TRANSCRIPTIONAL ANALYSIS OF MANTLE CELL LYMPHOMA

A GLOBAL SEARCH FOR CELLULAR ORIGIN AND THERAPEUTIC TARGETS

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Papers I-IV

Original papers

This thesis is based upon the following papers, which are referred to in the text by their Roman numeral (I-IV).

I Ek, S., Högerkorp, C-M., Dictor, M., Ehinger, M., Borrebaeck, C.A.K (2002)
"Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared to subpopulations of normal human B cells" Cancer Res 62(15): 4398-405.

II Ek, S., Björck, E., Högerkorp, C-M., Walsh, S.H., Thorselius, M., Rosenquist, R., Nordenskjöld, M., Porwit-MacDonald, A., Borrebaeck, C.A.K
"Mantle cell lymphomas overexpress CCL4, CCL5 and 4-1BB ligand and show evidence of an activated B cell origin" (submitted 2004)

III Ek, S., Björck, E., Nordenskjöld, M., Porwit-MacDonald, A., Borrebaeck, C.A.K
"Increased expression of Ki-67 in mantle cell lymphoma is associated with de-regulation of several cell cycle regulatory components, as identified by global gene expression analysis" Haematologica. In press, 2004.

IV Ek, S., Ortega, E., Borrebaeck, C.A.K
"Transcriptional profiling and assessment of cell lines as *in vitro* models for mantle cell lymphoma" (submitted 2004)

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Abbreviations

AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
Apaf-1	apoptotic protease activating factor-1
ATM	ataxia telangiectasia mutated
BCR	B cell receptor
CCL	centrocytic lymphoma
CDK4	cyclin-dependent kinase 4
cDNA	complementary DNA
CHOP	cyclophosphamide, doxorubicin, vincristine and prednisone
CLL	chronic lymphocytic leukaemia/lymphoma
CNS	central nervous system
DLBCL	diffuse large B cell lymphoma
EBV	Epstein-Barr virus
FDC	follicular dendritic cell
FL	follicular lymphoma
FM	follicular mantle
GC	germinal centre
GS	growth signal
HEV	high endothelial venule
HIV	human immunodeficiency virus
hTERT	human Telomerase Reverse Transcriptases
Ig	immunoglobulin
MCL	mantle cell lymphoma
MMP	matrix metalloproteinase
MZ	marginal zone
NHL	non-Hodgkin's lymphoma
pRb	retinoblastoma protein
RAG-1	recombination activating gene 1
V_H	immunoglobulin variable heavy chain gene

Chapter 1

Introduction

Cancer is the common name for diseases caused by continuously growing, and therefore malignant, cells. The name cancer refers to the way the cells spread and adhere to normal tissue. In Sweden, 45 000 new cases of cancer are diagnosed every year and approximately 50% of these individuals are cured from their disease (Socialstyrelsen, 2003). The treatment success rate is highly dependent on the type of cancer and the general condition of the patient. Overall, about 20% of all deaths in Sweden are due to cancer and the number is increasing, partly as a result of the ageing population, but also due to increased exposure to carcinogenic compounds and UV light.

The increased risk of developing cancer with age can be explained partly by the fact that malignant transformation is dependent on mutations or other genetic alterations circumventing the normal cellular mechanisms working to maintain homeostasis and that such mutations accumulate with age (Renan, 1993). However, some types of cancer are more frequent in childhood, thus excluding an absolute dependence on the sequential accumulation of somatic mutations during life for the development of cancer (Renan, 1993). The first evidence of an association between a genetic defect and the development of cancer was shown in 1960 when Nowell and Hungerford identified a deletion in chromosome 22 in patients with chronic myeloid leukaemia (Nowell *et al.*, 1960). After decades of research in the field of cancer, an overwhelming amount of information has been gathered. However, some believe that in the near future a few fundamental key-features, general to most types of cancer, will be discerned from the mass of complexity (Hanahan *et al.*, 2000).

The recent development of the expression profiling technology has contributed to the tremendous influx of information about different types of cancer. The technology has also revolutionised the process of categorising different malignancies into sub-groups to define molecular differences that may influence responsiveness to therapy and survival time. Thus, at its best, expression profiling may pinpoint the underlying molecular events and simplify both diagnosis and decisions regarding treatment (Davis *et al.*, 2002).

To improve overall success rates for the treatment of cancer, new therapies must be sought. The major problems with the available treatments are toxicity and induced drug resistance in some types of tumours. Thus, even if the patients respond to treatment, it is

not possible to give high-dose treatment over long periods of time to prevent relapse. A new trend using antibodies conjugated to cytotoxic or radioactive compounds reduces the toxic effects such as neutropenia (low white blood cell count) and anaemia, since the drug is concentrated to the tumour masses (Kuroki *et al.*, 2003). Antibodies against for example CD20 (rituximab), CD22 (epratuzumab) and human epidermal growth factor receptor 2 (trastuzumab) are already being used with promising results in the therapy of B cell non-Hodgkin's lymphoma (NHL) (rituximab and epratuzumab) and breast cancer (trastuzumab) (Kuroki *et al.*, 2003).

To increase the number of therapy-targets new cancer-associated receptors or antigens must be identified. The microarray technology is valuable for this task as tens of thousand of genes can be screened in large quantities of patient material, enabling the identification of over-expressed targets, such as cell surface associated antigens or genes coding for cell cycle regulatory proteins. After an initial filtering of the genome using microarrays, the corresponding proteins can be investigated.

In this thesis, mantle cell lymphoma (MCL), which is a B cell lymphoma with poor prognosis, has been investigated. The objectives of the studies have been to investigate the cellular origin in relation to normal B cells (paper I and II), and the molecular events involved in increased proliferation in MCL (paper III). The aim was to identify over-expressed genes and their corresponding proteins that could be used as targets for new therapy strategies for MCL. Furthermore, three different cell lines, established from patients diagnosed with MCL, were investigated to determine their applicability as *in vitro* models for MCL (paper IV).

The results obtained indicate that MCL derives from an antigen-activated B cell, which is in contrast to the previous belief that MCL derived from naïve B cells. A proliferative signature was further identified, involving genes associated with the increased proliferation of blastoid MCL. Interestingly, cyclin D1 - the hallmark of MCL - was further up-regulated in the high-proliferative MCL. In agreement with the somatic mutations found in a sub-group of MCL, one of the MCL cell lines examined was also found to be somatically mutated. The phenotype of this cell line was also slightly more differentiated than the other non-mutated MCL cell lines. Thus, it may be optimal to use different cell lines to perform functional studies of somatically mutated compared to unmutated MCL. However, all the MCL cell lines were readily distinguishable from the other lymphoma cell lines, indicating that the MCL cell lines have retained a MCL-specific gene signature.

Following two introductory chapters covering the microarray technology (Chapter 2) and basic knowledge concerning MCL (Chapter 3), the results of the different studies will be discussed in a broader context (Chapter 4-7).

Chapter 2

Microarray analysis

2.1 Development of gene expression profiling techniques – the pre-microarray era

Classical genetics has been a great tool for understanding diseases that are caused by the gain or loss of functionality in a single protein, encoded by a single gene (Liang *et al.*, 2003). However, the mechanisms of diseases, such as cancer, that are influenced by the expression of multiple gene products have been more difficult to understand using these simplified genetic models. During recent years functional genomics, the analysis of differential gene expression, has become one of the major technologies for discovering and understanding the mechanisms behind malignant transformation, as reviewed by Liang and Pardee (2003). After recombinant DNA technology started its development in the late 1970s, methods were invented for analysis of differentially expressed mRNAs using this new techniques. One of the first approaches to defining the differences between normal and tumour cells was performed using a technology called differential hybridisation. This technique uses radioactively-labelled complementary DNA (cDNA) probes from sample and control tissue, for example diseased and normal tissue (Sargent, 1987). Each of the probe sets is hybridised to one of two duplicate filters with cloned cDNA contained in phage. Uniquely expressed genes give rise to spots on one of the duplicate filters, but not the other. The complexity of the technique, i.e. the number of spots, was simplified considerably when Zimmerman and colleagues developed the subtractive hybridisation procedure that enabled only those genes that are uniquely expressed in one of the two samples to be hybridised (Zimmermann *et al.*, 1980). This was achieved by labelling the transcriptome of one of the samples and further hybridising it to the unlabelled transcriptome of the other samples. Only the unique, still single-stranded, labelled cDNAs could then be hybridised to and detected on the filter with cDNA phage plaques.

