on the NK cell subsets; CD3$^-$ CD56^{bright} cells and CD3$^-$CD56^{dim} cells after incubation for 24 hours with either DMSO (control), IL-2 100IU, oestrone 40pg/ml, lenalidomide 2µM, oestrone 40pg/ml and lenalidomide 2µM and IL-2 100IU, oestrone 40pg/ml and lenalidomide 2µM. The expression of the NK activating receptors on the cytokine CD3$^-$CD56^{bright} cells and cytotoxic CD3$^-$CD56^{dim} cells was compared to the untreated effector cells.

Overall there was a high baseline expression of the NKp46, NKp30 and NKG2D and moderate to low expression of NKp44 compared to the isotype controls on both subsets of NK cells.

No significant difference was found in the expression of all the NK activating receptors on the cytokine CD3$^-$CD56^{bright} cells and cytotoxic CD3$^-$CD56^{dim} cells between the untreated effector cells and effector cells treated with oestrone, lenalidomide or combination of oestrone and lenalidomide ($p>0.05$) (Figure 39 to 42). There was no significant difference in the expression of NK activating receptors between the genders in either the healthy controls or MCL patients ($P>0.05$). Interestingly, there was a trend towards lower expression of NKp30 on the cytotoxic NK subset CD3$^-$CD56^{dim} and cytokine NK subset CD3$^-$ CD56^{bright} in female controls, $p>0.05$ (Figure 35 D and 36 D). This trend was also observed in the female MCL patients, but was not as marked (Figure 37D and 37D). It is possible that female MCL have greater activation of these

receptors as there base line due to the presence of the MCL. Therefore these differences are observed between female controls and female MCL patients with regards to NKp30 receptor. There is no data on the NK cell receptors activation in patients with MCL who have been treated with lenalidomide, but other studies in myeloma patients have shown a reduction on NKp30 expression when the NK cells are exposed to lenalidomide and also in combination with dexamethasone (Carter & Wood, 2011). Therefore as oestrogens have steroid like properties it is possible that similar effects are seen as those in myeloma patients.

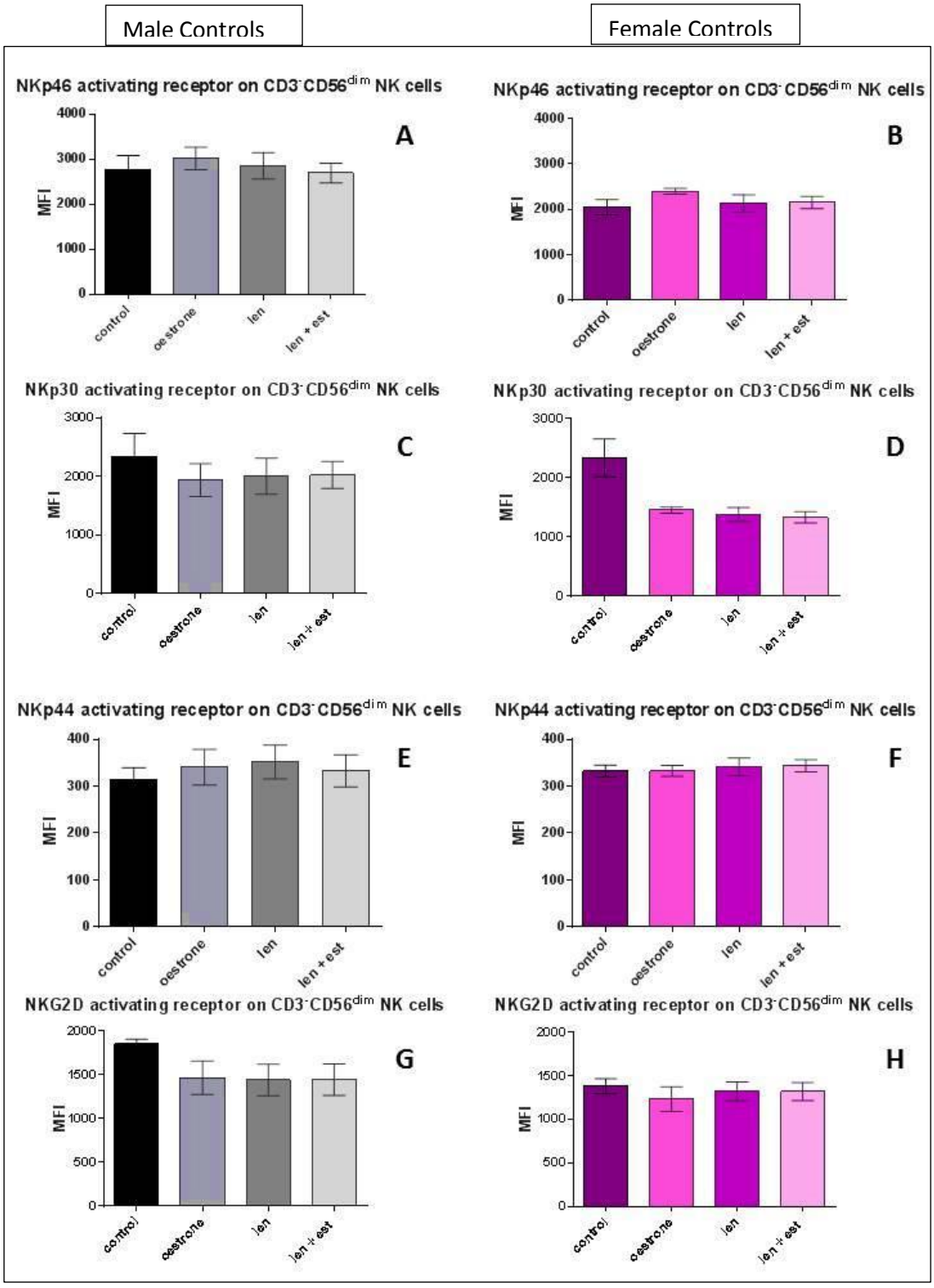


Figure 35: The expression of NK activating receptors NKp46, NKp30, NKp44 and NKG2D on NK cell subset: cytotoxic CD3-CD56^{dim} from healthy controls

PBMCs from 4 male (grey graphs A,C,E &G) and female (pink graphs B,D,F & H) healthy controls following incubation with either oestrone or lenalidomide or a combination of oestrone and lenalidomide (len + est). The results are presented as mean MFI +/- SE.

(len - lenalidomide; len+est - lenalidomide and oestrone)

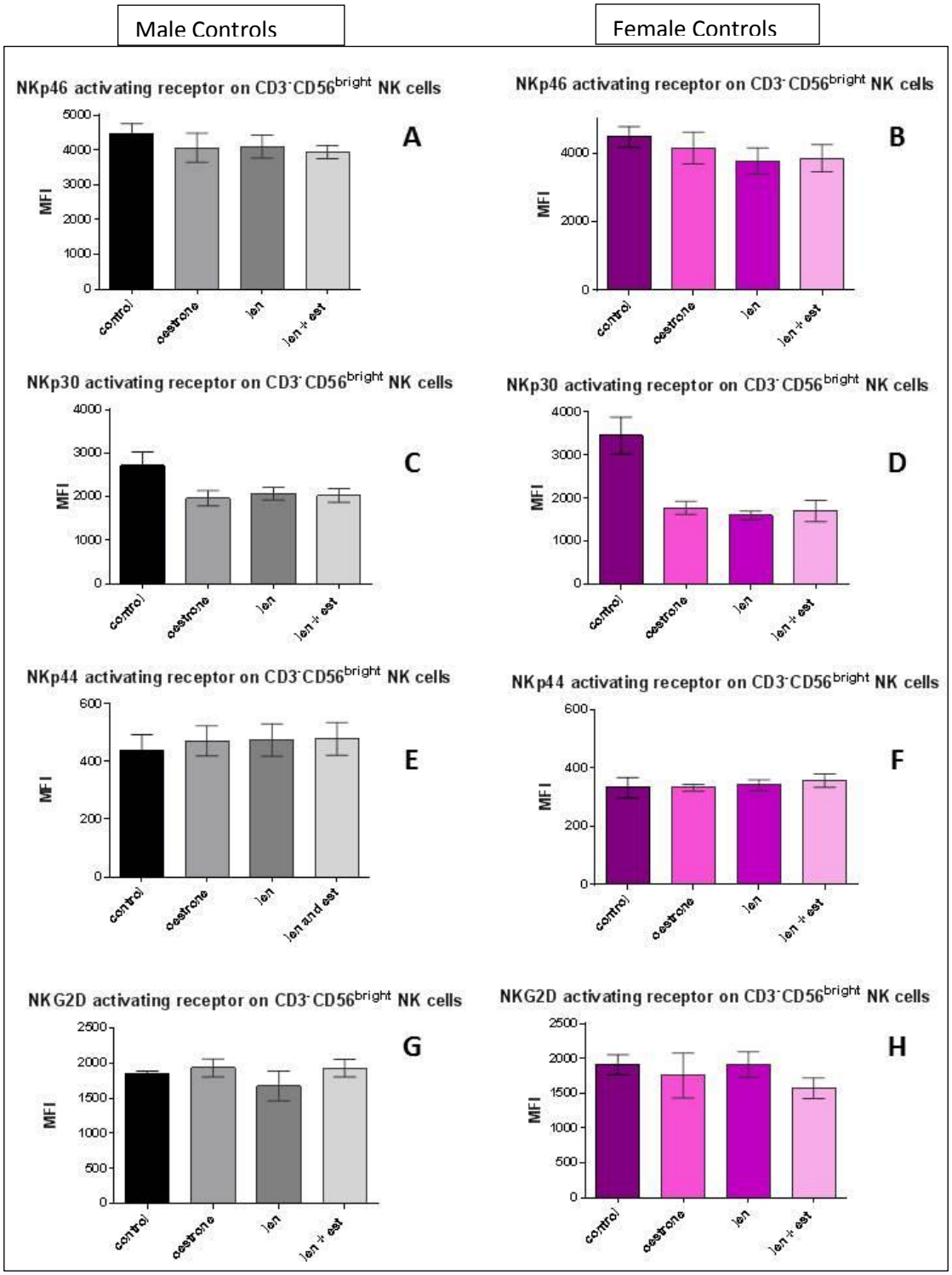


Figure 36: The expression of NK activating receptors NKp46, NKp30, NKp44 and NKG2D on NK cell subset: cytokine CD3$^-$CD56^{bright} of healthy controls

PBMCs from 4 male (grey graphs A, C, E &G) and female (pink graphs B, D, F & H) healthy controls following incubation with either oestrone or lenalidomide or a combination of oestrone and lenalidomide (len + est).

Expression of NK activating receptors are presented as the mean MFI +/-SE.

(len- lenalidomide; len+est – lenalidomide and oestrone)

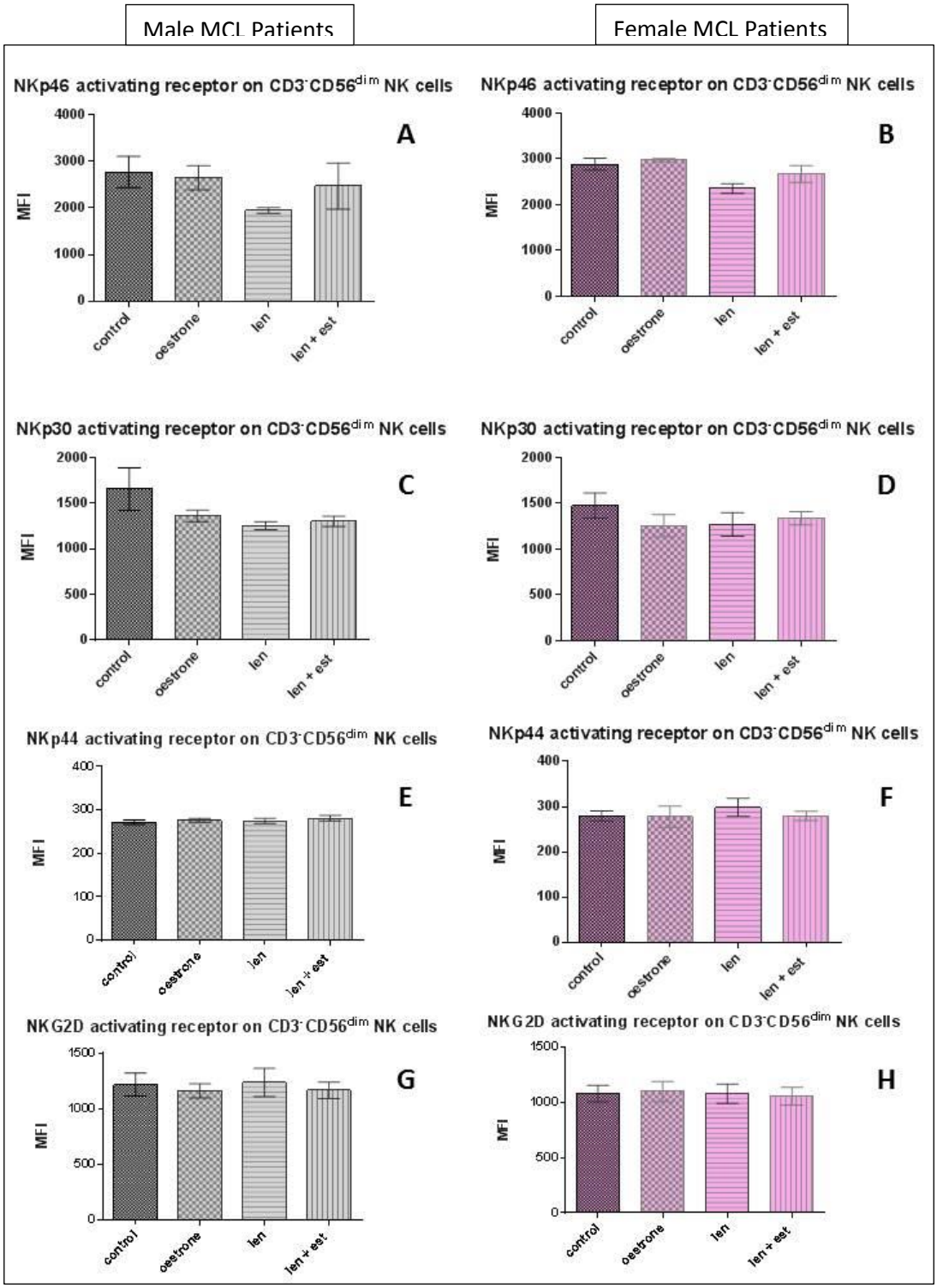


Figure 37: MCL patients: The expression of NK activating receptors NKp46, NKp30, NKp44 and NKG2D on NK cell subset: cytotoxic CD3$^-$CD56^{dim}

Effector cells from 3 male (grey graphs A,C,E &G) and female (pink graphs B,D,F & H) MCL patients following incubation with either oestrone or lenalidomide or a combination of oestrone and lenalidomide (len + est).

The expression of the NK cell activating receptors are presented as mean MFI +/- SE. (Len – lenalidomide; len+est – lenalidomide and oestrone)

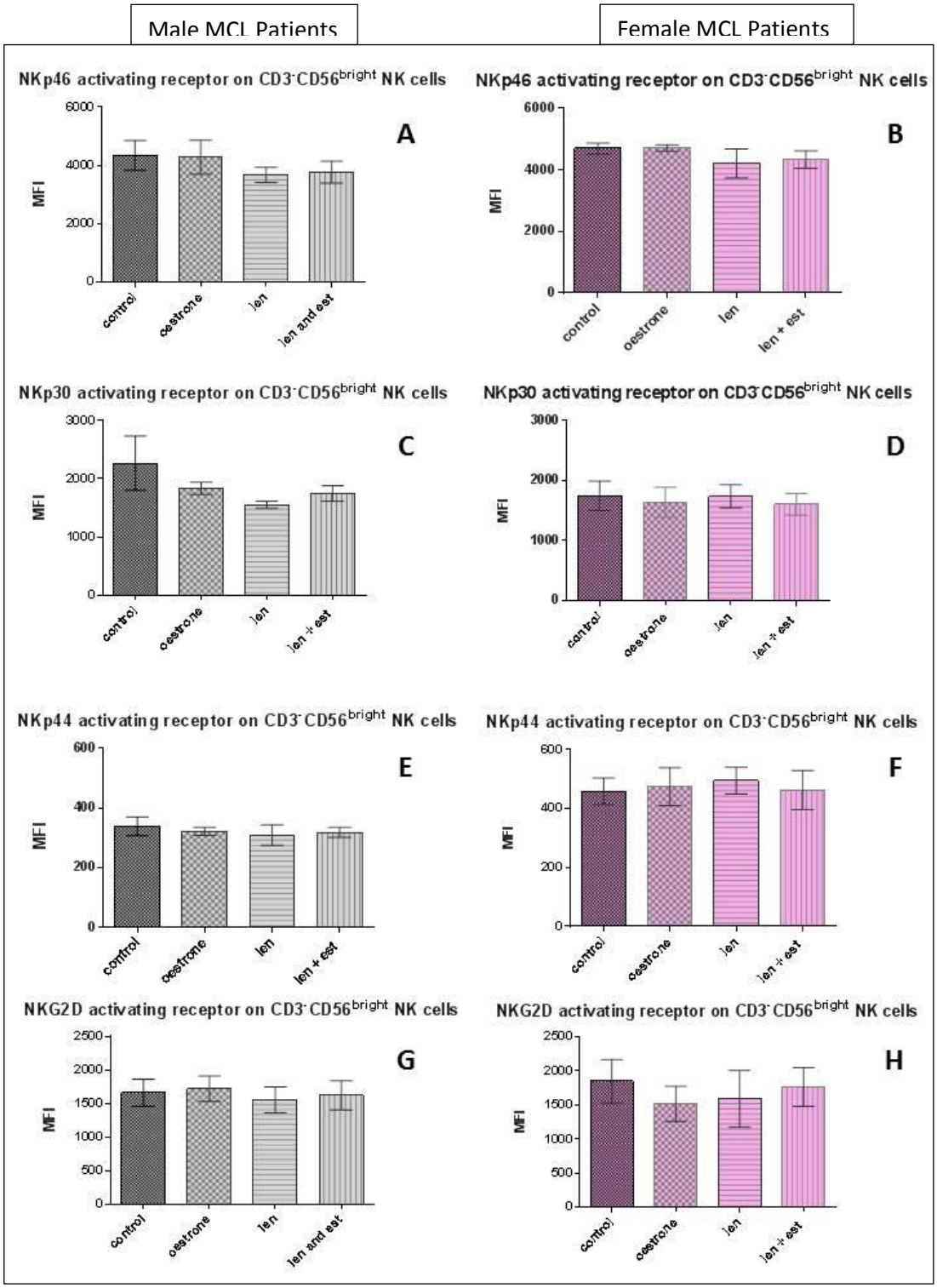


Figure 38: Expression of NK activating receptors NKp46, NKp30, NKp44 and NKG2D on cytokine NK cell subset CD3$^-$CD56^{bright} in MCL patients

Effector cells from 3 male (grey graphs A, C, E &G) and female (pink graphs B, D, F & H) MCL patients following incubation with either oestrone or lenalidomide or a combination of oestrone and lenalidomide (len + est).