2.2 Development of global gene expression profiling techniques – microarrays

In the mid-1990s, the microarray technology was developed, which revolutionised analysis of the transcriptome. The superiority of the technique lies in the possibility of monitoring the expression of mRNA from tens of thousands of genes simultaneously. The technique comprises two separate methodologies, the spotted cDNA and the synthesised oligonucleotide arrays. The cDNA microarrays were developed by Patrick Brown and David Botstein (Schena *et al.*, 1995) and the oligonucleotide arrays, also called GeneChip arrays, were launched by Affymetrix Inc. (Chee *et al.*, 1996). The cDNA microarray technology uses cDNA probes that are spotted onto a solid support such as glass-slides or nylon membranes. The GeneChip technology uses probes that are synthesised onto a solid support. The major differences between the techniques are that: (1) on cDNA arrays, one probe for each gene is used, while 16–20 probes, which together constitute a probe set, are used on the GeneChip arrays; (2) the probes on the cDNA arrays are normally 100–200 bp long, while the probes on the GeneChip arrays are 20–25 bp long. However, the whole GeneChip probe set covers 200–300 bp of the 3' end of the gene. (3) On cDNA arrays, the relative abundance of sample vs. control mRNA is measured. This is achieved by labelling mRNA from two separate samples with different fluorochromes, and further hybridising the two samples to the same array. In contrast, using GeneChip arrays, the absolute mRNA content of each sample is measured and compared to the mean intensity of the chip. However, all gene expression analysis must be based on the relation between groups of samples or other related data, as for example time series.

The two systems are complementary. While the main advantages of the cDNA- arrays are the low cost (per array) and flexibility, the GeneChip array system is a ready-to-use system with optimised selection of sequences, use of mismatch control, and with all software and protocols provided.

The fundamentals of cDNA arrays have recently been reviewed by Leung et al. (Leung *et al.*, 2003), while information concerning the Affymetrix GeneChip array can be found on the company website, www.netaffx.com (Liu *et al.*, 2003).

2.3 Microarray technology in cancer research

One of the first applications of microarray technology in cancer research was presented in a study by Golub *et al.*, who used the new gene expression profiling technique to distinguish tumour samples from patients with either acute myeloid leukaemia (AML) or acute lymphoblastic leukaemia (ALL) (Golub *et al.*, 1999). At that time, the procedure commonly used to separate these two types of leukaemia was a combination of several methods, including analysis of morphology, histochemistry, immuno-phenotyping and cytogenetic abnormalities. The work by Golub *et al.* resulted in the etablishment of a predictor comprising 50 genes, selected from a list of a thousand genes that were all highly correlated with the AML-ALL class distinction. The predictor can be used successfully to classify new samples into one of the two categories (Golub *et al.*, 1999). Hopefully, this

type of analysis will speed up diagnosis, thus saving time and money – or at least provide an additional tool for diagnostics.

Shortly after the publication of the AML-ALL class predictor, a trend-setting study on diffuse large B cell lymphoma (DLBCL) from Alizadeh and colleagues was published (Alizadeh *et al.*, 2000). The gene expression pattern of DLBCL but also follicular lymphoma (FL), chronic lymphocytic leukaemia/lymphoma (CLL) and various populations of normal B and T cells were revealed, using a cDNA "Lymphochip" with probes selected to be expressed in immune cells and/or cancer. The major finding was that DLBCL could be divided into two sub-groups depending on the expression of a germinal centre (GC) or *in vitro*-activated genotype. These sub-groups correlated with the heterogeneous response to treatment and the patients in the *in vitro*-activated-like group were found to have a worse prognosis than the patients in the GC-like DLBCL group (Alizadeh *et al.*, 2000). Thus, the microarray technology proved to be useful in identifying a genetic signature correlating the molecular mechanism behind the heterogeneity in clinical behaviour with clinical outcome for individual patients. This knowledge can be used to optimise treatment decisions for patients afflicted by DLBCL.

One last example of early studies of lymphoid malignancies, using expression profiling, is a study of CLL. CLL is a molecularly heterogeneous disease with 50% of the patients having rearranged Ig genes with extensive somatic mutations and the other 50% having leukaemic cells that are germline-encoded (Staudt, 2002). The differences in mutational status were found to be associated with clinical outcome. Thus, patients having tumours with mutated V_H genes have a stable disease, whereas the patients with unmutated V_H genes generally have a more aggressive disease. To find out whether CLL could be divided into sub-groups, the gene expression pattern was compared to that of other B-cell malignancies. The genes distinguishing CLL from the other malignancies were expressed equally in both mutated and unmutated CLL, indicating that CLL is a single disease with a common cell of origin and/or mechanism of transformation. However, large differences in gene expression were seen comparing mutated vs. unmutated CLL, but as little as three genes could be used to predict the mutational status of the samples (Staudt, 2002). Since the clinical differences in patients with CLL are associated with mutational status, it is important to categorise the patients according to this criterion. This can now be performed by analysing the expression of a few genes instead of more time-consuming sequencing of the V_H genes. The use of microarray analysis in a hypothetical case of lymphoma is shown in Figure 2.1.

As shown during recent years, microarray technology is an extremely useful tool, and is complementary to earlier technologies. The main advantage is the rapid screening of large numbers of samples and genes. I believe that the development of the technique will continue to be refined, but that more efforts will be put in the down-stream work, analysing the corresponding gene products and performing functional studies. The new challenge for technology is to find effective means of applying a throughput-screening system to this step also. For the interested reader, more extensive reviews of the use of microarrays in cancer research can be found elsewhere (Alizadeh *et al.*, 2001; Hampton *et al.*, 2003; Schmidt *et al.*, 2003).

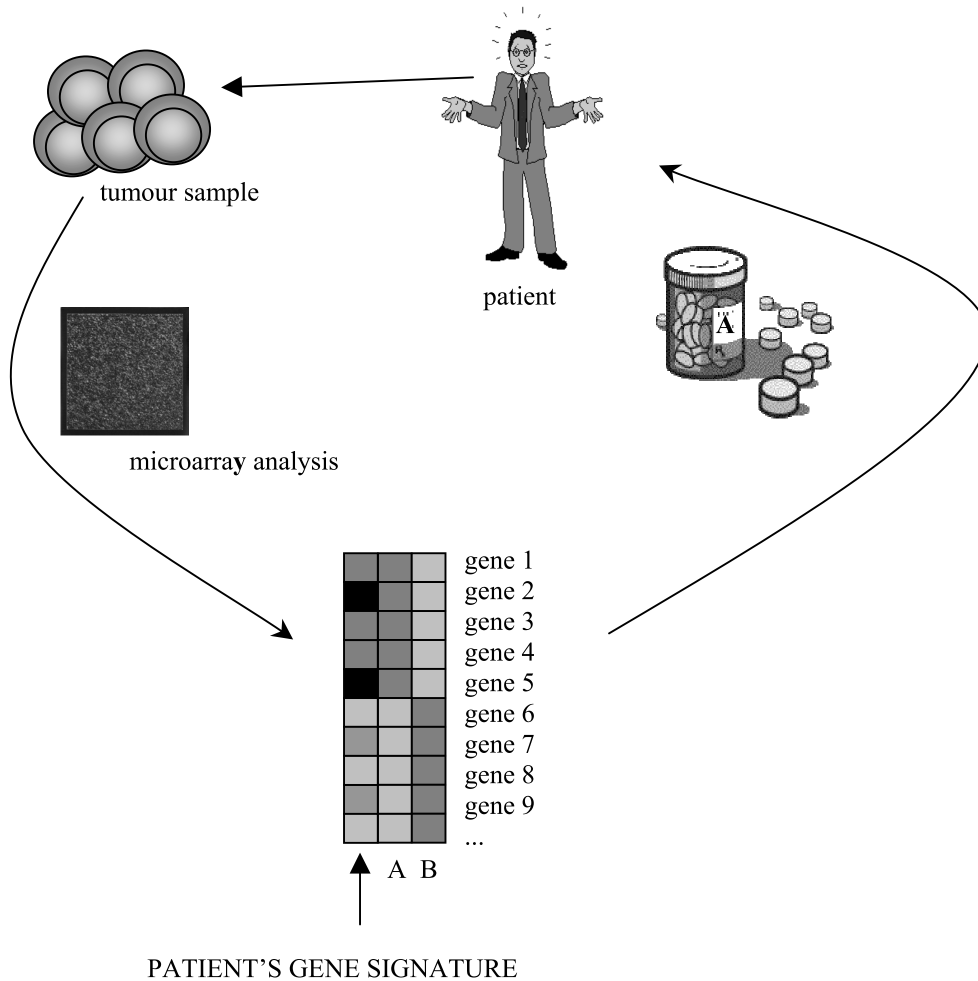


Figure 2.1. The usage of the microarray technology for individual treatment decisions is exemplified with a hypothetic case. A patient is afflicted by a certain type of lymphoma. From previous studies it is known that patients with the same diagnosis respond differently to treatment. In fact, it has been shown that patients with tumours with the gene signature designated "A" respond better to one type of treatment while patients with tumours with the characteristic gene signature "B" respond better to a second treatment type. Thus, by determining the gene signature of the patient, the correct treatment may be administered.

Chapter 3

Mantle cell lymphoma

3.1 B cell lymphomas

B cell lymphomas are the malignant counterparts of different developmental stages of normal B lymphocytes and are divided into precursor B cell neoplasms and mature/peripheral B cell neoplasms, according to the WHO classification (Harris *et al.*, 2000). The main groups of B cell neoplasms are precursor B-lymphoblastic leukaemia/lymphoma, CLL, plasma cell myeloma, extranodal marginal zone (MZ) B cell lymphoma of MALT type, FL, MCL, DLBCL and Burkitt lymphoma (Harris *et al.*, 2000).

B cell differentiation is strictly regulated by homeostatic controls; even so, malignant transformation occasionally proceeds unimpeded (Shaffer *et al.*, 2002). B cell malignancies, like other types of cancer, frequently affect individuals with an impaired immune system, such as patients suffering from acquired immunodeficiency syndrome (AIDS) or patients treated with immunosuppressive agents. Furthermore, several kinds of virus have been associated with the occurrence of NHL. These oncogenic viruses include Epstein-Barr virus (EBV), human T-cell lymphotropic virus type I, and human herpesvirus 8. The role of EBV infection in immunosuppressed individuals, for example after transplantation, and in individuals infected by human immunodeficiency virus (HIV), is particularly well documented (Grossbard, 2002).

Even the normal differentiation of B cells offers a number of opportunities for oncogenic hits to occur. The rearrangement of the immunoglobulin (Ig) genes during B cell development involves double-stranded DNA breaks, initiated by recombination-activating genes. These breaks are involved in the faulty joining of DNA strands, leading to the translocations t(14;18) and t(11;14), which are involved in FL and MCL, respectively (Willis *et al.*, 2000; Shaffer *et al.*, 2002).

Most lymphomas express a functional B cell receptor (BCR) due to successful rearrangement of at least one allele, despite the frequent occurrence of translocations involving the IgH locus on the other allele, indicating that – at least initially – the B cell was dependent on a functional BCR (Lam *et al.*, 1997). In addition, a high proportion of lymphomas show a biased immunoglobulin variable heavy chain gene (V_H) usage. Despite

this, it is unclear whether lymphomas depend on ongoing antigenic stimulations; only in one type of marginal-zone lymphoma has the antigen involved, that of *Helicobacter pylori*, been identified. Interestingly, the majority of marginal-zone lymphoma patients appear to be cured as a result of antibiotic treatment of their *Helicobacter pylori* infection, indicating that the malignancy was indeed driven by the antigenic stimulation (Wotherspoon *et al.*, 1993; Bayerdorffer *et al.*, 1995).

3.2 Characteristics, origin and diagnosis of MCL (1975-2004)

MCL has previously been designated intermediately differentiated lymphocytic lymphoma, centrocytic lymphoma (CCL) or diffuse small-cleaved cell lymphoma (Kurtin, 1998; Swerdlow *et al.*, 2002; Bertoni *et al.*, 2004). The name CCL was first used in the early 1970s and as the name indicates, the proposed origin for the malignancy was a GC centrocyte (Lennert *et al.*, 1975; Swerdlow *et al.*, 2002). The correlation between CCL and normal centrocytes was partly based on the similarity in nuclear morphology and presence of surface Ig (Tolksdorf *et al.*, 1980; Swerdlow *et al.*, 2002). In 1983, immuno-phenotypic studies confirmed the B cell origin of CCL (Swerdlow *et al.*, 1983). At the same time, it was suggested that the malignancy was derived from B cells that differed from the majority of cells found in a normal follicular centre, as the tumour cells lacked expression of both CD10 and peanut agglutinin, both of which are normally expressed in follicular centres. It was concluded that CCL was related to lymphoid follicles but “not easily placed into either the mantle or GC compartment” (Swerdlow *et al.*, 2002). In 1992, the International Lymphoma Study Group proposed a change in nomenclature from CCL to MCL because studies had now indicated that the follicular mantle (FM) was more likely to be the origin (Banks *et al.*, 1992). The change in nomenclature also highlighted the distinction between lymphomas with a GC phenotype and MCL.

MCLs grow in three different patterns in lymph nodes: mantle zone, nodular and diffuse. These growth patterns can be divided further into typical or blastoid variants (Kurtin, 1998). The characteristics of the different clinical, phenotypic and genotypic features of MCL were agreed on in 1994 (Zucca *et al.*, 1994). Morphologically, typical MCLs can have either a nodular or a diffuse growth pattern, with or without residual GCs, and are normally infiltrated with either loose or tight follicular dendritic cell (FDC) aggregates (Swerdlow *et al.*, 2002).

MCL most often arises in older adults. The median age is 60 years (range 18-86) at diagnosis, and the malignancy has a male predominance (male:female, 3:1) (Kurtin, 1998). Although the median survival period is short, only 3 to 5 years, and very few patients show long-term survival, a few features indicate a more favourable prognosis. These features include a proliferative index below 10% and arguably, a nodular growth pattern (Kurtin, 1998). Most patients show disseminated disease at the time of diagnosis, with 80% of patients in stage III or IV (Table 3.1), enlarged lymph nodes and frequent (70%) bone-marrow involvement. Involvement of the blood (25%) and gastrointestinal tract is also common (Swerdlow *et al.*, 2002), while infiltration of the central nervous system (CNS) is rarely detected and may be related to blastoid transformation (Raty *et al.*, 2003). Lack of

lymph node involvement with isolated massive splenomegaly occurs in some patients, but this is infrequent (Kurtin, 1998).

The main feature of MCL is over-expression of cyclin D1, which is due to the translocation of bcl-1 to the heavy chain locus (Donnellan *et al.*, 1998; Stacey, 2003). This t(11;14)(q13;q32) was identified in the 1970s as a cytogenetic event occurring in rare examples of B cell non-Hodgkin lymphoma; Tsujimoto and colleagues cloned the translocation breakpoint in 1984 (Tsujimoto *et al.*, 1984). Cyclin D1 is a positive regulator of the G1/S cell-cycle restriction point and the over-expression induces increased cycling of the cells. Even if the over-expression of cyclin D1 is the main feature of MCL, it is probably not the sole oncogenic feature, as over-expression of the gene in mice fails to induce lymphomagenesis (Lovec *et al.*, 1994).