The expression of the NK cell activating receptors is presented as mean MFI +/- SE.

(Len – lenalidomide; len+est – lenalidomide and oestrone)

IL-2 effect on NK activating receptors on NK cell subsets: cytokine $CD3^-$ $CD56^{bright}$ cells.

The expression of **NKp46** activating receptor exhibited no significant difference when compared to controls, $p>0.05$ in any of the groups. There was no significant difference observed between the genders or in the base line expression between healthy controls and MCL patients (Figure 39).

However, the **NKp30** activating receptor expression exhibited a significantly greater overall expression after incubation with IL-2 in all groups, $p=0.04$. A significant difference was seen in the female patients when comparing untreated with IL-2 treated effector cells, $*p=0.01$. There was a trend towards greater base line expression in healthy controls compared to MCL patients (Figure 39).

In addition, the **NKp44** activating receptor expression exhibited a significantly greater overall expression after incubation with IL-2, $p=0.002$, with the most significant difference in the male patients when comparing untreated with IL-2 treated PBMCs, $*p=0.01$. There was no significant difference observed in the base line expression of NKp44 between healthy controls and MCL patients. No significant difference was observed between the genders (Figure 39).

The **NKG2D** activating receptor expression in male patients exhibited a significant increase after IL-2 incubation, $*p=0.02$. PBMCs treated with IL-2 from male healthy controls and female MCL patients did not exhibit a notable increase in their NKG2D expression when compared to the control (untreated) effector cells. Similarly to the other receptors there was no significant difference observed in the base line expression between healthy controls and MCL patients (Figure 39).

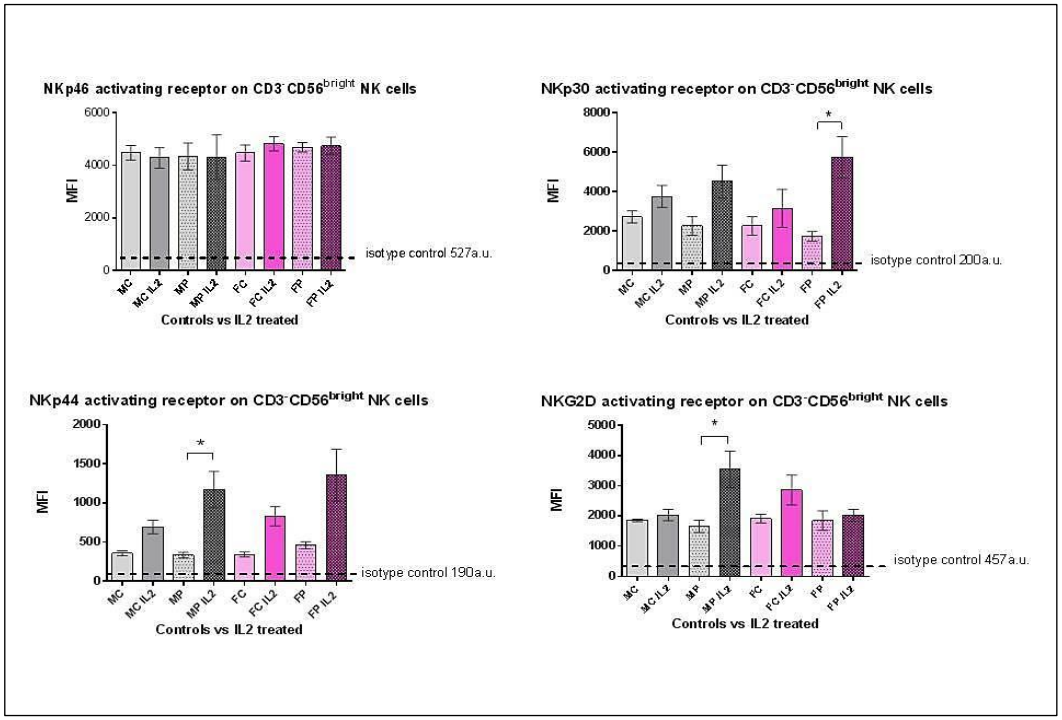


Figure 39: Expression of NK activating receptors NKp46, NKp44, NKp30 and NKG2D on cytokine *CD3$^-$CD56^{bright} NK cell subset* compared with control and after incubation with IL-2

The expression of NK activating receptors shown above is presented as MFI +/- SE.

(MC-male control group (n=4); MC IL-2- male control group incubated with IL-2 (n=4); FC-female control group (n=4) ; FC IL-2- female control group incubated with IL-2 (n=4); MP-male patient group (n=3); MP-IL2 male patient group incubated with IL2 (n=3); FP- female patient group (n=3); FP-IL2- female patient group incubated with IL2(n=3)).

IL-2 effect on NK activating receptors on NK cell subsets: cytotoxic $CD3^-$ $CD56^{dim}$ cells.

The expression of **NKp46** activating receptor exhibited no significant increase after IL-2 when compared to controls (P>0.05). No difference was observed between the genders in either of the groups. Nor was there a difference exhibited in the base line expression between the healthy controls and MCL patients (Figure 40).

Interestingly the **NKp30** activating expression exhibited no significant increase after IL-2 incubation. However there was a trend towards the patient group exhibiting a more notable rise in the expression of the NKp30 after incubation with IL-2. There was no significant difference with the expression of NKp30 after incubation with IL-2 between male and female healthy controls or MCL patients, P>0.05.There was a trend towards greater base line expression of NKp30 receptor in healthy controls compared to MCL patients (Figure 40).

The expression of **NKp44** activating receptor exhibited an overall significantly greater expression after incubation with IL-2 compared to the control arm, *p=0.01. There was a trend towards the male and female healthy controls exhibiting greater expression of NKp44 receptors compared to the male and female MCL patients respectively. There was no significant difference with the expression of NKp44 after incubation with IL-2 between male and female healthy controls or MCL patients, P>0.05). Similarly, there was a trend toward greater expression of NKp44 expression in healthy controls compared to MCL patients (Figure 40).

Additionally the **NKG2D** activating receptor expression exhibited a significant overall increase in expression after incubation with IL-2 in all four groups, p=0.04 and the most significant increase was observed in the male patient group, *p=0.04. Again, there was a trend toward greater expression of NKG2D expression in healthy controls compared to MCL patients (Figure 40).

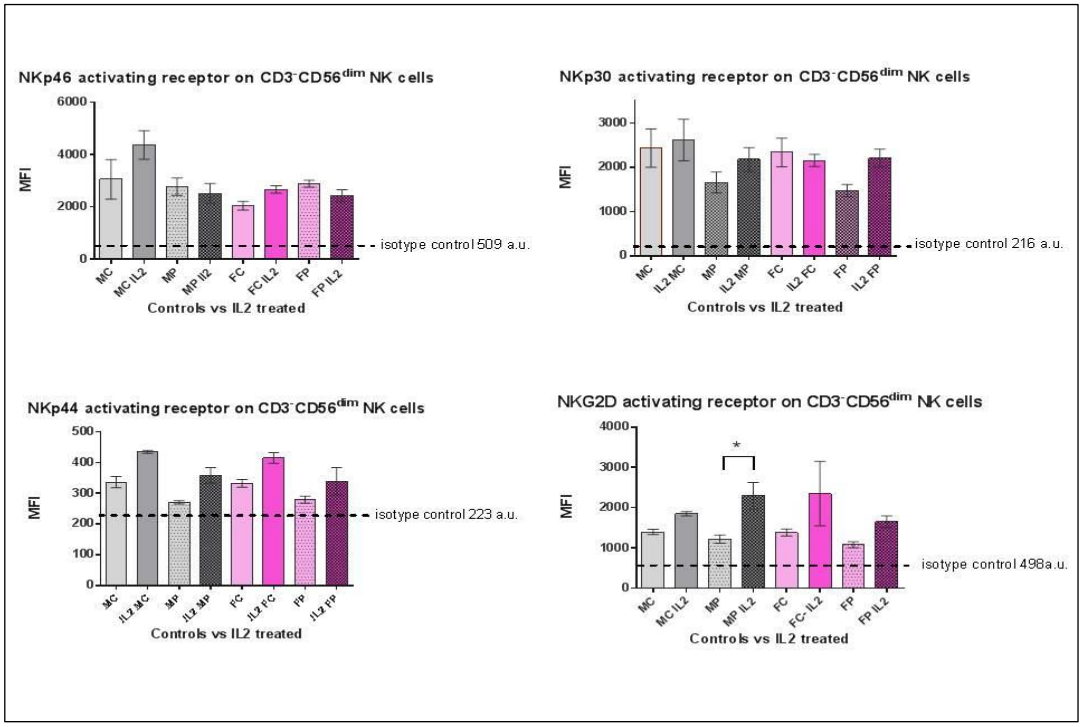


Figure 40: Expression of NK activating receptors NKp46, NKp44, NKp30 and NKG2D on *cytotoxic CD3$^-$CD56^{dim} NK cell subset* compared with control and after incubation with IL-2

The expression of NK activating receptors is presented as MFI +/- SE.
(MC-male control group (n=4); MC IL-2- male control group incubated with IL-2 (n=4); FC-female control group (n=4) ; FC IL-2- female control group incubated with IL-2 (n=4); MP- male patient group (n=3); MP-IL-2 male patient group incubated with IL-2 (n=3); FP- female patient group (n=3); FP-IL-2- female patient group incubated with IL2(n=3))

Summary of results

1. Both the male and female healthy controls exhibited a higher mean cytotoxic function compared to the respective patient subjects.

2. The natural cytotoxicity of female health controls was greater than male healthy controls as demonstrated on K562 target cells. However on Granta 519 target cells the cell mediated cytotoxicity was comparable between male and female healthy controls.

3. Incubation with lenalidomide or in combination with oestrone did not result in any significant difference in the cytotoxic function or enhancement of cytotoxicity in any of the groups.

4. Oestrone did not significantly affect the cell mediated cytotoxicity of any of the groups. However there was a small response seen in male MCL patients compared to female and healthy controls.

4. IL-2 enhanced the cytotoxic function of the effector cells from all subjects, except for the effector cells from the female patients with Granta 519 target cells.

5. IL-2 produced a significant increase in the NK activating receptors on both subsets of NK cells. NKp46 was unaffected by IL-2 incubation. Lenalidomide, and oestrone did not affect the expression of the activating receptors

7. Incubation of PBMCs with oestrone, lenalidomide or IL-2 and their combinations did not increase the quantity of the immune cells.

8. There were lower number of NK and T cells in the PBMCs of female MCL patients compared to male MCL patients.

4.11. Discussion

The function of PBMCs (effector cells) from male and female MCL patients and male and female healthy controls was evaluated by measuring their cell mediated cytotoxic activity through flow cytometry. The percentage of dead target cells was determined and taken as the measure of cell mediated cytotoxic activity of the effector cells. Comparisons were then made between healthy controls and MCL patients, between female and male MCL patients and female and male controls to determine whether there was a significant difference in their function.

4.11.1. Cell mediated cytotoxicity of effector cells from controls was superior to MCL patients

The results demonstrated an overall greater cell mediated cytotoxicity of effector cells from healthy controls compared to MCL patients. This trend was especially evident with Granta 519 target cells compared to K562 target cells.. This is observation could be attributed to K562 cells' high sensitivity to NK mediated cytotoxicity compared to G519 cells which have some expression of MHC-class 1 making it less sensitive to NK cell mediated cytotoxicity as described previously. These results correlate to other studies as discussed below

Cancer patients exhibiting an inferior immune response has been observed in other studies (Kim *et al*, 2013; Imai *et al*, 2000; Guven *et al*, 2003). For example, a study reported by Dhodapkar *et al*, observed reduced cytotoxicity activity and reduced IFN-γ production in isolated T cells from blood and bone marrow from myeloma patients against autologous myeloma cells. The T cells from the myeloma patients were capable of producing adequate IFN-γ in response to influenza virus peptide suggesting the lack of response was due to

the tumour and its microenvironment. In this study the T cells were stimulated with autologous dendritic cells with or without anti-syndecan-1 antibody (Syndecans are heparan sulfate proteoglycans (HSPGs) that are involved in cell adhesion, tissue morphogenesis, differentiation, and act as low affinity co-receptors of soluble growth factors) to enhance presentation of MHC class I products. In contrast to freshly isolated T cells, these stimulated T cells exhibited increase in secretion of IFN-γ in response to autologous tumour (Dhodapkar *et al*, 2002). IFN-γ is secreted by NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells, antigen presenting cells and B cells (Frucht *et al*, 2001; Lighvani *et al*, 2001). IFN-γ activated macrophages and induces the expression of MHC-class I, class II and co-stimulatory molecules on APCs (Frucht *et al*, 2001). Therefore they indirectly enhance the killing effects of cytotoxic T cells and macrophages against tumour cells. Macrophages are stimulated by IFN-γ and result in classically activated macrophage M1 which are perceived as pro-inflammatory, anti-microbial and anti-tumour (Foey, 2014). Patients with follicular lymphoma have also been shown to exhibit reduced immune response to cancerous cells. A study reported by Ramsay *et al*, demonstrated a significant reduction of T cell mediated cell cytotoxicity due to T cell dysfunction at forming F-actin synapses with cancer cells when compared to cells from healthy controls ($p<0.01$). This observation was thought to be due to the direct influence of the tumour cells as it was demonstrated that the T cells from healthy subjects exhibited a reduction of T cells synapses after a period of incubation with follicular lymphoma cells (Ramsay *et al*, 2009). There is a paucity of data to show the precise cytokines produced by Granta 519 cells. However, one of the futures additions to this study is to measure the amount of cytokines like IFN-γ and IL-2 secreted by the cells and also examine is Granta

519 cells secrete any inhibitory cytokines such as IL-4 (which enhances B cell proliferation), IL-13 and chemokines such as CXCL12 and 13 (adhesion molecules) by luminex technique which is a technology based on a 4 member solid phase sandwich immunoassay. Up to 100 different polystyrene beads, each of which possesses unique spectral properties and is coated with analyte-specific capture antibodies.

NK cells from healthy controls have been observed to demonstrate better function compared to those from patients with acute myeloid leukaemia. A study reported by Costella *et al*, demonstrated a reduction of at least 20-25% in in-vitro cell mediated cytotoxicity of NK cells from patients with leukaemia compared to NK cells from healthy controls. They summarised the observation was secondary to lower NK activation receptor expression on the NK cells from AML patients (Costello *et al*, 2011).

Another recent study has demonstrated patients with Hodgkin's disease exhibit a significantly lower cell mediated cytotoxicity compared to healthy controls due to the elevated levels of inhibitory soluble ligands in their serum (MICA/ BAG6) ($p=0.0001$)(Reiners *et al*, 2013).

The studies above show that tumours can have significant influence over the host's immune response through differing mechanisms of immune evasion such as presence of inhibitory ligands, modification of the tumour microenvironment and possibly other more complex mechanisms yet to be fully understood.

As mentioned above, one of the future directions of this study could be to investigate further the role of the MCL microenvironment. In-vivo method to grow MCL cells on fibroblast cells to simulate the microenvironment has been tried by our research group. This could be used to assess the presence of

various cytokines mentioned above and in chapter 1. Additionally, addition of various agents such as lenalidomide and osetrogens could be analysed to show their effects on the microenvironment. Interestingly IL-15 is which is trans-presented by macrophages, together with CD40 interactions with T_{FH} cells , may contribute to the proliferation of lymphoma cells by activating the STAT5 which could be investigated (Scott & Gascoyne, 2014; D Foey, 2015).

4.11.2. Female healthy controls display greater cell mediated cytotoxicity compared to male healthy controls on K562 target cells

Interestingly the female healthy controls demonstrated an overall greater cell mediated cytotoxicity compared to male healthy controls on K562 target cells. This observation could be due to lower numbers of cytotoxic T cells and NK cells in the PBMCs from male healthy controls when compared to the female healthy controls rather than the individual immune cell functionality. However, the difference in the cell numbers did not achieve statistical difference (figure 4.14 & 4.15). Additionally the expression of the activating receptors on the NK cells were similar between the male and female healthy controls thus supporting the notion that the difference in numbers probably accounted for the results observed.

When Granta 519 were the target cells the trend observed with the K562 target cells was not as evident. One of the possible reasons for this is that Granta 519 cells exhibit some expression of MHC class-1 and are therefore are less susceptible to NK cell mediated cytotoxicity (Wang *et al*, 2013) and although the numbers of both T cells and NK cells were higher in female healthy controls than male healthy controls the difference in the numbers of T cells was not as marked as that of NK cells.