Rare MCL-like B cell lymphomas lack cyclin D1 expression, but still have the same morphological and immunophenotypic characteristics (Yatabe *et al.*, 2000). These cyclin D1-negative MCL-like lymphomas have been shown to have a better prognosis and should be considered as an entity separate from MCL (Yatabe *et al.*, 2000; Rosenwald *et al.*, 2003). Other phenotypic characteristics of MCL are the expression of CD19, CD20, CD22, CD79a, CD79b and the lack of CD10 and CD23 expression (Kurtin, 1998). The lack of CD10 and CD23 distinguish MCL from FL and B-CLL, respectively. MCL cells also over-express Bcl-2 but lack t(14;18) (q32:q21), which is characteristic of FL. Although MCL cells mainly express sIgM/IgD, $C\gamma,\varepsilon,\alpha$ transcripts have been identified in MCLs carrying both mutated and unmutated V_H genes (Babbage *et al.*, 2004).

Thus, MCL is far from a homologous entity and even though the tumours share many characteristics, they can potentially be divided into sub-groups. Novel technologies such as microarrays can be used to identify the molecular differences involved in the heterogeneity in morphology, clinical behaviour and mutational status. This approach may form the basis of a therapy adjusted to each sub-type of MCL.

Table 3.1 Ann Arbor staging system (Grossbard, 2002)

Stage	Characteristics
I	Involvement of a single LN region
II	Involvement of two or more LN regions on the same side of the diaphragm
III	Involvement of LN regions on both sides of the diaphragm
IV	Extranodal organ involvement

3.3 Malignant transformation, progression and proliferation in MCL

3.3.1 Translocation of CCND1 in MCL

The translocation of cyclin D1 to the heavy chain locus seems to occur during an attempted primary D_H-J_H rearrangement in early B cells, although the translocation may also occur at a later stage during an attempted secondary rearrangement, as reported in

some cases of MCL (Welzel *et al.*, 2001). As the Ig genes become rearranged, double-stranded DNA breaks are created, a process that is dependent of RAG-1 (recombination activating gene 1) and RAG-2. It is believed that the translocation of cyclin D1 occurs if these breaks are resolved in a faulty manner (Willis *et al.*, 2000). Re-expression of RAG-1 and RAG-2 genes in GC B cells has recently been described. The ability of mature, GC B cells to once again rearrange their Ig genes is surprising but the benefits are obscure. One explanation, based on the work of Han *et al.* (Han *et al.*, 1997), is that B cells make a last attempt to replace a low-affinity receptor with a high-affinity receptor (Tarlinton, 1998).

Some believe that other oncogenic events precede the 11;14 translocation and allow it to occur unhindered. One mechanism for this could be the frequent mutation/deletion of ataxia telangiectasia mutated (*ATM),* which is characteristic of the initial events in MCL (Schaffner *et al.*, 2000; Fang *et al.*, 2003). ATM, which together with other genes is involved in the cellular responses to double-strand breaks, is normally expressed in pre-GC B cells and has been suggested that inactivation of the gene may be involved in malignant transformation (Starczynski *et al.*, 2003; Bertoni *et al.*, 2004).

3.3.2 Progression of MCL

MCLs may progress to a blastoid variant, as reviewed by Matolcsy (1999) and Muller-Hermelink *et al.* (2001), but this variant may also arise *de novo* (Raty *et al.*, 2003). The more aggressive blastoid variant is associated with decreased survival (Lardelli *et al.*, 1990; Raty *et al.*, 2002) and 26–70% of MCL patients have a morphological progression towards this variant, detected either during life or at autopsy (Norton *et al.*, 1995; Weisenburger *et al.*, 2000). Identical clonally rearranged Ig genes suggest that blastoid variants arise by clonal selection of the original neoplasm (Matolcsy, 1999). Factors that predict blastoid transformation are leucocytosis, elevated serum lactate dehydrogenase and high proliferative index (Raty *et al.*, 2003). The mechanisms involved in the morphological progression are far from elucidated, but a few reports have been published (Bea *et al.*, 1999; De Vos *et al.*, 2003). De Vos and colleagues found that several cell-cycle-related genes were de-regulated when normal MCLs were compared with blastoid MCLs, such as the gene for cyclin-dependent kinase 4 (CDK4), which was up-regulated in the blastoid MCLs (Bea *et al.*, 1999; De Vos *et al.*, 2003). CDK4 cooperates with cyclin D1 in the progression through the G1/S checkpoint. Other cytogenetic abnormalities that were found when comparing typical and blastoid MCLs were deletions of p53 and p16 (Bea *et al.*, 1999). Proliferation has been shown to be associated with decreased survival time (Rosenwald *et al.*, 2003) and, as discussed above, is also predictive of blastoid transformation (Raty *et al.*, 2003).

3.4 Somatic mutations and Ig V_H family usage

MCL was originally considered to be derived from naïve B cells and most tumours show unmutated V_H genes. However, recent studies have shown that 10–20% of MCLs have mutated Ig genes, indicating that a sub-group has passed through a differentiation stage involving somatic hypermutation of V_H genes (Pittaluga *et al.*, 1998; Laszlo *et al.*, 2000; Welzel *et al.*, 2001; Thorselius *et al.*, 2002; Camacho *et al.*, 2003; Kienle *et al.*, 2003). It is noteworthy that all MCL cells in an individual, including the ones that are V_H mutated, are clonally identical, indicating that the tumour cells are frozen at the stage where the malignant transformation took place (Shaffer *et al.*, 2002; Walsh *et al.*, 2003). Interestingly, it has been reported that B cells can mutate their V_H genes in the absence of GC but the mechanisms involved are far from understood (Matsumoto *et al.*, 1996; Weller *et al.*, 2001). Furthermore, a biased V_H usage has been described for MCL, which suggests that the transformation is antigen driven (Pittaluga *et al.*, 1998; Thorselius *et al.*, 2002; Camacho *et al.*, 2003; Walsh *et al.*, 2003). No antigen has been identified but V_H 3-21, which is one of the most commonly used V_H genes in MCL, has been used to produce rheumatoid factors and may recognise an auto-antigen (He *et al.*, 1995; Shaffer *et al.*, 2002).

3.5 Antibody-based therapy of MCL: the solution to increased survival time?

Most patients with MCL who show response to treatment, or even total remission, soon relapse with new tumours. Thus, more effective treatments must be sought. It is also essential to minimise toxicity, as most patients afflicted by MCL are 60 years of age or more and are likely to experience treatment-related complications (Porcu *et al.*, 1998). The standard treatment for MCL in most clinics is CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), but chemotherapy in combination with antibody-based therapy (rituximab) has recently been evaluated (Boye *et al.*, 2003; Hiddemann *et al.*, 2003). Preliminary results show increased response to this combined therapy, but the effect needs to be studied further in randomised trials with greater numbers of patients. In 10–15% of MCL patients, limited stage (I/II) disease (Table 3.1) is diagnosed. Since these patients only have a few localised tumours, regional radiation therapy has been shown to be a potentially curative treatment (Hiddemann *et al.*, 2003).

Chapter 4

B cell differentiation

4.1 From stem cell to mature peripheral B cell – and the pitfalls in between

B cells originate from pluripotent haematopoietic stem cells and proceed through several checkpoint-regulated pre-B cell stages before acquiring the immature B cell phenotype with rearranged Ig genes and displaying BCRs (μ) on the surface (Hardy, 2003). At this stage, the fate of the cells is uncertain as they may be eliminated, rendered anergic or their receptors may be edited if interacting with self-antigens. Thus, only a fraction of the immature, IgM^+, B cells generated are positively selected to leave the bone marrow for the spleen where they mature to IgM^+, IgD^+ B cells and acquire the ability to join the re-circulating pool of B cells (Pillai, 1999). Once in the periphery, the mature B cells patrol the secondary lymphoid system and are ready to recognise, bind and internalise antigens (Hardy, 2003).

In response to antigen, the B cells have the ability to differentiate further, either by becoming short-lived plasmablasts, secreting low-affinity IgM – but may also, when acquiring costimulation by T-cells, differentiate and participate in the formation of germinal centres (GC) (Sprent, 2003). In the GCs, the B cells are exposed to two different processes, somatic mutation and isotype switch, which change their Ig affinity/specificity and isotype. Following somatic mutation and isotype switch, massive apoptosis is seen, striking all B cells that have lost their ability to recognise the antigen or that bind with too-low affinity to efficiently compete for antigen (Hardy, 2003). B cells, which still bind, in particular those that have an increased affinity for the antigen, are positively selected by recognising antigen retained on the surface of FDCs. These B cells are further rescued from apoptosis by the survival and proliferation-promoting signals provided by FDCs and T cells (Liu *et al.*, 1996). However, the exact mechanisms involved in GC formation and hypermutation remain to be determined, and are probably more flexible than previously believed (Manser, 2004).

When analysing different stages of B cells, molecular markers specific for each stage are used to separate the populations. Mature B cells can be divided into different B cell stages based on the expression of CD23, CD38, CD77 and IgD (Pascual *et al.*, 1994). FM B cells

are IgD^+ and can be divided into naïve ($CD23^-$) and activated ($CD23^+$) pre-GC B cells. The GC B cells are characterised by their CD38 expression and can further be divided into centroblasts ($CD77^+$) and centrocytes ($CD77^-$). Memory B cells are negative for both IgD and CD38 (Pascual *et al.*, 1994; Nicholson *et al.*, 1995). These populations have been analysed for the usage of mutated V_H genes, and only the GC and the post-GC B cells were mutated relative to the germline sequences. Thus, the molecular events initiating the process of somatic mutation probably take place during the transition from an activated (IgD^+, $CD23^+$) to a GC B cell (Pascual *et al.*, 1994). The gene expression changes occurring during B cell differentiation have also been accessed using microarrays (Ma *et al.*, 2001; Klein *et al.*, 2003). The structure of the GC and the characteristics of the different B cell populations are shown in Figure 4.1a and b.

Lately, the association between somatic hypermutation and CD27 expression in normal B cells has been revealed (Klein *et al.*, 1998). Interestingly, when analyzing B cells for expression of CD27 and IgD, it is not only the naïve (IgD^+, $CD27^-$) or memory (IgD^-, $CD27^+$) B cell populations that are isolated, but also a $CD27^+$, IgD^{low} population. This population was initially suggested to be B cells in transition between a naïve and activated (memory) B cell stage (Agematsu *et al.*, 1997), and it has been shown that these B-cells mainly produce IgM upon stimulation (Agematsu, 2000). This population will be discussed further in chapter 4.3.

To understand the mechanisms involved in the growth of lymphomas and their response to external stimuli from soluble molecules or neighbouring cells, it is essential to determine and characterise the differentiation stage that the lymphoma is "trapped" in. The homing capability, resistance to apoptosis, the proliferation rate and the nature of the cells that interact with the B cells are drastically changed as the normal B cell differentiates. Most lymphomas show clonal identity, i.e. the differentiation process is terminated. In some cases, the aberrant termination of the differentiation process may actually be the cause of the cancer (Shaffer *et al.*, 2002). The different types of lymphomas, derived from mature B cells, have been assigned to specific B cell stages, primarily on the basis of a GC expression profile and the somatic hypermutation status, i.e. unmutated V_H genes, mutated V_H genes or ongoing somatic mutation. Most lymphomas have gone through somatic mutations of their V_H genes, indicating a GC or post-GC origin. CLL is believed to originate from an antigen-experienced B cell and a sub-group display mutated V_H genes (Gurrieri *et al.*, 2002). DLBCLs can be divided into two different sub-groups that each resemble GC B cells or *in vitro*-activated B cells (Alizadeh *et al.*, 2000). FLs display ongoing somatic mutation (Zelenetz *et al.*, 1992) and have a clear GC expression profile (Shaffer *et al.*, 2002), while Multiple Myeloma is the malignant counterpart of plasma cells (Seidl *et al.*, 2003). In contrast, MCLs do not show a GC expression profile and until recently were believed to use germline-encoded V_H genes. However, a subset of MCL has been shown to have mutated V_H genes, indicating that some MCLs have been exposed to the mutational machinery, thus excluding a naïve B cell origin for, at least, a sub-group of MCLs (Pittaluga *et al.*, 1998; Laszlo *et al.*, 2000; Welzel *et al.*, 2001; Thorselius *et al.*, 2002; Camacho *et al.*, 2003; Kienle *et al.*, 2003).

4.2 Determining the cellular origin of MCL

One of the main objectives of the project that resulted in paper I was to determine the normal counterpart of MCL. The Affymetrix microarrays, constituting a new, rather revolutionary technique, were in our hands. Our ambition was to use highly purified tumour material, as this would eliminate the detection of genes expressed in cells other than the tumour cells. Collecting fresh material for half a year left us with only 2 samples of purified MCL tumour cells, and it was necessary to also include previously frozen tumour material (n=5) that contained a fraction of non-tumour cells. For comparison, we used five flow cytometry-sorted B cell populations, including naïve (IgD^+, $CD23^-$, $CD38^-$) and activated B cells (IgD^+, $CD23^+$, $CD38^-$), centroblasts (IgD^-, $CD38^+$, $CD77^+$), centrocytes (IgD^-, $CD38^+$, $CD77^-$) and memory B cells (IgD^-, $CD38^-$). For each population, 2–3 biological replicates were used.

At that time, the proposed origin of MCL was a naïve B cell of the FM (Kurtin, 1998) and we were surprised to find that our observations using global expression analysis did not support a naïve B cell origin. The first observation was that, as judged from mRNA levels, the expression of several differentiation-associated surface antigens such as *IgD*, *CD23* and *CD62L* (L-selectin) was down-regulated compared to naïve B cells. Although the function of IgD still is obscure, the expression of it is characteristic of mature pre-GC B cells and is commonly used to define naïve B cells (Pascual *et al.*, 1994). CD23, which is the low-affinity receptor for IgE, is a B cell activation-associated antigen (Kikutani *et al.*, 1986) and is used to define the activated pre-GC population (Pascual *et al.*, 1994). L-selectin is involved in rolling and promotion of migration of lymphocytes from the bloodstream, across the walls of high endothelial venules (HEV), into lymphoid organs (Ager, 1997). Consistent with the inability of GC B cells to migrate (Roy *et al.*, 2002), *L-selectin* was expressed on naïve B cells but was down-regulated in the GC B cell populations (unpublished observations). Furthermore, *CD27* was found to be up-regulated in the MCL as compared to the pre-GC B cell populations. CD27 is a memory B cell marker (Klein *et al.*, 1998) and the expression correlates to somatic hypermutation in different B cell populations (Dono *et al.*, 2003). We observed high levels of *CD27* expression in both the GC populations and the memory B cells (unpublished observations). Thus, it is reasonable to believe that CD27 expression is induced during the transition from a pre-GC to a GC stage. Altogether, the expression levels of *CD27*, *IgD*, *CD23* and *L-selectin* suggested a more differentiated origin for MCL than previously proposed.

The second major observation was that the chemokine receptor expression pattern was altered compared to pre-GC B cells, indicating a more differentiated origin. Chemokines are involved in the organisation of the lymph node and are essential for the migration of immune cells into the correct lymphoid organs. The localisation of B cells in lymph nodes is influenced by CCR7, CXCR5 and CCR6 (Moser *et al.*, 2001; Casamayor-Palleja *et al.*, 2002; Reif *et al.*, 2002). The expression of CCR7 on B cells targets the cell for migration to lymph nodes, where extravasation is dependent on binding of CCR7 to CCL21 (SLC) or