One of the limitations presented here was the variation in numbers of NK and T cells numbers between the groups. Although the total number of effector cells was consistent between the experiments, the number of individual immune cell groups which make up the PBMCs could not be verified. Isolation of 'untouched' NK cells was attempted in this study using Miltenyi MACS separator as per manufacturer instructions. However the viability (less than 50%) and quantity of the NK cells isolated was poor and could not be utilised in any of the planned experiments. This is an area that was planned for further investigation following optimisation of the techniques used. In addition to isolation of NK cells, isolation of T cells to analyse their function would be interesting. The advantage of isolating immune cells is that it would enable the investigation of individual immune cell groups. It would also allow more consistent cell numbers to be used between the subjects thus enabling better comparison.

4.11.3. The cell mediated cytotoxicity function of effector cells incubated with oestrone, lenalidomide or combination of oestrone and lenalidomide demonstrated no significant effect or difference between the genders

As discussed previously a recent study demonstrated female MCL patients had a better response to lenalidomide compared to male MCL patients (Eve *et al*, 2012). In order to further investigate this observation, cell mediated cytotoxicity was evaluated in the presence of lenalidomide and oestrone. The overall trend observed with the cell mediated cytotoxicity of effector cells incubated with oestrone or lenalidomide or a combination of both, from healthy controls and MCL patients was comparable to the results exhibited by the untreated effector cells from the respective groups. This suggests that the addition of oestrone or lenalidomide did not have any significant effect on the cell mediated cytotoxicity of effector cells towards either of the target cells. Furthermore there was no

significant difference observed in the cell mediated cytotoxicity when male and female MCL patients were compared. A limitation to the assessment of oestrone was that the bioactivity of oestrone was not specifically measured. This could have been investigated by whethere addition of oestrone lead to increased activity of NK cells or T cells. For example measuring degranulation of NK cells by analysis of CD107a could indicate its activity on these cells.

Interestingly, the effector cells incubated with oestrone from the male MCL patients demonstrated a trend towards greater cell mediated cytotoxicity compared to the female MCL patients at E:T ratio of 50:1 (Figure 4.9), but this did not achieve statistical significance. On further analysis of this trend it was found that one of the male patients that presented with clinically indolent and limited stage disease exhibited a much greater cell mediated cytotoxicity compared to the rest of the male patients. This therefore affected the mean result due to individual variation. Discounting this result there was no notable difference between the male and female MCL patients of effector cells incubated with oestrone. Therefore it is not possible to conclude if incubation with oestrone influences cell mediated cytotoxicity. Furthermore, oestrone did not have any significant influence on the expression of NK activating receptor or proliferation of the immune cell subsets after a 24 hour incubation (figure 4.18 to 4.21). It is possible that the dose used in these experiments was suboptimal as oestrone can have a dose dependent effect (Straub, 2007). It is difficult to ascertain the exact concentration of oestrogens in the tumour microenvironment as there have been no studies published to date to demonstrate concentrations within this environment. Additionally the lack of significant effect of oestrone on the effector cells could be a result of the relative short incubation time of 24hrs. The activity of oestrone on G519 cells could be assessed by Ki67 a marker of

proliferation. This can be done by immunofluorescence technique. Studies by Yakimchuk et al, have reported reduction in Ki67 on MCL cell lines when treated with oestrogen Beta agonist. Additionally they also demonstrated by real time PCR decreased B cell activating factor (BAFF), which is a proliferation protein in B cell lymphoma (Herman *et al*, 2011; Scott & Gascoyne, 2014). Therefore when compared to control (unstimulated) G519 cells the activity of oestrone on G519 cells could be assessed (Yakimchuk *et al*, 2014, 2011a).

Lenalidomide is able to modulate the immune system, enhancing anti–tumour immune responses via activation and proliferation of NK cells (chapter 1). It does not activate the NK cells directly but instead acts by providing potent co-stimulatory signals to T cells, increasing IL-2 and IFN-γ production (Anderson, 2005). The results presented here are in contrast to other studies which have demonstrated an increase in immune cell activity against cancerous cells after treatment with lenalidomide (Richardson *et al*, 2010; McDaniel *et al*, 2012; Ramsay *et al*, 2009). A study reported by Richardson *et al*, demonstrated up to a 15-20% increase in cell mediated cytotoxicity of effector cells incubated with lenalidomide compared to untreated effector cells ($p<0.05$). This significantly enhanced cell mediated cytotoxicity was thought to be in part secondary to a significant increase in NK cells numbers after incubation with lenalidomide (Richardson *et al*, 2010). Another study has demonstrated repair of the dysfunctional synapse of T cells from patients with follicular lymphoma after incubation of T cells with lenalidomide resulting in restoration of the cell mediated cytotoxicity (Ramsay *et al*, 2009). A further study reported by Davies, *et al*, also demonstrated enhanced activation of T cells and NK cells when treated with lenalidomide. This again was not a direct effect on the immune cells but secondary to an increase in IL-2 and IFN-γ resulting in the improved

function of NK cells and T cells (Davies *et al*, 2001). They suggested that in patients who responded to lenalidomide had increased levels of IL-2 and IFN-γ in the serum resulting in increased number of NK cells and enhancement of their activity. It was also suggested that the increase in the IL-2 and IFN-γ was due to the direct effect of lenalidomide and thalidomide on T cells (Davies *et al*, 2001). To date there is no precise data on lenalidomide's effect on other cells such as macrophages and NK T cells. However it effect on the microenvironment as discussed in chapter 1 would lead to the assumption that other cells such as the macrophages could also be implicated in its actions. Lenalidomide has an inhibitory effect on pro-inflammatory cytokines and it is therefore no unreasonable to assume that M1 macrophages which are pro-inflammatory could have a protective function on MCL and thus may be augmented by lenalidomide. This is an area that would need further research and as mentioned before MCL cells grown in-vitro on fibroblast to stimulate the microenvironment could be a good platform to do these initial studies. One of the limitations of this study was that IL-2 and IFN-γ was not done at the time. This was in the planning for the next phase of the experiments but due to limitations of patient material it was difficult to do it in this context. Furthermore a better plateform to perform this assay would be in the MCL simulation of the microenvironment by growing MCL on fibroblast beds or stromal cell beds.

The results presented here are in contrast to other studies probably due to the shorter time effector cells were incubated with lenalidomide and differences in the dose used (Hayashi *et al*, 2005b; Richardson *et al*, 2010; Chanan-Khan & Cheson, 2008). The results also demonstrated no significant increase in the number of immune cell subsets after incubation with lenalidomide or its combination with oestrone. This again was in contrast to other studies which

have reported increase in the immune cells numbers after incubation with lenalidomide (Richardson *et al*, 2010; Chanan-Khan *et al*, 2006). Again this could be a reflection of the dose and incubation times used in these other studies. Hence, these results suggest a lack of immunomodulation effect by lenalidomide on its own or when combined with oestrone within the time frame of the incubation period. It should be noted that we selected this dose based on the likely physiological dose achieved *in vivo*.

To date there have been no other studies to evaluate the better response rate of female MCL patients to lenalidomide compared to male MCL patients. In order to fully investigate this observation these experiments would need to be repeated with longer incubation times of at least 72 hours and possibly higher concentrations (possibly doubling the concentrations) of lenalidomide and oestrone. The rationale for this period is based on the clinical observation where the effects or benefits of the treatments are not seen for at least 5 days on treatment with lenalidomide. This could be a cumulative effect and periodic experiments may aid to describe this observation.

4.11.4. IL-2 enhanced cell mediated cytotoxicity

IL-2 plays a pivotal role in the immune response. It mediates T cell growth and proliferation, promotes cytotoxic T cells and NK cell (LAK cells) cytolytic activity which is essential for activation-induced cell death. It modulates naïve CD4+ T cell differentiation to T helper 1 and 2 cells. It also influences the development and maintenance of T regulatory cells (Liao *et al*, 2013). Therefore as expected, incubation with IL-2 demonstrated increased cell mediated cytotoxicity function in both control and patient groups. These results correlate to other studies in which IL-2 demonstrated an increased activation and function of the immune

cells against cancerous cells (Higuchi *et al*, 1989; Grimm *et al*, 1982; Gottlieb *et al*, 1990). Incubation of effector cells with IL-2 resulted in NK cells exhibiting an increase in the expression of the NK activating receptors which in turn may imply that the NK cells were better primed to carry out the cell mediated cytotoxicity (section 4.10) (Hudspeth *et al*, 2013). Interestingly, there was no significant increase in the number of immune cell subsets after incubation with IL-2 when compared to the untreated effector cells (figure 4.14 to 4.17). This is probably accounted for by the relative short incubation time as discussed above.

The positive effects of IL-2 on immune cell function have been demonstrated in other malignancies such as renal cancers, melanoma and lymphomas (Coventry & Ashdown, 2012; Rosenberg, 2014; Liao *et al*, 2013; Hombach *et al*, 2005; Foa *et al*, 1991). A study reported by Foa *et al*, demonstrated a functional increase of immune cells from a basal level of 12% to 42% ($p<0.005$) after incubation with IL-2 (Foa *et al*, 1991). Another study in with B cell lymphomas including mantle cell lymphoma, demonstrated a significant rise in NK cell activity (up to 60% increase in cell mediated cytotoxicity) following IL-2 administration. This study reported the rise in activity correlated in part to the expansion of the NK cells numbers after IL-2 treatment (Gluck *et al*, 2004).

Although there was an increase in cell mediated cytotoxicity of IL-2 incubated effector cells from MCL patients, their cell mediated cytotoxicity activity was still lower than the healthy controls. This observation suggests impaired function of the immune cells from patients with MCL which may be due to the MCL. A study reported by Wang *et al,* similarly demonstrates reduced function of the effector cells from MCL patients. They demonstrated that cytotoxic T cells in MCL was

significantly inhibited by the inhibitory effects of the lymphoma related ligand B7-H1 (Wang *et al*, 2013a). This suggests that cancerous cells can evade the immune system by inhibiting the activation of the immune cells despite the presence of indigenous IL-2.

Another possible reason for the lower cytotoxicity function of effector cells from MCL patients could be that these effector cells possess a lower base line activity. Therefore despite exhibiting an increase in the cytotoxicity function from their base line function they are unable to mount a similar response to that of healthy effector cells. This could be a result of ineffective release of cytolytic granules as demonstrated in a study reported by Danielou-Lazareth et al (Danielou-Lazareth *et al*, 2013), where although they demonstrated an increase of the cytotoxicity after IL-2 therapy, the extent of increase was approximately 15-20% less than that of healthy controls, similar to the results presented here.

Interestingly the effector cells from female MCL patients did not exhibit a significant increase in the cell mediated cytotoxicity after incubation with IL-2 as seen with the effector cells from male MCL patients (section 4.4). Further evaluation of this result suggested that this trend could be accounted for by the greater number of NK cells and T cells quantified in the effector cells of the male patients compared to the female patients (figure 4.14 to 4.17). Interestingly the NK cells from male MCL patients exhibited a greater and more significant rise in the expression of the activating NK cell receptor NKG2D after incubation with IL-2 (figure 4.18 and 4.19). This could also support the greater cell mediated cytotoxicity observed in male MCL patients compared to the female MCL patients. As discussed previously, further analysis of the results revealed unexpectedly greater cytotoxicity function in one of the male MCL

patients compared to the rest. This male patient exhibited a clinically indolent MCL compared to the rest of the male MCL patients which may also account for this discrepancy. It is also possible that the cohort of female MCL patients investigated here presented with clinically aggressive MCL. This could have resulted in them possessing functionally impaired immune cells as previously discussed (Danielou-Lazareth *et al*, 2013). However, this trend observed in the female MCL patients needs to be further investigated to determine if it is consistent when greater numbers of MCL patients are investigated.

One of the main limitations of this study is the small number of participants. As mentioned above individual variations in cell function and numbers can significantly impact on the overall mean result. More participants would be valuable to evaluate some of the trends that have been observed. This will allow for individual variations and for more robust statistical analysis.

It would be interesting to measure the cytokine levels of IL-2 and TNF-α in the supernatant of the culture media by enzyme linked immunosorbent assay (Davies *et al*, 2001). This could provide further information to determine if effector cells from female MCL patients produce greater quantities of these cytokines compared to male MCL patients.

In addition identification of ligands in the serum of patients would be interesting to investigate. This may be possible using methods which involve flow cytometry. The level of ligands such as B7-H1, MICA and MICB would perhaps enable us to identify inhibitory effects of ligands from MCL cells on the function of the T and NK cells.

Although oestrone is the most abundant female hormone in postmenopausal women, there is still presence of other oestrogens. These oestrogens may have

different immunomodulatory effects on the tumour microenvironment which may help to explain the gender difference in the incidence of MCL. The cell mediated cytotoxicity experiments could be evaluated with other female hormones such as oestradiol or oestriol to determine if they have a significant effect. In addition the research group were in the process of obtaining patient serum for analysis of various cytokines and hormones and this was about to get ethical approval which would have been a good additional anaylsis to supplement these work.

The function of immune cells in the peripheral blood could be different to that of the cells present in the tumour microenvironment such as in an affected lymph node. Evaluation of immune cells after fine needle aspiration of affected lymph nodes would be interesting to analyse. Firstly to examine their cell mediated cytotoxicity compared to the immune cells from the peripheral blood and secondly to ascertain if there is a gender difference.

Clinically indolent MCL in now an increasingly recognised subgroup of MCL (Hsi & Martin, 2014). The patients included in this thesis exhibited both clinically aggressive and indolent MCL. In chapter 3 we presented data which exhibited greater number of immune cells in the circulation of patients with clinically indolent MCL. Unfortunately due to the limited numbers we could not compare the gender difference between the two clinical subtypes. Furthermore patients with clinically indolent MCL can be monitored for an extended period of time without the need to commence treatment and these patients do not exhibit any evidence of disease progression during this monitoring phase. This suggests that the immune system holds in ‘check’ the cancer according to the immunoediting principle (Gross *et al*, 2013). This is an area that would be interesting to study further and investigate whether there is a difference in

function of the immune cells between the two subtypes of MCL and at the same time between the genders in each of the subtypes of MCL. A national observational study in MCL that is going to collect clinical data and obtain blood and tissue samples for a research Biobank is due to be launched by the haematology research group here in Plymouth. This would provide a vital source of participants and material to enable further studies as described above.

4.12. Conclusion

Healthy controls exhibited greater cell mediated cytotoxicity compared to the MCL patients.

Incubation with IL-2 enhanced the cell mediated cytotoxicity of the effector cells in both healthy controls and male MCL patients. It also enhanced the expression of NK cell activating receptors; NKp44, NKp30 and NKG2D in all groups.

There was no significant effect of lenalidomide, oestrone and their combination on the cell mediated cytotoxicity of effector cells to result in a meaningful difference between the genders or when compared to untreated effector cells. In addition there was no increase in immune cell numbers or expression of the NK activating receptors when PBMCs were incubated with lenalidomide, oestrone or the combination of lenalidomide and oestrone.

These results do not exhibit a significant difference in the cell mediated cytotoxicity of effector cells from male and female MCL patients.

Chapter 5

Results

Enumeration of NK, T and T regulatory cells in the lymph node biopsies of patients with Mantle Cell Lymphoma and identification of oestrogen receptors α and β on MCL cells in the lymph node biopsies of patients and in Granta 519 cell line

5.0. Background

Lymph nodes are vital to the immune system. They comprise of most subtypes of immune cells which have greater presence in certain areas of the lymph nodes. The B cells are mainly located in the cortex and are arranged into follicles in the lymph nodes. The T and NK cells are located in the paracortex area and around the parafollicular and inter follicular areas (Willard-Mack, 2006)(Figure 41 and 42).

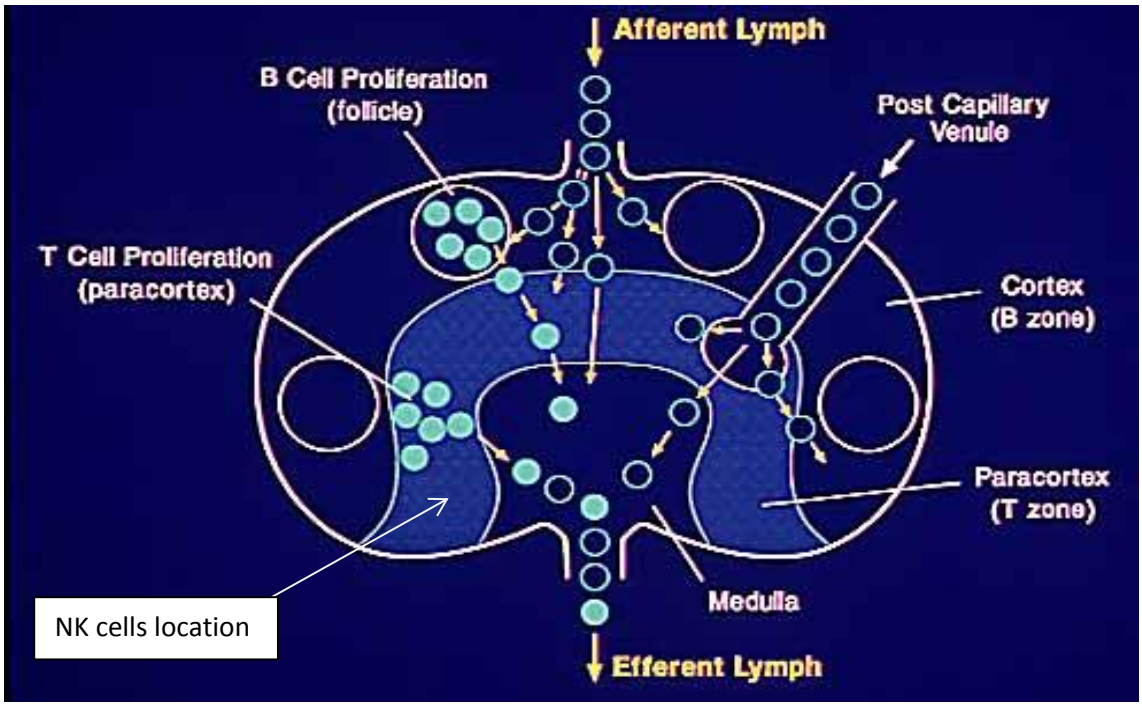


Figure 41: **Diagrammatic representation of a normal lymph node**
The cortex is mainly composed of B cells arranged in follicles which develop a central germinal centre when B cells are activated. The Paracortex mainly consists of T cells and other immune cells such as the NK cells and macrophages. The medulla contains medullary sinuses, plasma cells, memory B-cells, some T-cells, and histiocytes. *Adapted from pathpedia.com*

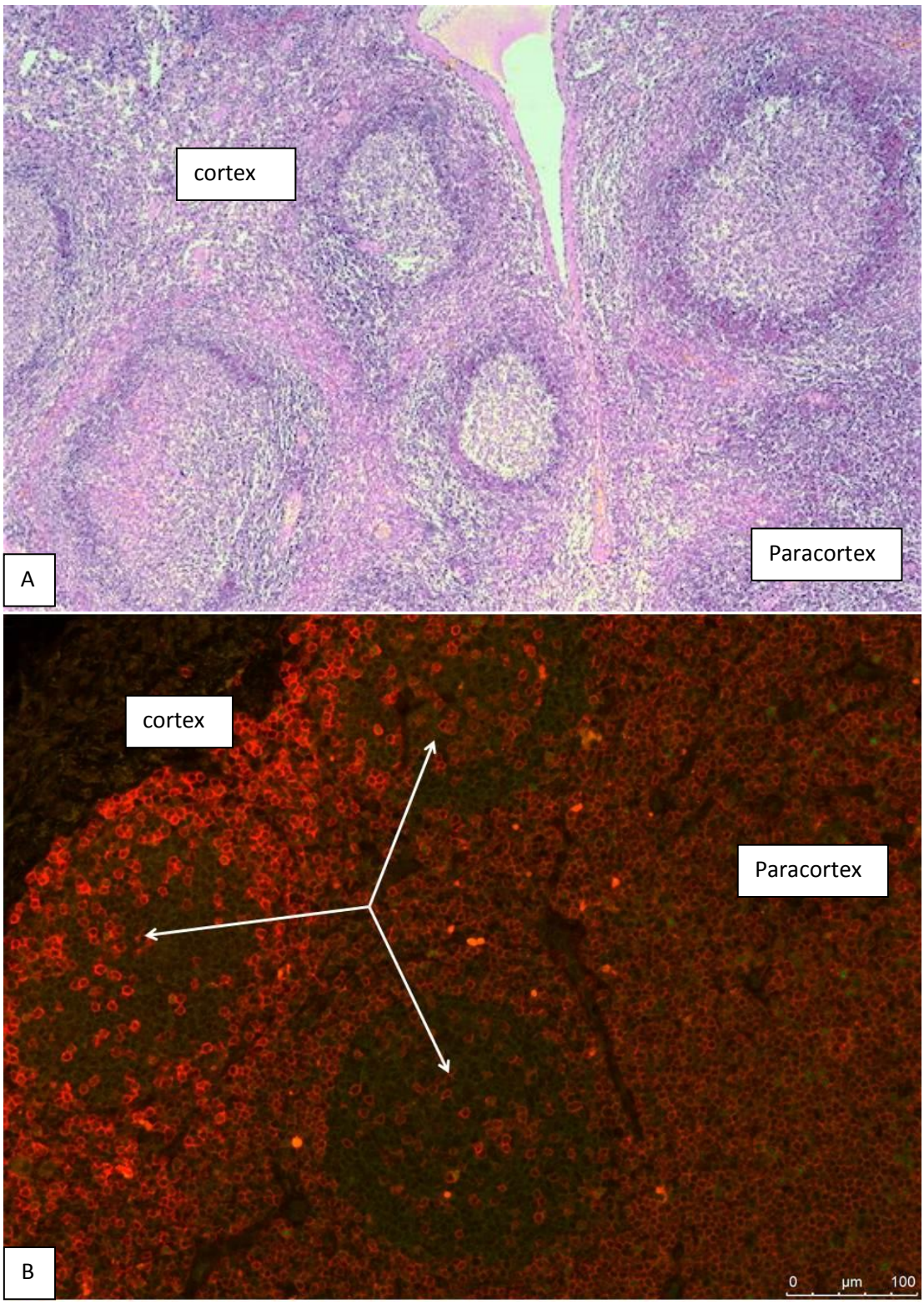


Figure 42 A & B: Lymph node structure from normal healthy control

Figure A; Haematoxylin and eoisin (H&E) stain of lymph node from a healthy control (magnification x 100). Demonstrating follicles in the cortex of the node and to the left of these is the paracortex which is rich in T cells.

Figure B exhibiting the follicles largely composed of B cells (white arrows), surrounded by para follicular T cells with $CD3^{+}$ (red) cytoplasmic staining by immunofluorescence (magnification x 100). Right of the follicles exhibit the paracortex area which have no follicles in that area.

Lymph nodes harbour approximately 40% of all the lymphocytes and there are a greater number of T and NK cells within the lymph nodes compared to the peripheral blood. These lymphocytes 'home' to the lymph nodes and subsequently migrate to their selected compartments within the lymph node. Movement of the immune cells into the lymph nodes is mediated by series of chemokine gradients, which facilitate the specific recruitment of select immune cells based on their chemokine receptor expression. B cells 'home' to the lymph node by the coordinated expression of integrins (leukocyte function-associated molecule – LFA) and adhesion molecules such as the vascular cell adhesion molecule (VCAM-1), as well as chemokines (CCL19 and CCL21) on the endothelial cells of the high endothelial venules (HEVs) (Von Andrian & Mempel, 2003). The expression of cell adhesion molecules such as P-selectin and E-selectin also assist in the movement of immune cells into the lymph node. Movement of the B cells between zones of the lymph nodes is achieved via the expression of CXC chemokine receptor 4 (CXCR4) or CXCR5 on the B cells and the increase of their respective ligands CXCL12 and CXCL13(Von Andrian & Mempel, 2003; Mueller & Germain, 2009) . The migration of other immune cells such as T cells, NK cells and macrophages into the lymph node is via a similar mechanisms of that described in the B cells above. These cells have homing receptors and in the T cells these are VCAM-1 integrin receptors, the NK cells express intercellular adhesion molecule (ICAM – 1,2) and VCAM-1, and macrophages have similar expression to that of NK cells (Delves *et al*, 2011). In addition naïve lymphocytes and dendritic cells, express the CCR7 chemokine receptor and are therefore also directed into the peripheral lymph nodes due to the HEVs expressing chemokines CCL19 and CCL21 on their luminal surface (Von Andrian & Mempel, 2003). Large number of dendritic cells

are also transported to the affected lymph node which in turn encourages immune cells to migrate into the lymph node as they release the chemokines to facilitate this (Girard *et al*, 2012).

The B cells proliferate within the follicles of the lymph node when activated by antigens presented to them. The T cells and T regulatory cells proliferate after they encounter their antigens presented by the dendritic cells. Upon stimulation the T cells secrete IL-2 which in turn stimulates the NK cells that are amongst the T cells in the lymph nodes. This interaction bridges the innate and adaptive immune systems and switches on the cytolytic activity of the NK cells and T cells to eliminate the diseased or cancerous cell (Ferlazzo *et al*, 2004; Fehniger *et al*, 2003; Willard-Mack, 2006). As discussed in the previous chapter 3, the results have demonstrated that females have lower number of T cells and NK subset $CD3^{-}CD56^{dim}$ cells in the peripheral blood and we hypothesised that females with MCL could possess a higher number of immune cells within the lymph node compared to the male patient with MCL. This may in part explain why prognosis is better in females as they may have more cytotoxic cells at the site of lymphoma and within the tumour microenvironment. This may enable the female MCL patients to mount a greater immune response due to the increased numbers to eliminate tumour cells.

5.0.a. Oestrogen can influence prevalence of cancer

An epidemiological study reported by Nelson *et al*, has demonstrated that in women who have used oral contraceptives had a significantly lower risk of intermediate or high grade lymphoma when compared to women who had never used these compounds. The increased oestrogen levels was thought to be the reason for this observation. Additionally, this study also demonstrated that

postmenopausal women were at a greater risk than premenopausal women of developing NHL (Nelson *et al*, 2001). Further epidemiological studies have corroborated these findings in lymphoma where a significant risk reduction in women treated with hormone replacement therapy or oral contraceptives was observed (Lee *et al*, 2008a).

These epidemiological studies have also been supported by observations reported by Yakimchuk *et al*, who demonstrated that T cell lymphomas grew faster in male versus female mice. The female protection was abolished by ovariectomy, implying that female hormones are involved in the protection. This was further confirmed when proliferation of these tumours was markedly reduced when mice were treated with an oestrogen receptor agonist (Yakimchuk *et al*, 2011a).

As previously discussed in chapter 1, section 1.3, the effects of oestrogen is via the two oestrogen receptors ERα and β. They have opposite effects with the ERα eliciting a proliferative and the ERβ exerting an apoptotic and anti-proliferative effect on certain cells when stimulated (Gruber *et al*, 2002). Recently the expression of ERs in immune cells have been described (Yakimchuk *et al*, 2013). $CD4^+$ T cells have been shown to exhibit a much higher level of ERα compared to $CD8^+$ T cells which exhibit both ERα and ERβ equally but at much lower expression levels (Phiel *et al*, 2005). However, there is limited data on the expression of ER on T regulatory cells. The ERβ is primarily expressed on the B cells lymphocytes (Shim *et al*, 2006). The expression of ERs, have been described on other immune cells. Monocytes and NK cells express more ERβ (Komi & Lassila, 2000) and macrophages tend to express more ERα (Mor *et al*, 2003). The effects of oestrogens on these cells

vary according to the more abundant ER on the cell type. Oestrogens have been known to affect the immune cells response (Straub, 2007). Oestrogens also affect the lymphopoiesis and also exert suppressive effects on T and B cells (Yakimchuk *et al*, 2013). Oestrogens have been shown to cause thymic involution negatively affecting both the CD4/CD8 populations (Straub, 2007). Furthermore the effects of oestrogen on the innate immune cells such as neutrophils, NK cells have been shown to increase their activity which probably relates to the difference found in the innate immune reactivity between males and females (Pennell *et al*, 2012). Other studies have demonstrated ERβ expression in Burkitt cell lines and PBMCs from CLL patients. These studies have observed activation of the ERβ receptor by an agonist, DPN had an inhibitory effect on the tumour survival (Yakimchuk *et al*, 2012a, 2011a). The expression of ERβ on lymphoid malignancies is not surprising as it has been demonstrated that there is high expression of ERβ on normal B cells and in particular, in the B cells in the mantle zone of the lymph node follicle (Phiel *et al*, 2005). Therefore the expression of ERβ on MCL was investigated.

The aims of this chapter were to:

1. Determine if the numbers and/ or distribution of the NK, T and T regulatory cells in the lymph node biopsies differ between male and female patients with MCL.

2. Investigate the expression of oestrogen receptor α and β on Granta 519 MCL line and MCL in lymph node biopsies from male and female patients.

Statistical analysis

The results of the enumeration of the NK cells ($CD3^-CD56^+$), T cells ($CD3^+$) and T regulatory cells ($CD3^+$ $FoxP3^+$) were analysed on GraphPad Prism version 6.0. The results were presented at the mean with +/- standard error of the mean (SEM). Non-parametric analysis was performed using Mann-Whitney U testing when comparing two groups, i.e. female MCL patients versus male MCL patients.

5.1. Distribution of MCL depicted by CD20 and Cyclin D1 in the lymph node

Using immunofluorescence microscopy six lymph node biopsies from 3 male and 3 female patients with MCL (patient characteristics shown in chapter 2) and one control were examined for the presence of MCL cells (defined as $CD20^+$ with nuclear $cyclinD1^+$); NK cells (defined as $CD3^-$ $CD56^+$); T cells (defined as $CD3^+$) and T regulatory cells (defined as $CD3^+$ with nuclear $Foxp3^+$).The results demonstrated a diffuse infiltration of mantle cell lymphoma cells ($CD20^+$ cyclin $D1^+$) in all of the 6 biopsies from the MCL patients. There was no normal lymph node architecture present in any of the biopsies examined (Figure 43).

Lymph node from healthy control *Lymph node from patient with MCL*

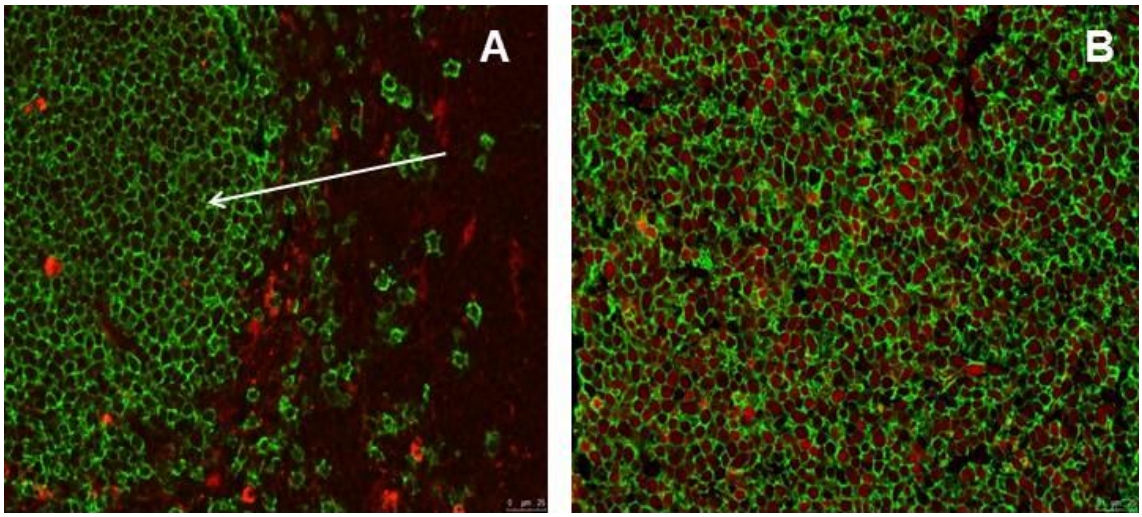


Figure 43: Panel A and B illustrating the distribution of B cells and MCL cells

A. Immunofluorescence demonstrating distribution of B cells in a normal lymph node from a healthy control. Arrow shows the CD20 (green) B-cells mainly distributed within the lymph node follicle. There was no nuclear staining present in this particular biopsy to exhibit cyclin D1 presence (magnification x200).

B. Immunofluorescence with CD20 (green) and cyclin D1 (red) on a lymph node biopsy section of a male patient with MCL. CD20 is represented by surface staining of the CD20 (green) and Cyclin D1 (red) is represented by nuclear staining. The majority of the cells were $CD20^+$ and Cyclin $D1^+$ (MCL cells) demonstrating the loss of normal architecture of the lymph node by diffuse infiltration of the MCL cells. This figure is representative of all 6 biopsies from MCL patients (magnification x200).

5.2. Distribution of NK, T and T regulatory cells in the lymph node biopsy of male and female patients with MCL

In the normal lymph node the distribution of NK, T and T regulatory cells was found to be largely around the follicle. There were a few T cells and T regulatory cells scattered within the B cell follicle as illustrated by Figure 44 below.

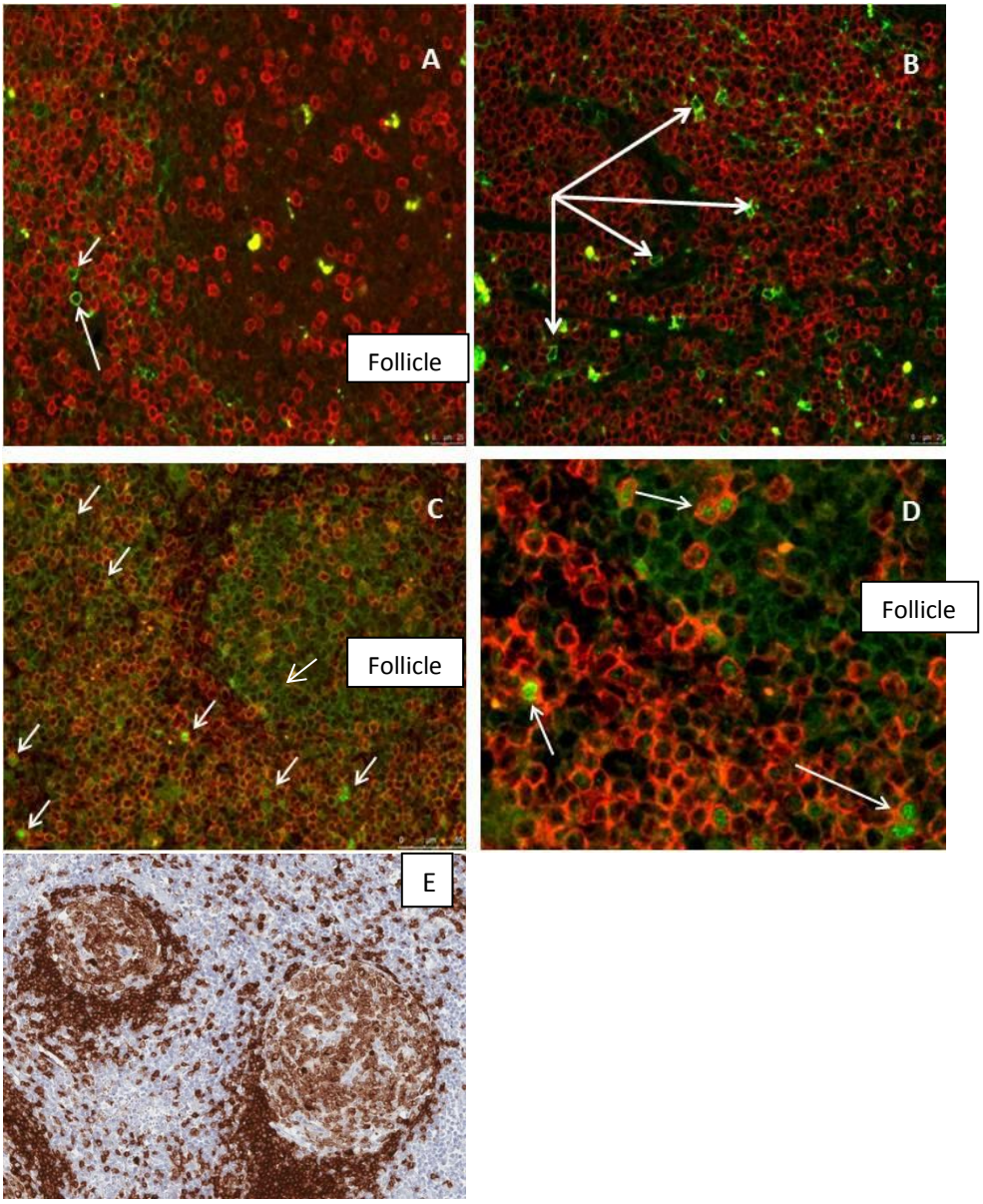


Figure 44: **Distribution of NK cells, T cells and T regulatory cells within the lymph node from a healthy control (Magnification x200 unless otherwise stated)**

Cells of interest are shown with the white arrow. **Panel A & B**: Representative images from sections stained with CD3 (red) and CD56 (green). **Panel C & D**: Representative images from sections stained with CD3 (red) and Foxp3 (nuclear green). **Panel E** is H&E stain depicting CD20 B cells distribution (brown)(magnification x100).