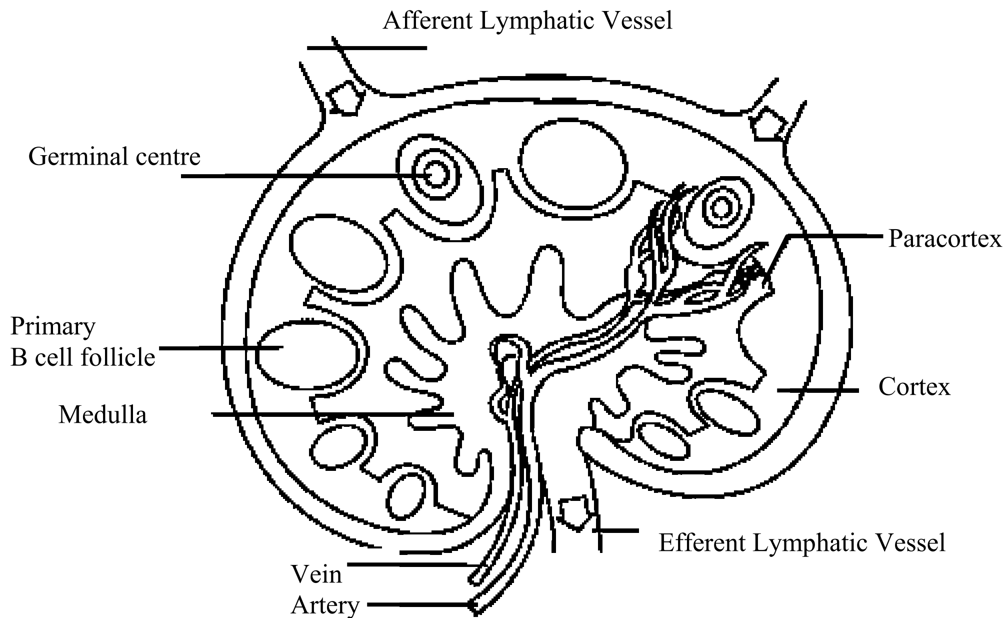
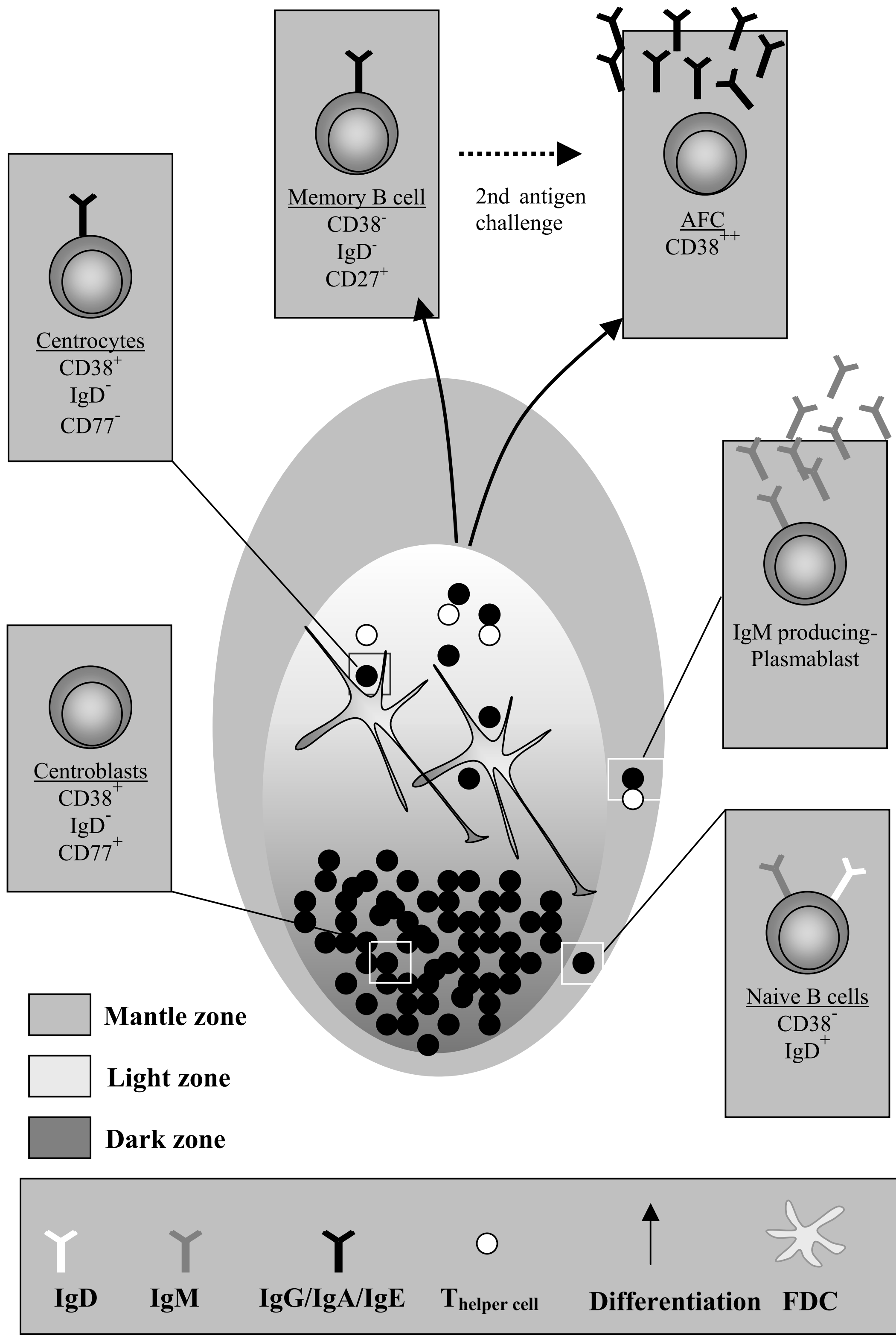


Figure 4.1a. Schematic presentation of a lymph node. Germinal centres evolve from a few proliferating B cells in the primary B cell follicle after antigen stimulation.

Figure 4.1b. The different areas of a germinal centre constitute cells with different properties. The mantle zone, which surrounds the GC, is mainly populated by naïve B cells. The GS are further divided into a dark and light zone. In the dark zone, B cells with low or no expression of B cell receptor (BCR) proliferate and acquire somatic mutations of their V_H genes. In the light zone, B cells are rescued from apoptosis by BCR-mediated recognition of antigens. Most of the available antibody-antigen complexes are retained on the surface of FDCs, and the competition for the limited amount of available antigen is keen. Further survival signals are mediated by T_{helper} cells present in the GC. After stimulation by T cells, the B cells may switch their Ig isotype, but may also go through additional affinity maturation and expansion in the dark zone. The remaining B cells that do not display a BCR or lack affinity for antigens present are cleared by tingible body macrophages, also present in the light zone. B cells that have high affinity for the antigens present and that have also acquired antigen restricted T cell co-stimulation may leave the germinal centre, and recirculate as memory B cell. Some B cells differentiate to antibody forming cells (AFC), which home to the bone marrow and secrete high affinity antibodies. The memory B cell may differentiate to AFC and may thus rapidly produce high affinity antibodies after a second antigen challenge.


Memory B cell
CD38-
IgD-
CD27+
2nd antigen challenge
AFC
CD38++
Centrocytes
CD38+
IgD-
CD77-
IgM producing-Plasmablast
Centroblasts
CD38+
IgD-
CD77+
Naive B cells
CD38-
IgD+
Mantle zone
Light zone
Dark zone
IgD
IgM
IgG/IgA/IgE
Thelper cell
Differentiation
FDC

CCL19 (ELC) on HEVs (Till *et al.*, 2002). CCL21 is expressed by the endothelial cells but CCL19, which is produced within T cell- and B cell-rich zones of the lymphoid organs, needs to be transcytosed to the luminal surface of the endothelium (Muller *et al.*, 2003). Furthermore, once in the T-cell zone the expression of CXCR5 overrides and prevents migrations toward CCL21/CCL19 and promotes migration of B cells toward CXCL13 (BCA-1) (Reif *et al.*, 2002). CXCL13 is produced by FDCs and stromal cells and attracts $CXCR5^+$ cells to the B cell follicles (Baekkevold *et al.*, 2001; Okada *et al.*, 2002; Muller *et al.*, 2003). After antigen encounter, the B cells in the B cell follicle relocate once more and move towards the border between the T-cell zone and the B cell follicle, thereby enabling B and T cell contact and subsequent co-stimulation (Reif *et al.*, 2002). Antigen stimulation of the BCR also induces down-regulation of CCR6 (Krzysiek *et al.*, 2000; Casamayor-Palleja *et al.*, 2002). Thus, GC B cells lack expression of CCR6 (Krzysiek *et al.*, 2000; Liao *et al.*, 2002). When comparing MCL with pre-GC B cells we observed down-regulation of *CXCR5*, while the expression of *CCR7* was unchanged, suggesting an antigen-activated origin for MCL (paper I). This was further supported by the down-regulation of *CCR6* (paper I). The normal GC B cell populations showed low or undetectable levels of *CXCR5* and *CCR6* expression (unpublished observations). Thus altogether, the expression of *IgD*, *CD23*, *CD62L*, *CD27* and a number of chemokine receptor genes clearly indicated that the MCL cells are arrested in the transition between a pre-activated B cell and a GC B cell. This was also confirmed in the second study performed, using a larger cohort of patients (paper II). In this extended study, new findings consolidated the idea that MCLs are most likely derived from an antigen-activated B cell. One of the genes that most strongly supported an activated origin was the over-expression of *CCL4*. CCL4 has been shown in two separate studies to be detected in different human B cell populations after stimulation of the BCR (Bystry *et al.*, 2001; Lu *et al.*, 2003), indicating a direct relationship between the chemokine and antigen-activation in B cells. CCL4 mainly attracts $CD4^+$ T cells and the secretion of CCL4 after antigen encounter may normally contribute to the interaction between rare antigen-specific B and T cells (Krzysiek *et al.*, 1999). Similarly, antigen-activated B cells in mice have been shown to attract regulatory T cells via CCL4 secretion (Bystry *et al.*, 2001). Thus, the expression of CCL4 in MCL further supported an antigen-activated origin for these tumours. The regulation of the different chemokines is shown in Figure 4.2.

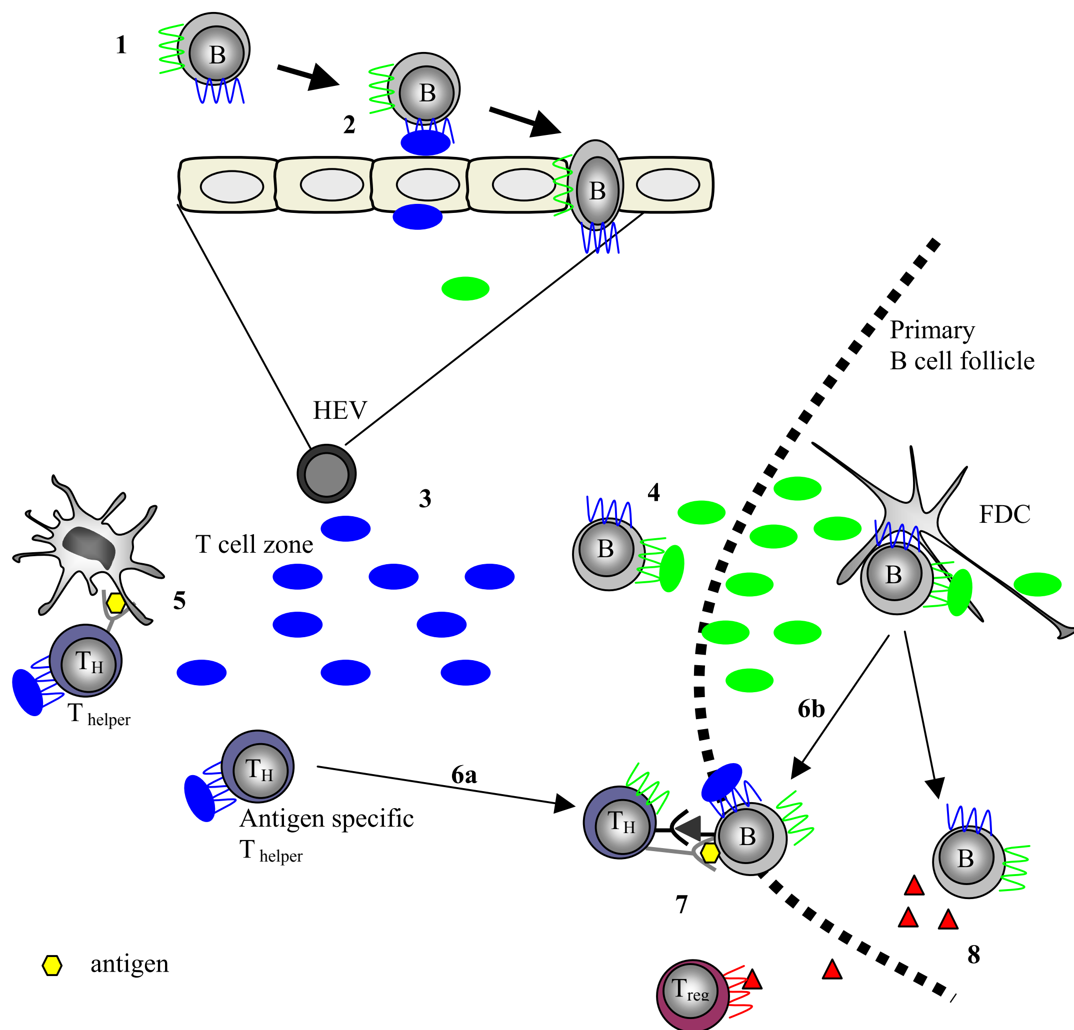


Figure 4.2 Tethering and rolling of naïve lymphocytes over the surface of high endothelial venules (HEV) are mediated by L-selectin. However, activation, which is induced by chemokines, is necessary to promote firm adhesion (1). The recruitment of lymphocytes to secondary lymphoid organs is promoted by the binding of CCR7 to CCL19 and CCL21 (both in blue) on the surface of endothelial cells lining the HEV (2). In the lymph node, B and T cells are positioned depending on the expression of the chemokine receptors CXCR5 (green) and CCR7 (blue) (3). Naïve B cells are highly chemotactic for CXCL13 (green), which is produced by FDCs, and migrate towards the B cell follicle (4), although some B cells that encounter antigen form foci of proliferating AFC, producing low affinity ab (not shown). The T cells are chemotaxtic for CCL19 and CCL21, which are produced by stromal cells, and are arrested in the T cell zone where the ag-specific T cells are primed by DCs (5). Some of the ag-activated T_{helper} cells up-regulate the expression of CXCR5 and migrate towards the B cell follicle (6a). Similarly, ag activated B cells in the follicle are highly chemotactic for CCL19/CCL21 and re-migrate towards the T cells zone (6b). Thus, at the boundary between the T cell and B cell zone, rare antigen-specific B and T cells meet (7). The T cells provide co-stimulatory activation of ag-specific B cells, which enables the formation of GCs. Furthermore, it has been shown that CCL4 (red) is produced by B cells after ag activation and that CCL4 secretion attracts T_{reg} cells (8).