T cells ($CD3^+$) are shown by the red surface staining and are largely distributed around the follicle with some scattered cells within the follicle as shown in **panel A, B and D**.

NK cells ($CD3^-CD56^+$) are shown by the green surface staining amongst the T cells and scattered around the follicle as shown **by panel A and B (arrows).**

T regulatory cells ($CD3^+Foxp3^+$) are demonstrated by the red surface and green nuclear immunofluorescence staining for $CD3^+$ and $Foxp3^+$ respectively (arrows). These cells are amongst the T cells parafollicular regions and occasionally within the follicle as shown in **panel C and D. Panel D** is an enlargement (magnification x400) of a section of Panel C demonstrating the surface staining of $CD3^+$(red) and nuclear staining of $Foxp3^+$ (green) as highlighted by the arrows.

All 6 lymph nodes from MCL patients examined demonstrated a complete loss of normal lymph node architecture due to the diffuse infiltration by MCL cells as shown in the below figure 49. This resulted in the NK, T and T regulatory cells scattered through the lymph node with loss of their normal pattern of distribution (Figure 44)

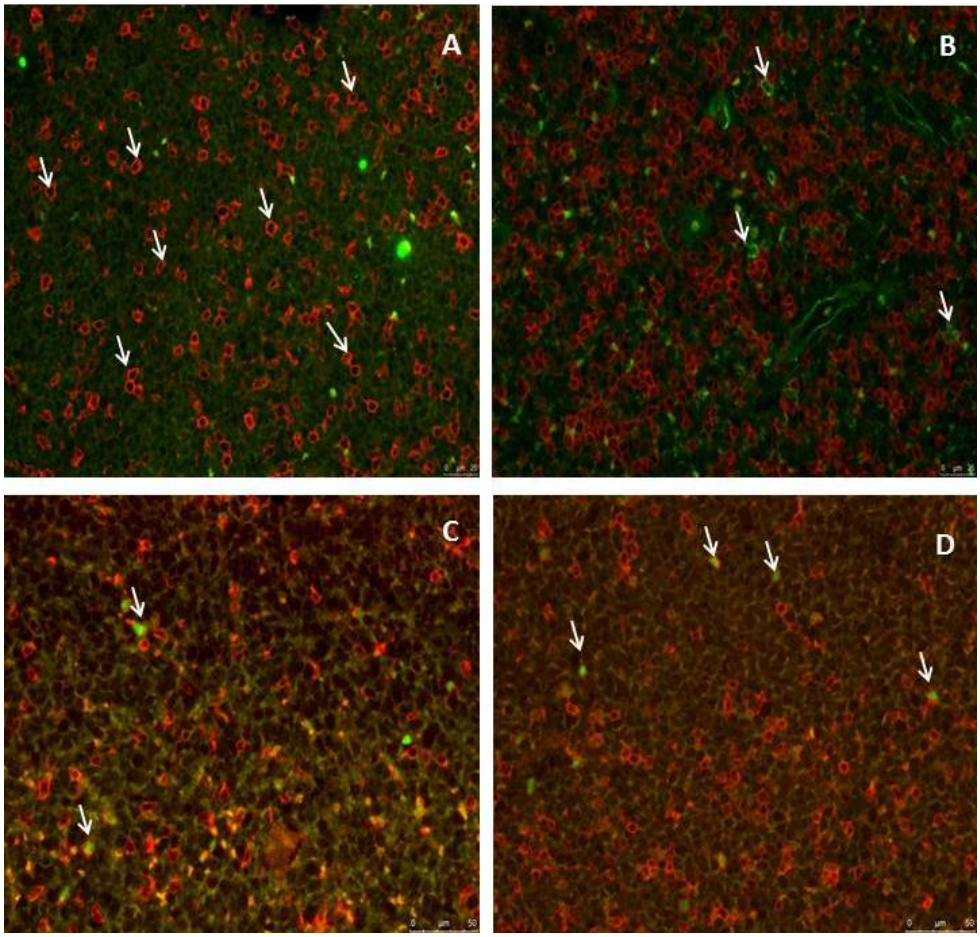


Figure 45: **Distribution of the NK, T and T regulatory cells within the lymph node from a patient with MCL (magnification x200)**
Cells of interest are shown with the white arrow. Panel A & B: Representative images from sections stained with CD3 (red) and CD56 (green). Panel C & D: Representative images from sections stained with CD3 (red) and Foxp3 (nuclear green).
T Cells ($CD3^+$) are demonstrated in red and are seen scattered throughout the lymph node specimen in **panel A (arrows).**
NK cells ($CD3^-CD56^+$) are shown in green amongst the T cells (red) and scattered throughout the lymph node **panel B (arrows).**
T regulatory cells ($CD3^+Foxp3^+$) are demonstrated by the red surface and green nuclear for $CD3^+$ and $Foxp3^+$ respectively. These cells are amongst the T cells ($CD3^+$ $Foxp3^-$) as shown in **panel C and D (arrows). Panel C and D** are lymph nodes biopsy from male and female MCL patient respectively. Due to the limited lymph node tissue available dual staining with CD20 was not done. However majority of the unstained cells were CD20 B cells surrounding the NK, T or T regulatory cells shown above and as demonstrated in figure 43 B.
All six biopsies had a similar presentation as represented here.

5.3. The enumeration of NK, T and T regulatory cells within the lymph node of male and female patients with MCL

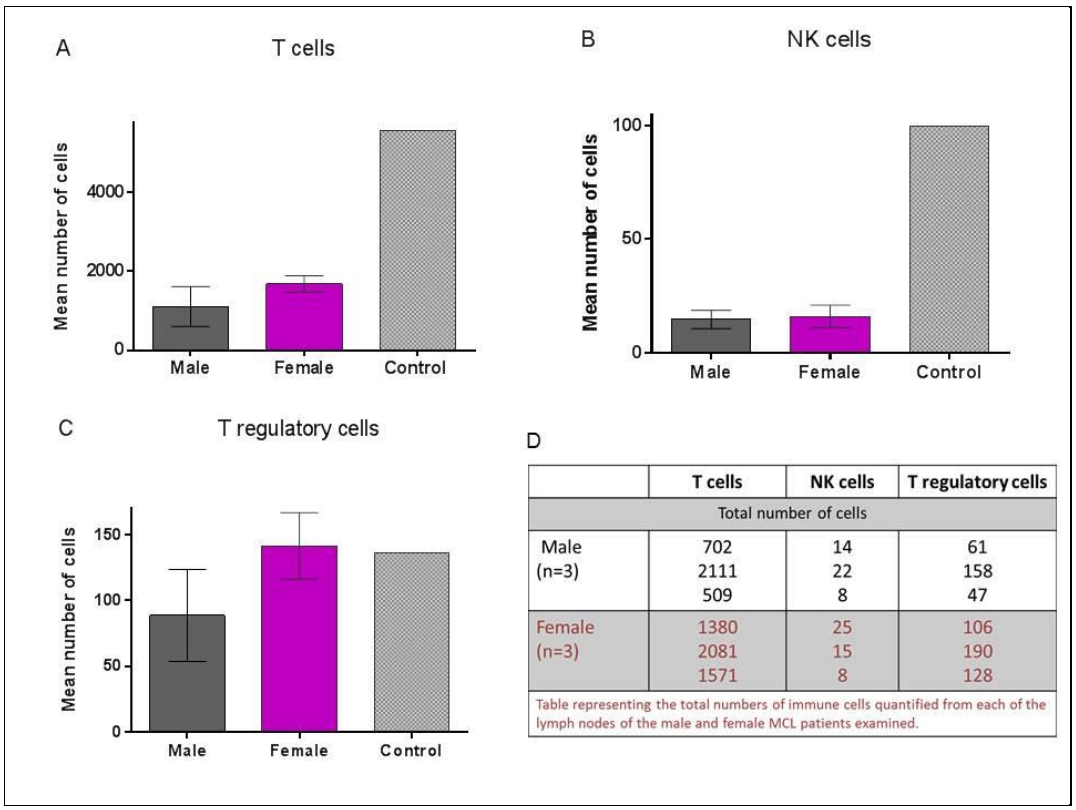
To determine if there was a difference in the numbers of immune cells in the lymph nodes of male and female MCL patients the quantification of the NK, T and T regulatory cells in male (n=3) and female patients (n=3) with MCL was performed on images taken from 13 randomly chosen sites from within each biopsy section at x200 magnification. Quantification was performed using the Leica LAS AF software.

The total number of NK and T cells were lower in both the male and female patient samples, when compared to the control lymph node (n=1; figure 50). However, there was a trend towards higher numbers of T cells in the female patients when compared to the male patients, although this did not reach statistical significance (Figure 46 A & D). The total number of T cells ($CD3^{+}$) from all 3 lymph node samples in female patients was 5032 cells compared with 3322 cells in the male patients in 13 random fields of view per specimen at a magnification of x100. One male patient was observed to have a greater number of T cells in the lymph node compared to the rest of the male patients (Figure 50 D). On further analysis this patient at diagnosis presented with a clinically aggressive behaving MCL. However, his history was suggestive of a more indolent course where by the patient noticed an enlarged lymph node in his axilla for greater than 12 months preceding his presentation at diagnosis. This may explain his higher T cell count as the history is suggestive of an indolent initial course.

Male patients exhibited a reduced number of T regulatory cells when compared to the control. Although the difference between males and females did not differ

significantly, the female patients had comparable numbers of T regulatory cells to the control subject (Figure 46C).

Overall, the results appeared to suggest that within this cohort of patients, there are a greater number of immune cells in the lymph nodes of female patients when compared to the male patients.



	T cells	NK cells	T regulatory cells
Total number of cells			
Male (n=3)	702 2111 509	14 22 8	61 158 47
Female (n=3)	1380 2081 1571	25 15 8	106 190 128

Table representing the total numbers of immune cells quantified from each of the lymph nodes of the male and female MCL patients examined.

Figure 46: Comparison of the immune cells subsets numbers in the lymph nodes

A. Represents the mean number of **T cells** in the lymph node of male (1107 cells +/- 505) and female (1677 cells +/- 209) MCL patients and control subject (5548 cells).Female exhibit a higher number of T cells in the lymph nodes compared to the male MCL patients (p=0.70).

B. Represents the mean number of **NK cells** in the lymph node of male (14 cells +/- 4.06) and female (16 cells +/- 4.93) MCL patients and control subject (100 cells) (p=0.80).

C. Represents the mean number of **T regulatory cell**s in the lymph node of male (88 cells +/- 34.9) and female (141 cells +/- 25.1) and control subject (136 cells). Females tend to exhibit higher numbers of T regulatory cells compared to the male MCL patients (p=0.40).

D. Table to present the individual total mean number of immune cells per lymph node between male and female MCL patients. Female patients exhibit a consistently higher T cells and T regulatory cell count compared to the male MCL patients.

(male (n=3), and female (n=3) MCL patients and a control (n=1)).

5.4. The presence of oestrogen receptor β on mantle cell lymphoma and Granta 519 cell line

Identification of ERα and ERβ was undertaken by immunohistochemistry method as described in chapter 2. The lymph node biopsies from 3 female and 3 male MCL patients undergoing routine diagnostic biopsies were utilised for analysis. The expression in the MCL cell line, Granta 519 target cells used through this study were also assessed. One male nodal biopsy was excluded due to a technical error during the staining process. Due to the limited lymph node material avialable additional slides could not be made.

A neuronal tissue from neuroblastoma was the positive control that was utilised to confirm that the ERα antibody was working under these IHC conditions (Cao *et al*, 2015)(Figure 47 A). Using the same conditions it was demonstrated that the ERα was absent from both the Granta 519 cells and mantle cell lymphoma cells in all the lymph node biopsies examined here (Figure 47 B & C respectively).

A histological section of an ovarian cancer biopsy was utilised to confirm that the ERβ antibody was working under these IHC conditions (Figure 48 A and B). In contrast to the ERα receptor expression, ERβ was expressed on both the Granta 519 (Figure 48 C &D) and on MCL cells (Figure 48 E, F) of all the lymph nodes examined. There was marginally less ERβ staining on the lymph node biopsy compared to the Granta 519 cells probably due to the other immune cells amongst the MCL cells not taking up the antibody.

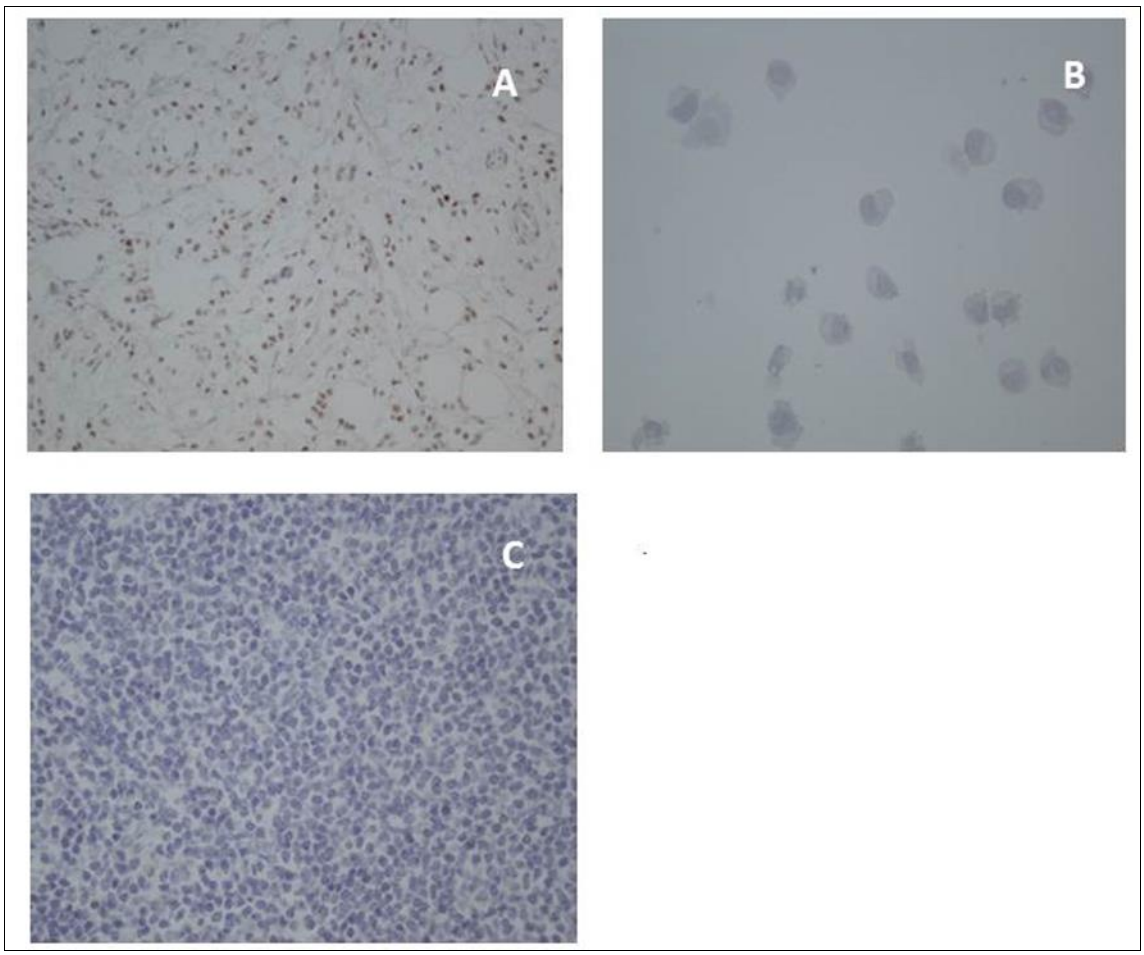


Figure 47: **ERα in Granta 519 cells and MCL cells in lymph node biopsy**

A. Neuronal tissue used as a positive control to demonstrate the ERα presence. ERα was demonstrated by the brown nuclear staining pattern on the control tissue (**panel A**) (x 200 magnification).

B. No positive staining with ERα antibody was present on Granta 519 cells. This represents the lack of ERα expression on Granta G519 cells (**panel B**) (x 200 magnification).

C. No positive staining with ERα was present on the MCL cells in the lymph node biopsies of 5 MCL patients (males n=2 and female n=3). This represents the lack of ERα receptor on the MCL cells present in these biopsies. (Panel C is representative of all five lymph node biopsies)(**panel C**) (x 200 magnification).

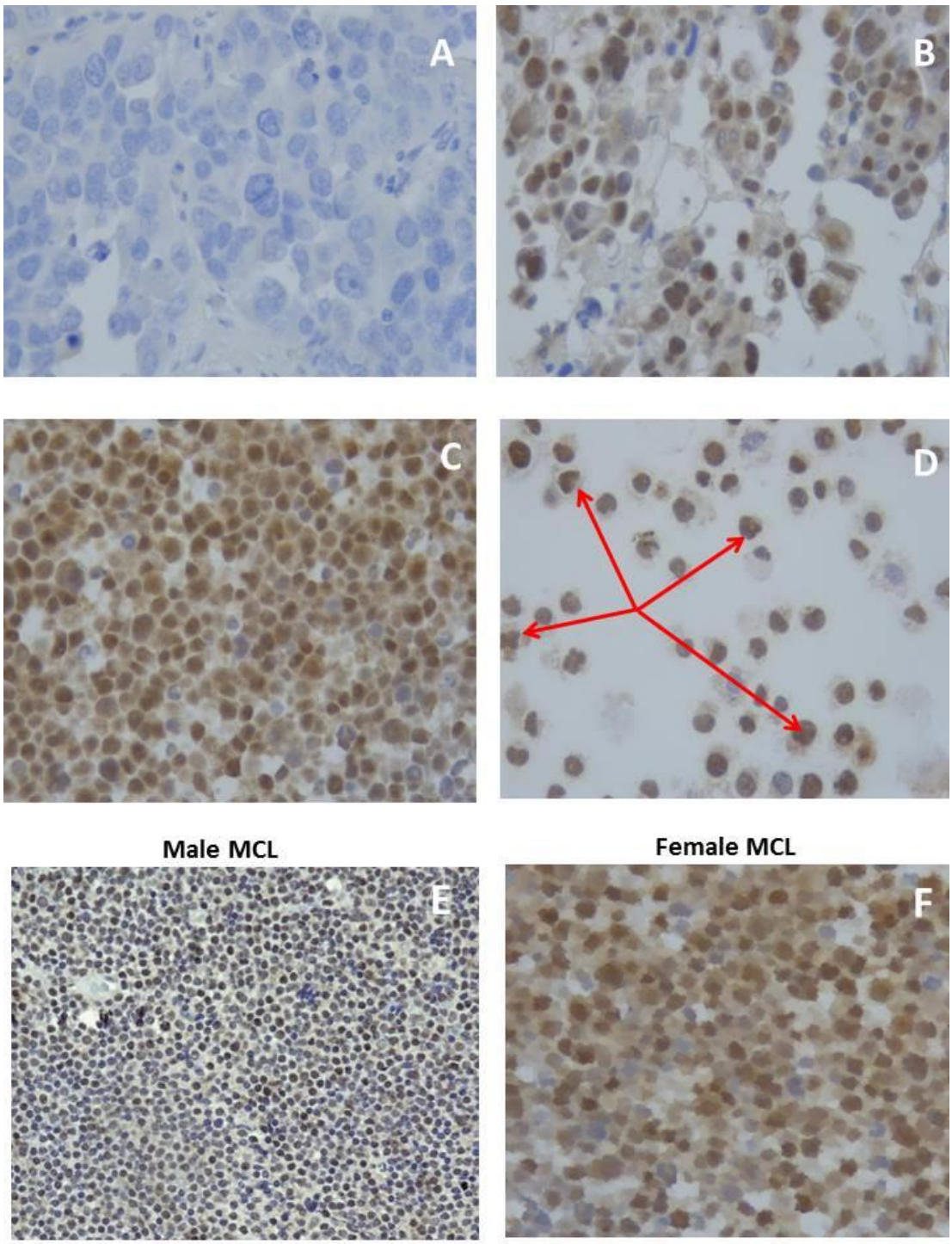


Figure 48: **ERβ expression on Granta G519 cells and MCL cells**

A. Negative control achieved by having no ERβ antibody present for staining on ovarian cancer biopsy (magnification x 200).
B. Positive control achieved by ERβ antibody on ovarian cancer biopsy depicted by the brown nuclear staining (magnification x 200).
C & D. Brown nuclear staining of the Granta 519 cells representing positive expression of nuclear ERβ expression on these cells (red arrows) (magnification x 200).
E & F. Lymph node sample from male and female patient with diffuse infiltration of MCL exhibiting positive staining for ERβ by exhibiting brown nuclear staining (**E.** magnification x 100 and **F.** magnification x 200), 5 MCL nodes (3 female and 2 males) all stained positive for ERβ with no significant difference in the distribution of ERβ.

Summary of results

1. There was a trend towards female MCL patients exhibiting greater number of immune cells compared to male MCL patient.

2. Oestrogen receptor β was predominately expressed on MCL cells in both male and female patient samples and Granta 519 cells.

5.5. Discussion

Lymph nodes of patients with MCL were examined to establish whether B-cell and T-cell populations within the lymph nodes differed in males and females. The patients evaluated were from a homogeneous group all presenting with clinically aggressive MCL (defined as patients presenting with B-symptoms and requiring immediate treatment without any period of observation). It was found that all the lymph node biopsies exhibited a diffuse pattern of infiltration by MCL characterised by a loss of the normal lymph node architecture. The normal architecture whereby the follicle of the lymph node typically consists of mainly B cells and the parafollicular and interfollicular areas consist of mainly T cells and NK cells was lost in the lymph nodes from these MCL patients. This loss of architecture correlates to the typical loss of normal architecture described in the histology of lymphomas (Feller & Diebold, 2004). Therefore it is not surprising that due to the diffuse infiltration of MCL within the lymph nodes that were examined, lower numbers of all the immune cells were present compared to the control lymph nodes The immune cells were ‘scattered’ amongst the MCL cells without any formal structure. In addition there is no specific description of immunoglobulin production by MCL. In other B cell malignancies like CLL, it has

been described reduced production of immunoglobulins, but in mantle cell lymphoma there has been no precise description of this however it has been described that although there is production of immunoglobulins in some cases their function is impaired (Kuppers, 2005; Gribben, 2010)

Interestingly the general trend was for the female MCL patients to exhibit a greater number of NK, T and T regulatory cells in the lymph nodes when compared to the male MCL patients. However, this did not achieve statistical significance which may be accounted for in part by the limited number of samples and patients examined.

T cells

The results presented in chapter 3 demonstrated that the female patients had significantly lower numbers of T cells in the peripheral circulation compared to the male patients. It was speculated that this observation could be due to higher number of T cells infiltrating the lymph node to kill MCL cells in females, who typically have better overall survival and response to therapy. Greater numbers of T cells were indeed observed in the lymph nodes of female patients when compared to male patients, although this did not reach significance. These trends observed here suggest that in context of the tumour microenvironment female MCL patients may elicit a greater immune response compared to male MCL patients due to the greater number of relevant potentially tumour targeting immune cells present within their lymph nodes (Gajewski *et al*, 2013). Other studies support this suggestion. For example, a recent study by Nygren *et al*, demonstrated higher numbers of $CD4^+$ & $CD8^+$ T cells in the lymph node of patients with MCL was associated with a more favourable outcome and patients with an indolent behaving MCL were observed to have a greater number of T

cells in their lymph nodes. Notably, the results presented here agree with their observation that female patients exhibited significantly greater number of T cells ($CD3^+$) in the lymph nodes compared to the male patients (p=0.009)(Nygren *et al*, 2014). As has already been discussed this disease is more prevalent in men when compared to women and those women who do get MCL, frequently have a more indolent form of the disease. An alternative explanation for the higher number of immune cells within the lymph node could be that the female patients have a reduced tumour burden compared to male patients and so preserve more of the normal architecture of the lymph node. In turn, this may be related to the females when compared to males being able to mount a better immune response and eliminate more cancerous cells within the immediate vicinity of the immune cells stopping MCL spread. However, the lymph nodes examined here from 3 male and female MCL patients did not have a distinct difference in the pattern of infiltration of MCL cells on any of the nodes form either gender. They all exhibited a diffuse infiltration of MCL cells with a complete loss of architecture. It would require a larger number of patients and lymph node biopsy to achieve any formal conclusions. Additionally, it could also be secondary to females being able to eliminate MCL cells more effectively in the periphery again preventing the spread of MCL. Additionally, the effects of oestrogen in the female environment may further inhibit the cancerous cells directly via the ER or indirectly through stimulating the immune cells. The exact mechanism by which females may be able to eliminate cancerous cells more effectively is not yet identified but one of the methods to investigate whether females have more effective immune cells in the lymph nodes or peripheral blood is to isolate the immune cells from each compartment by B cell depletion to leave predominately T and NK cells and then compare the difference in cell mediated cytotoxicity of

the immune cells from the lymph nodes and peripheral blood and from male and female MCL patients. Extraction of cells from the lymph nodes can be achieved by either fine needle aspiration of cells or excision of the whole node followed by extraction of cells by needle aspiration (Lotze & Thomson, 2005). Once the cells have been extracted similar techniques as described above can be utilized to assess the activity of these immune cells. The control lymph nodes studied in Nygren et al, also demonstrated higher number of T cells compared to the patient lymph nodes. This and the trend observed in the results may explain the reason why female MCL patients tend to have a more favourable outcome.

It would be interesting to further analyse the T cell subsets; $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ cytotoxic T cells in the nodal biopsies to correlate the results reported in the above mentioned study.

T Regulatory cells

The overall trend demonstrated female patients exhibiting a greater number of T regulatory cells in the lymph nodes compared to the male patients, although this did not achieve significance. This was also observed in the quantification of T regulatory cells in the peripheral blood of the female MCL patients compared to the male patients as discussed in chapter 3. Although there have been no specific studies to date examining the gender difference with T regulatory cells in MCL, other studies have demonstrated that greater number of T regulatory cells, such as in diffuse large B cell lymphoma correlate to a better overall survival. In follicular lymphoma higher tumour infiltrating T regulatory cells have shown to be associated with better survival (Lee *et al*, 2008b; Carreras *et al*, 2006). This has also been observed in classical Hodgkin's lymphoma ($p=0.004$)(Tzankov *et al*, 2008). Generally, T regs suppress the immune

response as discussed in chapter 1. However they are able to directly suppress B cells by reducing the expression on AID (activation induced cytidine deaminase) and inhibiting their Ig class switch recombination and immunoglobulin production (Lim *et al*, 2005a). Additionally T regulatory cells may suppress MCL cell proliferation via the up-regulation of perforin and granzymes to induce B cell cytotoxicity and cell death (Zhao *et al*, 2006). It is possible that the gender difference observed in MCL is due to the female MCL patients exhibiting greater number of T regulatory cells which may have a direct inhibitory effect on the MCL cells. However further larger studies are needed with clinical correlation to establish the significance and function of T regulatory cells in MCL as it is generally accepted that T regulatory cells appear to have an inhibitory effect on T cells (Josefowicz *et al*, 2012).

NK cells

The number of NK cells in the lymph nodes examined, were comparable between the male and female MCL patients. The quantity of NK cells in the patients was lower when compared to the control subject. This could be accounted for by a reduced migration of the NK cells into the affected lymph node. Migration of NK cells into lymph nodes is facilitated by endogenous IFN-γ and dendritic cells expressing chemokine ligands CXCR3 (Wendel *et al*, 2008; Watt *et al*, 2008). As shown above the normal lymph node architecture in patients with MCL examined here is completely distorted and largely comprises of MCL cells. Therefore there are fewer endogenous NK cells and possibly dendritic cells, resulting in less IFN-γ and chemokine expression within the lymph nodes to facilitate migration of NK cells into the affected lymph nodes. Immunomodulatory drugs such as lenalidomide have been shown to increase

NK cell migration into the tumour microenvironment by modifying dendritic cell responses and altering their chemokine expression profile, supporting the above hypothesis that reduced migration of NK cells may be due to the presence of MCL (Reddy *et al*, 2008).

It was not feasible to analyse by immunohistochemistry the subsets of NK cells; $CD56^{bright}$ and $CD56^{dim}$ due to the lack of suitable antibodies and the inability to reliably quantify expression levels. In order to determine whether the affected nodes from males and females exhibit greater numbers of $CD56^{dim}$ NK cells (direct cytotoxicity activity) compared to the $CD56^{bright}$ NK cell (cytokine mediated cytotoxicity) a fine needle aspiration from the affected lymph nodes would be required. The cells could then be analysed by flow cytometry to examine the specific subsets.

5.6. Oestrogen Receptor β present on MCL

As discussed earlier, studies have suggested that there is probable influence of oestrogen on the prevalence of NHL in males and females (Nelson *et al*, 2001). To understand whether there is a direct effect of the female hormones on the lymphoma cells, the two ER α and β were examined to establish their expression on the MCL cells in the lymph node biopsies of male and female MCL patients. Normal B cells express the ERβ and a study by Shim et al, has demonstrated that B cells in the mantle zone of the follicles in the lymph nodes express primarily ERβ (Shim *et al*, 2006). As this is the site from which MCL cells are derived it is conceivable that they may also express the ERβ and therefore possible that female oestrogens could have a greater direct influence on the cell characteristics through these receptors.

The results confirmed the presence of ERβ on Granta 519 cells and mantle cell lymphoma cells in the biopsies from both the male and female MCL patients. The expression of ERβ on the MCL cells from both the male and female patients had similar pattern of distribution and expression with no significant difference. This is consistent with other studies of MCL and B cell lymphoproliferative disorders (Yakimchuk *et al*, 2014, 2012b). A study as demonstrated that activation of ERβ with the oestrogen receptor agonist diarylpropionitrile (DPN) and KB9520 significantly inhibit the proliferation of engrafted Burkitt lymphoma cells by at least 50% ($p<0.001$) in NOD SCID mice (Yakimchuk *et al*, 2011a). Other Studies involving mouse models have also demonstrated tumour regression in grafted T cell lymphoma with DPN, an ERβ agonist (Yakimchuk *et al*, 2011b). However, it was not clear whether the hormone sensitivity reflected in the tumours was due to a direct effect on the MCL cells or an effect on the tumour microenvironment. An attempt has been made to determine whether the tumour microenvironment comprising of immune, endothelial and stromal cells which provide regulatory signals to lymphoma cells are influenced by the ERβ receptor they possess or whether the ERβ on the lymphoma cells have a direct effect. ERβ-/- mice, treated with ERβ agonist DPN, when compared with wild type mice grafted with ERβ positive murine EG7 T cell lymphoma cells showed a significant reduction of the EG7 T cell lymphoma in both groups of mice but there was no significant difference in the response of the lymphoma growth between the two groups suggesting that the ERβ agonists did not have much of an effect on the tumour microenvironment, but more on the tumour itself. However, further investigations are needed to determine whether ERβ expression in cells of the microenvironment have a role in lymphomas other than murine EG7 T cell

lymphoma tumours, as immunocompromised mice deficient in ERβ expression are not widely available (Shim *et al*, 2003; Yakimchuk *et al*, 2014). Once this is established the subsequent investigations that could be considered include investigation into the molecular pathways affected and the plethora of cellular genes involved in cell proliferation / inhibition after activation of ERβ.

Additionally, the ERβ has two splice variants; denoted as receptor β1 and β2 (Yakimchuk *et al*, 2013, 2012a; Shaaban *et al*, 2008).The oestrogen receptor β2 splice variant is capable of antagonising the proliferative effect of ERα and it can heterodimerize with oestrogen receptor β1 and enhance the anti-proliferative activity (Pettersson & Gustafsson, 2001). It has also been described to inhibit ERα thus altering the cellular signalling and survival of the cells (Zhao *et al*, 2007). Therefore, it would be interesting to further identify the oestrogen receptor subtype variants present on the MCL cells and determine whether expression of particular splice variants would impact on the direct action of the oestrogen receptor on MCL, or if males and females differentially express the splice variants.

The results presented certainly provide a good platform from which to further investigate the role of oestrogens on MCL. It would however be important to confirm these findings in other biopsies from a larger cohort of MCL patients. A further confirmation of the expression of ERs with flow cytometry and either western blot or PCR may be warranted. Testing other MCL cell lines such as REC1 and JVM2 cell lines for the oestrogen receptors would be valuable to confirm whether there is any uniformity on the receptor expression in different cell lines. Pursuing experiments with oestrogen receptor β agonists and antagonists would be interesting to determine if ERβ could be a therapeutic target in the treatment of MCL.

The presence of ERβ on MCL cells provides some insight into the gender difference observed in prevalence and response to treatment in MCL. Further studies are needed to understand the role of ER in MCL. These findings correlate to others studies and provide an area to investigate further.

5.7. Conclusion

There was a trend towards greater number of immune cells in affected lymph nodes of the female MCL patients compared to male MCL patients in this cohort. This could result in female exerting a greater immune response within the tumour microenvironment therefore being able to exhibit better responses to therapy. In relation to the immunoediting concept females may be able to keep the MCL in check more effectively than males. One of the limitations of this study was that it didn't assess the distribution of the other immune cells that form the microenvironment milieu such as the macrophages, dendritic cells, NK-T cells, NK subsets. Further studies with a greater inclusion of other immune cells would be needed to fully appreciate any gender bias which is related to the immune cells of the MCL microenvironment.

Additionally, MCL and Granta 519 cells both express nuclear ERβ. The ERα was not expressed on either of the Granta 519 cells or the MCL cells in the lymph node biopsies examined. Although it is not clear whether ER have a definite role in MCL, however it provides another potential area to investigate. As described in chapter 4, exposure of G519 cells to oestrone did not result in any direct cell death and there was no increase in cell mediated cytotoxicity when PBMCs where exposed to oestrone as well. As mentioned earlier, there is no data on the exact concentration or action of oestrogens in the microenvironment of MCL and it may be that the doses used in the experiments

were inappropriate or that other oestrogens such as oestradiol could have more of an influence.

These observations may aid to explain the gender difference observed in MCL. However, larger and broader studies are needed to further investigate these findings.

Chapter 6

Final Discussion

The aim of this project was to examine the gender difference displayed in MCL. MCL is predominant in males with a 3:1 ratio (males: females). A recent study has demonstrated female MCL patients have a better response rate to lenalidomide, an immunomodulatory drug, than male MCL patients (Eve *et al*, 2012). In addition, epidemiological studies have shown women exhibit a lower incidence of B and T cell lymphomas and have a better prognosis (Nelson *et al*, 2001; Römer & Pfreundschuh, 2014; Cook *et al*, 2011). Lymphomas are not generally perceived to be hormone controlled but a study reported by Nelson et al suggested oestrogen may have a protective effect against lymphoma (Nelson *et al*, 2001). It is generally recognised that women mount a stronger immune response compared to men and the concept of immunosurveillance and immunoediting is a well described mechanism by which the immune system plays a vital role in eliminating cancer (Swann & Smyth, 2007).

6.0. Higher number of T cells in male MCL patients compared to female MCL patients

A major finding in chapter 3 was the significantly greater number of T cells ($CD3^+$) in the peripheral blood of male MCL patients compared to female MCL patients. The T cells subsets; T helper ($CD3^+CD4^+$) and cytotoxic T cells (CD3+CD8+) also exhibited a trend towards greater numbers in the peripheral blood of male MCL patients compared to female MCL patients.

This finding was contrary to the hypothesis that female patients with MCL would exhibit greater numbers of T cells in the peripheral circulation so as to mount a greater immune response against cancerous cells compared to the male patients. A possible reason could be that greater number of T cells remain within or move into affected lymph nodes. In order to examine this concept enumeration of T cells (and NK and T regulatory cells) in the lymph nodes of six

MCL patients (3 male and 3 female) all presenting with clinically aggressive disease was undertaken (chapter 5). The results exhibited a notable trend towards greater number of T cells in lymph nodes of female MCL patients compared to the male MCL patients. This observation of T cells numbers in the nodal biopsy of MCL patients agreed with a recent study reported by Nygren et al who demonstrated significantly higher number of T cells in nodal biopsies of female MCL patients when compared to male MCL patients (P=0.0009). This study also demonstrated that patients with greater number of T cells in the nodal biopsy exhibited a better OS (p=0.043) (Nygren *et al*, 2014). Together these studies suggest that female MCL patients may have a better clinical outcome due to a higher number of T cells observed in their lymph node biopsies.

It is possible that the total function of the T cells present in the tumour microenvironment may also differ between the genders. This could be either due to the difference in the number of T cells and / or their inherent function within the tumour microenvironment. There have been no studies to date in MCL to evaluate the gender difference with regards to the T cell function within the tumour microenvironment. However a study examining the function of the T cells in follicular lymphoma demonstrated reduced overall functionality of T cells infiltrating the lymphoma. These T cells were found to have a much lower signalling response to IL-4, IL-10 and lL-21 cytokine stimulation indicated by low phosphorylation of STAT5 and STAT6 measured by phospho-flow cytometry (mean fold change of phosphorylation of STAT6 of 0.79 in T cells of FL patients compared to 2.18 in healthy controls). They also evaluated T cells from MCL patients and although these T cells infiltrating the MCL tumour were found not to have as significantly reduced function as those in follicular lymphoma, there

was a trend towards lower function compared to the normal T cells from healthy controls (Myklebust *et al*, 2013). Therefore it would be interesting to evaluate in additional larger studies the functional difference of T cells from the MCL microenvironment between the genders. The T cells could be isolated from fresh excised lymph nodes or by fine needle aspiration of the affected nodes. Their functional activity could then be compared with the T cells from the peripheral blood and between the genders.

6.1. NK cells

As with T cell numbers, there was a trend towards the male MCL patients exhibiting higher number of NK cells (chapter 3) in the peripheral blood compared to the female patients and the NK cell ($CD3^{-}CD56^{+}$) numbers were greater in the nodal biopsy of female MCL patients compared to the male MCL patients (chapter 5). The migration of NK cells between the affected lymph node and peripheral blood is similar to the T cells. It is possible that there is greater migration of NK cells into the affected lymph node in response to the cancerous cells resulting in a lower number of NK cells in the peripheral blood of female MCL patients. A study reported by Eve, *et al*, exhibited an initial reduction of NK cell numbers in the peripheral circulation of patients with MCL when treated with lenalidomide with restoration of these numbers after a period of time. They suggested that this observation could be a result of NK cells migrating into the affected lymph node which may have been facilitated by lenalidomide's immunomodulatory effect on the tumour microenvironment (Eve *et al*, 2012).

A further notable finding was the trend towards greater numbers of the NK cell subset: $CD3^{-}CD56^{dim}$ in the peripheral blood of male MCL patients compared to

the female MCL patients (chapter 3). This trend could be due to more clinically indolent MCL patients in the male cohort compared to the female cohort. It was not possible to compare the clinically indolent MCL patients between the genders due to the limited numbers, however the difference between immune cell numbers between male and female MCL patients with clinically indolent and aggressive disease would be an interesting area to further evaluate. A national study coordinated by University of Plymouth, Clinical Trials Unit and Derriford Hospital is currently in place to collect clinical data and biological material including PBMCs and nodal biopsies on clinically indolent MCL patient. This would be a valuable potential source to enable this further study.

The NK subset $CD3^{-}CD56^{dim}$ has been described to have direct cell cytolysis without the need for prior activation (Vivier *et al*, 2012a). Evaluation of their NK activating receptors (NKp44, NKp46, NKp30 and NKG2D) demonstrated no significant difference between the male and female MCL patients or the healthy controls (chapter 3). It has been recognised that the expression of these activating receptors correlates to the activation of NK cells (Sivori *et al*, 1999).Therefore these results suggest that these NK cells did not exhibit a difference in their activation status between the male and female MCL patients in the peripheral blood. However, this does not rule out differences of activation status between the genders within the tumour microenvironment and this warrants further investigation. A further limitation of this study is that the inhibitory receptors of NK cells were not evaluated. It is possible that higher expression of inhibitory receptors may be present in MCL patients. Evaluation of the inhibitory receptors by flow cytometry on NK cells would be interesting to examine both in the blood and in biopsies.

Within the affected lymph nodes representing the tumour microenvironment, it was not possible to evaluate and quantify the subsets of NK cells; $CD3^{-}CD56^{dim}$ and $CD3^{-}CD56^{bright}$ due to the lack of suitable antibody that could identify them accurately with IHC technique. One of the methods suggested earlier was to obtain cells via a fine needle aspirate of the affected node and using flow cytometry identify and quantify the NK cell subsets. It would be interesting to examine whether the female MCL patients would exhibit greater number of NK subsets; $CD3^{-}CD56^{dim}$ and $CD3^{-}CD56^{bright}$ within the affected lymph node compared to the male MCL patients. At the same time NK activation receptors on these cells could be analysed by flow cytometry to determine whether they have a greater expression compared to the peripheral blood NK cells and whether there is a difference between the genders. This may provide some insight into whether female MCL patients have more activated NK cells in the microenvironment compared to the male MCL patients.

6.2. T regulatory Cells

There was a trend towards female MCL patients exhibiting greater number of T regulatory cells in the peripheral blood and lymph node biopsies compared to male MCL patients (chapters 3 and 5). There are no studies to date that have examined the difference of T regulatory cells between the genders in MCL. However, studies with other B cell lymphomas have shown greater number of T regulatory cells to be associated with a better outcome (Eve *et al*, 2012; Kelley & Parker, 2010; Tzankov *et al*, 2008).

T regulatory cells suppress their target cells by releasing inhibitory cytokines such as IL-10, TGF-β (inhibits T cell proliferation by disturbing IL-2) and IL-35 (suppresses T cell proliferation and stimulates T regulatory cells to proliferate

and produce IL-10). Interleukin-10 induces suppression of both T helper and cytotoxic cells and down regulates the expression of co-stimulatory molecules, adhesion molecules and MHC-class II on APCs (Steinbrink *et al*, 2002). In some studies of B cell malignancies better survival has been shown when there is a high number of tumour-infiltrating T regulatory cells (Tzankov *et al*, 2008; Lee *et al*, 2008b). It may be that T regulatory cells have a double role in lymphoma. T regulatory cells on one hand could suppress the immune T cells therefore facilitating tumour growth but on the other hand they could act to suppress or even kill the tumour cells (Lindqvist & Loskog, 2012; Lindqvist *et al*, 2011; Mittal *et al*, 2008). A study by Mittal *et al*, demonstrated autologous malignant B cells to promote T regulatory cell proliferation. They incubated malignant B cells from lymphoma patients with $CD25^-$ T cells. This resulted in a strong induction of the $CD25^-$ T cells to develop into T regulatory cells ($CD4^+CD25^+FOXP3^+$) and subsequently expansion of T regulatory cell numbers was observed. This study suggested that the presence of the lymphoma stimulated the expansion of the T regulatory cells perhaps in order to inhibit the T helper cells allowing the lymphoma to proliferate (Mittal *et al*, 2008).

In contrast a study reported by Lim *et al*, demonstrated direct suppression of B cells by incubating T regulatory cells with B cells in an otherwise T cell free condition. This study demonstrated reduction of IgG and IgA production from B cells as a result of the action of T regulatory cells. However it was highlighted in this study that direct T regulatory contact was needed in order to suppress the activity of the B cells (Lim *et al*, 2005b). Another study reported by Likuni *et al*, demonstrated direct cytotoxicity action of T regulatory cells on B cells. They co-cultured T regulatory cells with autologous B cells from patients with systemic

lupus erythematosus and demonstrated B cell death by flow cytometry using Annexin V and 7-AAD staining (Likuni *et al*, 2009).

If the role of T regulatory cells in MCL lymphoma involves direct killing of lymphoma cells then it could be that the higher number of T regulatory cells in the peripheral blood and lymph node biopsies of female MCL patients compared to the male patients could in part explain why females do better than males. Further investigations are needed to clarify the role and function of T regulatory cells in patients with MCL and to determine whether they influence the gender bias.

6.3. Healthy controls exhibit greater cell mediated cytotoxicity compared to MCL patients

After the enumeration of the immune cells in the peripheral blood, the functional activity of these cells was evaluated by examining the cell mediated cytotoxicity. The major finding was an overall reduced cytotoxic activity of the effector cells from MCL patients when compared to the healthy controls on Granta 519 target cells (chapter 4). One of the possible reasons for this could be the presence of inhibitory ligands produced by cancerous cells to evade or inhibit the immune response. A study reported by Wang et al, demonstrated the presence of B7-H1 ligand which is a member of the B7 co-inhibitory / co-stimulatory ligand in MCL. The presence of B7-H1 inhibited T cell proliferation, impaired antigen specific T cell response and inhibited T cell mediated cytolysis. Blocking the action of the B7-H1 ligand allowed for the restoration of T cell function. (Wang *et al*, 2013a). Other studies of B cell malignancies have also demonstrated ligands to activate PD-1 inhibitory receptors on T cells. For example in a study of CLL the tumour cells exhibited a marked increase in PD-1 ligand (PG-L1) compared to non-malignant B cells from healthy controls (Ramsay *et al*, 2012). Activation of the

PD-1 (anti-programmed cell death 1) results in immunosuppressive signals that inhibit the kinases that are involved in the activation of T cells through phosphatase SHP2. These ligands also have been known to inhibit NK cells (Paulos & June, 2010; Nunes *et al*, 2012; Ramsay, 2013).

Inhibitory ligands on T cells such as the B7-H1 ligand mentioned above could be potential future targets for therapy. If other ligands such as Anti-cytotoxic T lymphocyte associated antigen (CTLA4 / CD152) could be identified in MCL, then these could potentially be inhibited by an antagonistic CTLA4-targeted antibody (ipilimumab) which has shown promising clinical responses in NHL (Ansell *et al*, 2009). Anti-PD-1 monoclonal antibody such as the CT011 antibody is currently being explored in haematological malignancies with some promising results. In addition, antibodies and small molecule inhibitors targeting numerous immune molecules which include inhibitory ligands B7-H3, B7-H4 (VTCN1), T-cell immunoglobulin and mucin domain containing molecule 3 (TIM-3) are now entering clinical trials (Ramsay, 2013).

6.4. No significant difference was observed in the cell mediated cytotoxicity of effector cells from male and female MCL patients

In examining the cell mediated cytotoxicity of effector cells from male and female MCL patients, there was no significant difference observed. In addition to the potential reasons for this observation described in chapter 4, this result could also be due to the effect of the lymphoma itself. Once the lymphoma has managed to escape the immune surveillance any gender difference could be diminished by inhibitory ligands produced by the tumours. One of the limitations of this study is the small number of participants. To fully evaluate and validate these results, studies with larger number of participants are needed. There are plans to continue recruitment of MCL patients by collaborating with multiple

sites which would result in a larger number of participants. Also the imminent launch of the MCL observational and Biobank national study coordinated by the University of Plymouth, Clinical Trials Unit will allow further participants for these studies to be recruited.

6.5. Incubation with IL-2 results in increased cell mediated cytotoxicity

Expectedly, incubation of PBMCs with IL-2 demonstrated a significant increase in cell mediated cytotoxicity in all groups except female MCL patients, where the rise in cell mediated cytotoxicity was not as pronounced as in the other groups (chapter 4). The increase in cell mediated cytotoxicity was probably in part due to the activation of NK cells by IL-2 which was supported by the results that demonstrated increased expression of NK cell activating receptors (NKp44, NKp30, NKG2) (chapter 4).

The increase in cell mediated cytotoxicity observed largely in male MCL patients correlates to a study by Carter *et al*, which demonstrated murine T cells inhibited by PD-1 ligands overcame their inhibition by exogenous IL-2. This study suggested that the inhibition of T cell activity was due to the low production of indigenous IL-2 which was thought to be secondary to the inhibitory pathways activated by the PD-1 ligand (Carter *et al*, 2002). However, there is no clear explanation as to why the female MCL patients exhibited a lower cell mediated cytotoxicity compared to the male MCL patients when exogenous IL-2 was added. It is possible that the lower number of immune cells present in the PBMCs of female MCL patients may have resulted in the lower cell mediated cytotoxicity observed in these patients (chapter 4). In addition there was a greater number of T regulatory cells in female MCL patients samples which in turn could have had an inhibitory effect on the cytotoxic T cells if the IL-2 effect on the T regulatory cells would have caused a

disproportionate expansion. The results observed in MCL patients could also be due to the clinical subtype of MCL present in the patients. The effector cells from male MCL patients that were examined were from patients with clinically less aggressive MCL compared to the female MCL patients. Although these 'less aggressive' patients did not meet the strict definition of clinically indolent disease (MCL in asymptomatic patients who did not require immediate therapy and were observed for more than one year) they did have an initial period of monitoring without requiring immediate therapy.

Either way this observation is worth following up with further larger studies. If it can be demonstrated that the activity of the immune cells is enhanced with exogenous IL-2 in all MCL patients, this could be exploited as an additional therapy for selected patients. A phase one trial in which relapsed / refractory NHL patients received weekly rituximab (an anti-CD20 antibody) therapy with IL-2 infusions demonstrated an overall 29.4% clinical response. They also demonstrated an expansion of the NK cell numbers and an increase in antibody dependent cellular cytotoxicity (ADCC) activity which was sustained with IL-2 administrations (Gluck *et al*, 2004).

6.6. Lenalidomide and Oestrone did not alter cell mediated cytotoxicity in male and female MCL patients

Addition of lenalidomide or oestrone or a combination of lenalidomide and oestrone to effector cells did not result in a significant difference in cell mediated cytotoxicity between the genders. These results were in contrast to other studies in which there was an increase in cell mediated cytotoxicity and number of effector cells after incubation with lenalidomide (Davies *et al*, 2001; Richardson *et al*, 2010; Chanan-Khan *et al*, 2006). One of the main reasons for these observations is probably the shorter incubation time (24 hours) and

differing doses when compared to other studies. Additional plans to extend the incubation time and repeat the cell mediated cytotoxicity experiments should be considered to further investigate these observations.

6.7. Oestrogen receptor β is present on MCL

The presence of ERβ on MCL as described in chapter 5 correlates to other studies exploring oestrogen receptors on lymphoid malignancies and MCL (Yakimchuk *et al*, 2014, 2011b, 2013). ERβ is thought to have an anti-proliferative and apoptotic effect when activated (Yakimchuk *et al*, 2011a; Nilsson & Koehler, 2005), but as yet the exact role of ERβ in MCL has not been fully defined or described. However, a recent study has provided some useful insights. Using the ERβ agonist DPN, they demonstrated that it could inhibit Granta 519 cell proliferation when engrafted in mouse models. They also showed inhibition of vascularisation of the lymphoma with a reduction of vascular endothelial growth factors (VEGFs) and LYVE-1; a lymphatic endothelial marker (Yakimchuk *et al*, 2014).

To further explore whether ERβ has a role in influencing the gender difference observed in MCL, it would be interesting to investigate the effects of the other female hormones such as oestradiol and oestriol on the proliferation of MCL. This could be achieved by incubating Granta 519 cells over a period of time with varying concentrations of these hormones and utilising CSFE to monitor Granta 519 cell proliferation by flow cytometry. Subsequently mouse models could be utilised to further explore this. Male mice engrafted with Granta 519 cells could be injected with the female hormones (oestrone, oestradiol and oestrial) at varying concentrations and time periods to determine whether they have an effect on the lymphoma growth. Klanavo et al, described their method of

engrafting established MCL cell lines like G519 cells in immunodeficeint non obese diabetic mouse models with success (Klanova *et al*, 2014). This model with ERβ$^{-/-}$ mouse models could be utilised. However it has to be acknowledged that the availability of this type of mouse model would be difficult to source, but collaboration with other research groups could potentially aid this research which have successfully engrafted MCL cells onto mouse models (Iyengar *et al*, 2015).

As mentioned before ERβ could prove to be a potential therapeutic target. Further studies with ERβ agonist such as DPN and KB9520 and antagonists like PHTPP (4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol) are needed to establish the role of ERβ receptor on MCL (Yakimchuk *et al*, 2013).

ERβ presents an area that could be explored further. A study with mouse models has demonstrated suppression of engrafted Burkitts lymphoma (which express ERβ) in non-obese diabetic SCID mice by treatment with ERβ agonist DPN compared to vehicle control treated mice (Yakimchuk *et al*, 2013). In the context of clinical applications further studies are needed but initial data from studies already carried out is promising.

6.8 Further Studies

As already addressed earlier, acquisition of more samples from MCL patients would be critical to pursue this work further. It would also be interesting to group the patients in two groups, with one group having clinically indolent MCL and the other clinically aggressive MCL, followed by comparing the gender difference in the respective clinically indolent and aggressive groups. This would be possible by the recently approved National Cancer Research Network

(NCRN) UK mantle cell lymphoma observational trial where access to diagnostic materials from MCL patients would be available for further research.

This study was limited in the number of immune cells that were investigated. Other immune cells such as NKT cells, macrophages and T cells such as T_{FH} cells could also be analysed between the genders to develop a more complete description of the immune cells between the MCL genders. It would also be beneficial to explore the difference of the NK inhibitor receptors (KIR, KIRL) between the groups as it was done for the activating receptors NKp46, NKp44, NKp30 and NKG2D. Unfortunately due to the limited diagnostic and patient material it was not possible to pursue the isolation of NK and various T cells from the peripheral blood. With further patient material this is an area that would benefit from further investigation as PBMCs do not produce accurate data on individual immune cells.

This study used peripheral blood for analysis, as this was easy to access and minimally invasive for the patients and control. However, cells from the microenvironment i.e aspiration form the affected nodes would be interesting to analyse. Plans for this were in place, but due to the rarity of this disease and more so the availability of patients with safely accessible lymph nodes to aspirate was difficult. Nonetheless it is an area which would be interesting to research. Furthermore at the same time cytokines and chemokines (IL-2, IFN-γ, IL-6, IL-13, CXCR5, CXCR7) from patient serum should also be analysed from MCL patients and between genders to ascertain and gender difference.

One of the key investigations needed here is to simulate the microenvironment by creating an in-vitro microenvironment followed by analyses of the MCL cell growth when exposed to various oestrogen, ERβ agonists such as DPN, and

treatments with lenalidomide. This would provide a closer data to the in-vivo environment of MCL. This may be achieved by either growing MCL cell lines or primary MCL on pre-stablished confluent of stromal or fibroblast cells. Once established further research may be done by introducing various cells like NK cells, T cells or treatments as described above to analyse the effects (Medina *et al*, 2012). This would also provide the basis of the first research paper with this work.

Having shown that Granta 519 cells and MCL cells from patients exhibited ERβ receptor additional experiments with ERβ agonists and antagonist would be interesting as described above. This would provide initial data on whether ERβ is functional on these cells and subsequently down-stream proteins as discussed earlier in chapter 1 could be analysed to demonstrate further their functionality.

6.9. Final Conclusion

This study has demonstrated a significantly higher number of circulating T cells in male patients with MCL. The significance of this finding needs to be investigated further through larger studies.

Although there was no significant difference in the cell mediated cytotoxicity demonstrated by effector cells between male and female MCL patients, the overall cell mediated cytotoxicity was impaired in patients with MCL compared to healthy control subjects, suggesting MCL could have an overall suppressive effect on the immune system.

The difference in prevalence of MCL between the genders could be related to the presence of ERβ on the MCL cells. However, this is only a suggestion and is an area which would particularly benefit in further research as it may provide another pathway to target when treating MCL.

An area of increasing interest is the epigenetics in MCL and cancer. This is an area that could be explored further at the same time as the other suggested studies. Epigenetics are heritable changes in gene expression that are not due to an alteration in the DNA sequence (Esteller, 2008). The most common epigenetic marker is DNA methylation (Esteller, 2008). Studies which have included genome-wide screening have addressed the potential inactivation of specific tumour suppressor genes by methylation in MCL (Leshchenko *et al*, 2010). A recent study identified five frequently methylated genes in primary MCL cells (SOX9, HOXA9, AHR, NR2F2 and ROBO1). This study demonstrated methylated SOX9 or HOXA9 was associated with a high proliferation index Ki-67 ($p<0.002$) and a shorter overall survival. This suggests that epigenetics changes may play an important role in the pathogenesis of these tumours (Enjuanes *et al*, 2011). This concept can be used to identify whether different epigenetics between male and female MCL patients occur. With the development of rapid sequencing technology this can be done rapidly and more economically (Morozova & Marra, 2008). If a difference is found this may provide further insight into the gender difference in MCL and potential targets for new tailored therapies.

In conclusion the greater prevalence of MCL in males compared to females remains to be explained. The processes involved in the development and elimination of lymphoma are extremely complex and are continued to be studied

and understood. However this thesis provides an insight into a few of the possible mechanisms that may be involved.

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Publications during the MD study period

(copies of the publications are in the pouch at the back)

International Journal of Hematologic Oncology. 2014; 3(1): 31-40
Management perspective for mantle cell lymphoma
Shah N, Rule S.

Abstract:
Mantle cell lymphoma (MCL) is genetically characterized by the t(11;14) (q13;q32) translocation resulting in the overexpression of cyclin D1. It generally has an aggressive clinical course with a poor prognosis. However, there is now a recognized subgroup with clinically indolent MCL. Management of MCL can be challenging. Early recognition of young and fit patients for potential intensive therapy and autologous stem cell transplant is important. Combination of rituximab with high-dose cytarabine should be used in upfront therapy for appropriate patients. In elderly and/or less fit patients, chemoimmunotherapy may be considered. Novel agents offer promising potential in the management of MCL and are likely to change the way it is treated.

Expert Rev Hematol. 2014 Oct; 7(5):521-31
Ibrutinib for the treatment of mantle cell lymphoma.
Shah N, Hutchinson C, Rule S.

Abstract:
Mantle cell lymphoma (MCL) is a rare and aggressive form of non-Hodgkin's lymphoma. It can follow a heterogeneous clinical course but generally patients relapse early after standard immunochemotherapy regimens and develop resistance to subsequent therapies. For younger patients, intensive approaches followed by autologous stem cell transplantation offer excellent long-term disease control but with the possible exception of an allogenic stem cell transplant, MCL is an incurable condition. As MCL principally affects older individuals, the majority of patients are not candidates for such intensive approaches. Ibrutinib is an orally active, Bruton's tyrosine kinase inhibitor. It inhibits signaling pathways downstream of Bruton's tyrosine kinase that appear critical for the proliferation and survival of MCL. As a single agent it has shown extremely promising activity in relapsed and refractory MCL patients with an excellent side-effect profile. The exact role for ibrutinib in the treatment of MCL is yet to be established; however, it is likely to fundamentally change the way we treat this disease.

Leuk Lymphoma. 2014 Nov 20:1-12 epub ahead of print.

How applicable is fludarabine, cyclophosphamide and rituximab to the elderly?

Shah N, Tam C, Seymour JF, Rule S.

Abstract:

The combination of fludarabine, cyclophosphamide and rituximab (FCR) has been widely used in the treatment of lymphoproliferative disorders, and is now considered as the standard first-line therapy for fit, young patients with chronic lymphocytic leukemia (CLL). However, in routine practice, the majority of patients with lymphoproliferative disease are over the age of 70 years, and most studies involving FCR have included younger, "fitter" patients, on average in their sixth decade of life. It is not easy to extrapolate the results of these studies to routine practice. In general, the impression is that FCR is less well tolerated in more elderly patients (> 70 years) with good organ function. However, there is a relative paucity of evidence to support this. In this review we aimed to critically examine evidence of the efficacy and toxicity of FCR in the elderly patient

Presentations at Scientific meetings during the MD study period

Significant gender differences in the number and distribution of T cells in patients with Mantle Cell Lymphoma.

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Mantle cell lymphoma (MCL) is a rare B cell neoplasm that accounts for approximately 4-8% of non-Hodgkin's lymphomas (NHLs). The median age at diagnosis is 65 years with a male to female predominance of 3:1. T cells are central to the adaptive immune system which is part of the initial defence against cancerous cells. It is perceived that females mount a greater immune response compared to males. As part of our investigations to account for the gender difference in MCL, we examined the T cells (CD3+) and their subsets; Helper (CD3+CD4+) and cytotoxic T cells (CD3+CD8+).

Peripheral blood of twelve untreated MCL patients (6 male and 6 female patients) was evaluated using flow cytometry to enumerate the T cells and the subsets.

The male patients exhibited a significantly greater total mean number of T cells compared to the female patients (2488 cells/µl vs.1245 cells/µl, respectively)(P=0.04) and both the helper T cells (1229 vs 737 cells/µl, male vs females, respectively) and cytotoxic T cells (789 vs 375 cells/µl, males vs. females, respectively) were observed to be higher in the male patients ($p>0.05$). We subsequently analysed T cells numbers in the lymph node biopsies from a clinically homogeneous group of 3 male and 3 female treatment naive MCL patients. Conversely, the female MCL patients had a greater total number of T cells in the lymph nodes compared to male patients (5032 cells vs 3322 cells, respectively)($p>0.05$).

These findings suggest that female patients may be able to mount a greater immune response compared to male patients by exhibiting greater number of T cells within the tumour microenvironment. Further larger studies are needed to correlate these findings with clinical parameters.

Accepted as a poster presentation at the British Society of Haematology Annual Scientific Meeting April 2015.

Single centre outcome of mantle cell lymphoma over the past decade

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Mantle Cell lymphoma (MCL) accounts for 6–8% of all non-Hodgkin lymphomas. It has a heterogeneous clinical course with poor prognosis and a challenge to treat. We retrospectively analysed 51 MCL patients diagnosed and managed in Plymouth between 2000 and 2010. 45/51 patients were included. Patient characteristics, therapies and biological markers were evaluated with regards to overall survival rate (OS). Up-front treatment regimens were categorised into treatment within clinical trials or physician's choice. The median age was 68 years (range: 40–88) and 58% (26/45) of the patients were older than 65 years (45% and 95% OS at 3 years in patients >65 and <65 years, respectively, p = 0.0001). There was a 3:1 predominance of male to female patients. The median follow up as 5 years and the median OS was 59 months. The OS at 2, 4 and 6 years were 71%, 57% and 38%, respectively. 15% (7/45) patients had initial treatment deferred resulting in a 57% OS at 3 years. 38% (17/45) of the patients were treated up-front within a trial. The OS at 3 years was 65% vs. 60%, p = 0.603, in patients treated within and outside clinical trials, respectively. There was no difference in the 3 year OS between patients with a monocyte count greater or less than 0.5 9 10*9/l (p = 0.873). However, there is a significant difference in OS at 3 years when correlated to MCL Prognostic Index (MIPI). 100% and 45% OS with low plus intermediate and high MIPI, respectively (p = 0.004). In 2004, a specialist service for MCL was developed. The 3 year OS for patients diagnosed and managed after this date showed a significant improvement (82% vs. 47%, p = 0.04). This unselected group of patients demonstrate significant improvement in OS over the past decade correlating to advances made in available treatment and notably development of specialised services for MCL.

Poster presentation at British Society of Haematology Annual Scientific Meeting April 2014.
Br J Haematol. 2014 April; 165 (s1)

A phase I study of the oral Btk inhibitor ONO-4059 in patients with relapsed/refractory B-cell lymphoma

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Bruton's tyrosine kinase (Btk) is a critical kinase in B-cell receptor (BCR) signal transduction and recent studies support that targeting Btk is effective in the treatment of B-cell malignancies. ONO-4059 is a selective, highly potent oral Btk inhibitor that has demonstrated anti-tumour activity in pre-clinical models. This Phase I study was initiated to determine the safety, tolerability, dose-limiting toxicity (DLT), pharmacokinetics and pharmacodynamics of ONO-4059 given as monotherapy (QD) to patients with relapsed/refractory NHL. We present the safety and efficacy data on 14 evaluable patients (mantle cell lymphoma n = 7, follicular lymphoma n = 3, plasmablastic-DLBCL n = 1, ABC-DLBCL n = 1, small lymphocytic lymphoma n = 1 and Waldenstrom's macroglobulinaemia n = 1), with a median age 64 years (range 48–88). Patients received a median of 3 prior therapies (range 2–8) and 93% (13/14) having prior exposure to a rituximab- containing regimen. Patients received ONO-4059 at doses ranging from 20–160 mg (cohorts 1–4). ONO-4059 was well tolerated, with no DLTs. A total of 18 ONO-4059-related adverse events were reported in 6 out of 14 patients; 15 adverse events were CTCAE-V4.0 G1 (n = 10) and G2 (n = 5). Three G3 haematological toxicities were reported in 2 patients; thrombocytopenia(x2) and anemia. No ONO-4059-related G4 events, or SAEs or infections were reported. Responses have occurred at doses of 40, 80 and 160 mg, with a best overall response rate of 42%; with 5 PR, 4 SD, 2 PD (both
MCL). Three MCL patients have achieved PR resulting in a best ORR of 50%. Almost all patients experienced clinically meaningful rapid reductions in lymphadenopathy observed within the first cycle. 10/14 patients are still on study with a median progression-free survival
of 93.5 days [Range 8–268]. In conclusion, ONO-4059 is a selective, highly potent oral Btk inhibitor showing a favourable safety profile with promising efficacy in this difficult-to-treat patient population. The study is currently ongoing with additional dose escalation cohorts.

Br J Haematol. 2014 April; 165 (s1)

Poster presentation at British Society of Haematology Annual Scientific Meeting April 2014.

The bruton's tyrosine kinase (BTK) inhibitor ono-4059: promising single agent activity in patients with relapsed and refractory NHL

F Morschhauser, L Terriou, M Dyer, C Hutchinson, S Rule, N Shah, G Salles, L Karlin, C Fegan, J Bagshawe, G Cartron, H Honda, A Nishimura, J Sharpe, T Ohno, T Yoshizawa, T Yasuhiro, J Birkett

Haematologica 2014: 99 (s1) - Poster presentation at European Hematology Annual meeting 2014.

A Phase I Study Of The Oral Btk Inhibitor ONO-4059 In Patients With Relapsed/Refractory B-Cell Lymphoma

Simon Rule, Nimish Shah, Gilles Andre Salles, Lionel Karlin, Franck Morschhauser, Louis Terriou, Martin JS Dyer, Claire Hutchinson, Chris Fegan, Guillaume Cartron, Tomasz Knurowski, James G Wright, Andrew J Saunders, Hideyuki Honda, Andrew Mazur, Toshio Yoshizawa, Kazuhito Kawabata, Joseph TP Birkett

Blood 2013; 122 (21):4397

Poster presentation at the American Society of Heamatology Annual meeting 2013

A Phase I Study Of The Oral Btk Inhibitor ONO-4059 In Patients With Relapsed/Refractory and High Risk Chronic Lymphocytic Leukaemia (CLL).

Gilles Andre Salles, Lionel Karlin, Simon Rule, Nimish Shah, Franck Morschhauser, Louis Terriou, Martin JS Dyer, Claire Hutchinson, Chris Fegan, Guillaume Cartron, Tomasz Knurowski, Andrew J Saunders, James G Wright, Hideyuki Honda, Bsc, Andrew Mazur, Toshio Yoshizawa, Kazuhito Kawabata, Joseph TP Birkett

Blood 2013 122 (21):676

Presentation at the American Society of Heamatology annual meeting 2013

Natural killer cells and their clinical correlation in mantle cell lymphoma (MCL)

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Natural Killer cells (NK) are essential for innate immunity with evidence that they can recognise and control some tumour growth. We have shown that in MCL, the activity of the immunomodulatory

drug lenalidomide, correlates with NK cell numbers. As such we undertook an observational study monitoring NK cells in unsolicited patients with MCL, over a 5 year period and retrospectively correlated this with clinical findings. Using flow cytometry (BDmultikit CD3 CD16+ CD56+), peripheral blood was used to identify NK count (normal range 160–660 cells/lL). Patients with more than two sequential NK counts were selected. A total of 19 patients (median age 65 years, 1:5-female to male ratio) where evaluated. Eighty-nine percent (17/19) of patients had relapsed MCL (1–4 prior regimens, including rituximab, ofatumamab, thalidomide, lenalidomide and one patient had an allogenic stem cell transplant). Eleven percent (2/19) had clinically indolent MCL were treatment naive with normal baseline NK counts. Forty-two percent (8/19) of the patients had a significant drop in the NK cell count

(<100 cells/lL) at relapse, of which 50% had no therapy prior to relapse for at least 2 months (1–8) and 50% relapsed while on treatment with lenalidomide or Ibrutinib. Patient who responded clinically

to therapy after relapse showed a rise in NK cell numbers. Twenty-five percent of the patients with reduced NK cells had refractory disease. Twenty-one percent (4/19) of the patients presented

with massive splenomegaly, of which 75% had NK counts greater than 1000cells/lL. These patients showed a normalisation of NK cell counts in response to treatment. This unique data correlating NK counts with clinical findings suggests that in a significant number of patients, relapse can be heralded

by a fall in NK cell number. This opens the possibility of using this as a simple means to monitor disease activity.

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