4.3 Genetic signature of MCL with somatically mutated vs. unmutated V_H genes

Our finding that MCLs originate from a B cell stage in between a pre-activated B cell (IgD^+ $CD23^+$) and a centroblast (IgD^-, $CD38^+$) made us interested in the studies showing somatic mutations of V_H genes in a subset of MCL (Pittaluga *et al.*, 1998; Laszlo *et al.*, 2000; Welzel *et al.*, 2001; Thorselius *et al.*, 2002; Camacho *et al.*, 2003; Kienle *et al.*, 2003). When our extended study using 21 MCL samples was started, information about the frequency of V_H somatic mutations and V_H usage in the different tumours was gathered. One of the objectives of the second study (paper II) was to define the differences between MCL cells with somatically mutated and unmutated V_H genes. Surprisingly, the genes found to distinguish somatically mutated from unmutated MCLs did not correlate with the genes that differentiate pre-GC from GC B cells. For example, in contrast to normal B cells, the expression of *CD38* did not correlate with somatic hypermutation (paper II). This is in agreement with the fact that CD38 is also a poor predictor of mutational status in CLL (Thunberg *et al.*, 2001; Vilpo *et al.*, 2003). The expression of *CD27* that, as discussed above, is highly correlated with the frequency of somatic mutations in normal B cell populations, also failed to correlate with mutational status (paper II). This was consistent with frequent expression of CD27 in a number of B cell lymphomas known to carry unmutated V_H genes (Dong *et al.*, 2002). The explanation for the dissimilarities in gene regulation between normal somatically mutated and unmutated B cells and somatically mutated and unmutated MCLs could be dual: (1) the somatically mutated MCL cells are either not really mutated (the frequency is very low), or (2) the mechanism involved is distinct from the normal somatic hypermutational machinery that takes place in the GC. Contrary to the first explanation, it has been shown that a 2% cut-off in mutational frequency is enough to avoid counting polymorphism and PCR errors (Matsuda *et al.*, 1993). Furthermore, T cells from MCL patients who have tumours with mutated V_H genes have been shown to have 100% homology to the germline sequence (Walsh *et al.*, 2003), indicating that polymorphisms and PCR errors are infrequent. This suggests that even MCL with a lower frequency of somatic mutations than 2% may have somatically mutated V_H genes, thus ruling out the first explanation for the lack of overlap between genes separating somatically mutated from unmutated MCL and GC from pre-GC B cells. The second explanation for the differences in gene expression is supported by the finding that B cells can mutate their V_H genes in the absence of GCs, caused by the absence of a functional CD40L or lymphotoxin-α, as shown in humans and mice respectively (Matsumoto *et al.*, 1996; Weller *et al.*, 2001). However, the mechanisms involved in this alternative hypermutational pathway are not known. Consistent with our findings, other studies have indicated that a subset of DLBCL, with low frequency of hypermutation (>2%), also lack a GC genotype but resemble *in vitro*-activated B cells (Alizadeh *et al.*, 2000). It has been proposed that these tumours may be derived from a GC-independent $CD27^+$ B cell lineage, as the cells have the same phenotype as the somatically mutated $CD27^+$/IgM^+/IgD^+ B cells seen in patients with a defective CD40L (Weller *et al.*, 2001; Weller *et al.*, 2003). Interestingly, Dono et al. describe an IgM^+/IgD^{low} sub-

population that uses both unmutated and mutated V_H gene segments, in contrast to the normal IgM-only mantle zone B cells that display unmutated V_H gene segments (Dono *et al.*, 2000; Dono *et al.*, 2003). This sub-popoulation is derived from human tonsil and it is defined as being sub-epithelial B cells, which show phenotypic (Dono *et al.*, 1996) and functional (Dono *et al.*, 1996) characteristics distinct from those of FM B cells and GC B cells. It has also been speculated that this population may have acquired somatic mutations in the MZ (ex-GC) after T cell-independent antigen encounter (Dono *et al.*, 2003). As we reported in paper I, MCLs over-express CD27 as compared to pre-GC B cells, and others have shown that mutated MCLs, like normal $CD27^+/IgM^+/IgD^{low}$ B cells (Weller *et al.*, 2001), have a lower frequency of somatic mutation than GC-derived lymphomas (Walsh *et al.*, 2003). Thus, as the phenotype of MCL ($CD27^+/IgM^+/IgD^{low}$) correlates with this GC-independent $CD27^+$ B cell lineage and little overlap was seen comparing the genes distinguishing mutated vs. unmutated MCL with the genes distinguishing GC vs. pre-GC B cells, it is possible that the mutations seen in somatically mutated MCL may have been acquired outside GCs (paper II). The characteristics of some of the populations are shown in Figure 4.3.

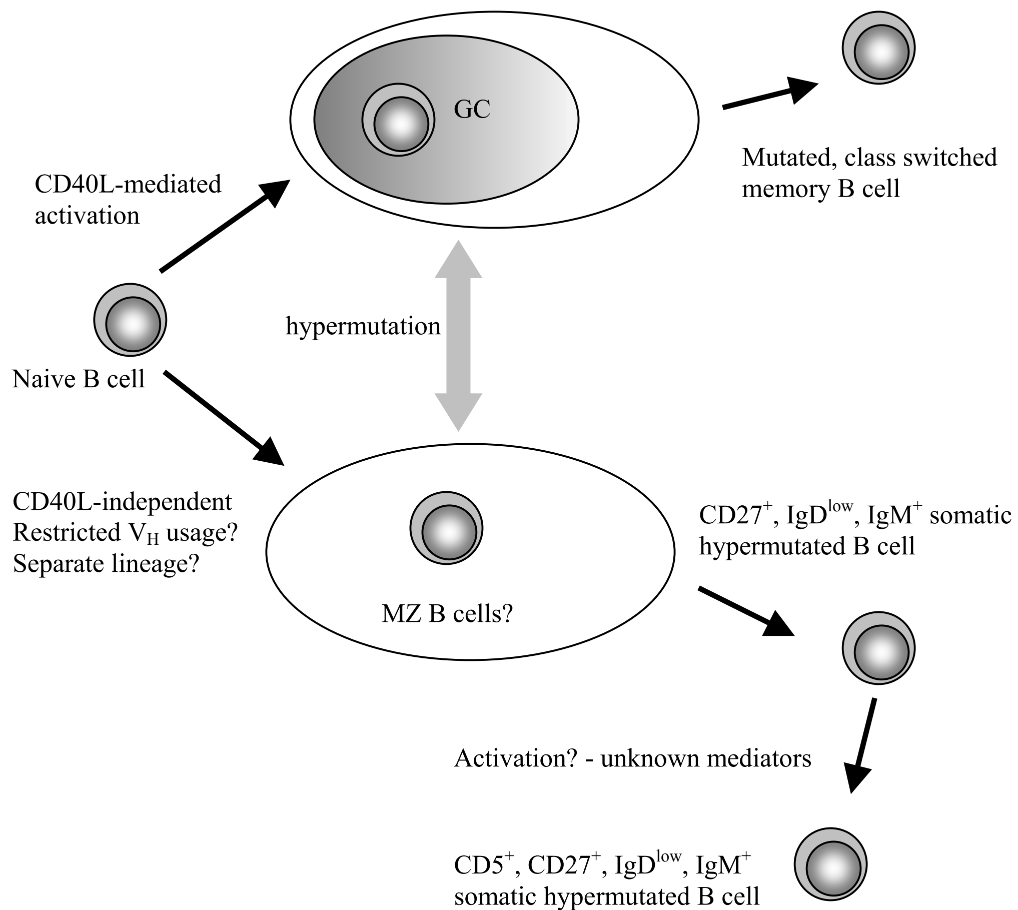


Figure 4.3 B cells form GC after stimulation by antigen and co-stimulation mediated by T_{helper} cells. Patients with X-linked hyper IgM syndrome lack a functional CD40L and lack GCs. The only somatically mutated cells found in the peripheral blood from these patients have a characteristic phenotype; they are IgM^+, IgD^+, $CD27^+$ B cells. When normal tonsil B cells are fractionated using different Percoll gradients, unactivated IgM^+/IgD^+ cells, classified as MZ cells, are found in the dense Percoll fraction. The expression of CD27 in these cells correlate to mutational status. In the less dense Percoll fractions, more activated cells are found. A similar population of MZ B cells is found that in addition to expressing IgM, IgD and CD27 (correlating to mutational status) also express CD5.

4.4 Restricted V_H gene usage in MCL

Together with reports of somatic mutations in the V_H genes of MCL, a restricted V_H usage has also been shown, with V_H4-34 and V_H3-21 being most frequently used (Camacho *et al.*, 2003; Walsh *et al.*, 2003).

A restricted V_H usage indicated that the malignant transformation might be antigen driven, which also is proposed for other types of lymphoid malignancies that use a restricted V_H repertoire, such as CLL (Tobin *et al.*, 2003), Sjögren's Syndrome (Bahler *et al.*, 1998) and multiple myeloma (Kosmas *et al.*, 2000). Interestingly, as indicated previously, V_H 3-21 is used in other diseases to produce rheumatoid factors and may recognise an auto antigen (He *et al.*, 1995; Shaffer *et al.*, 2002).

We attempted to find the genetic differences between MCL using V_H 3-21 as compared to MCL using random V_H genes. However, no differences in gene expression between these MCL-subgroups were detected (unpublished observations). This is probably due to that the antigen-induced change in gene expression is similar independent of the antigen recognised. Thus, MCL show related gene expression profiles, independent of the V_H genes used.

Chapter 5

Escaping homeostasis

Normal cells are in various ways restricted in their capacity to proliferate, as excellently reviewed by Hanahan *et al.* (Hanahan *et al.*, 2000). Thus, for a malignant cell to conquer homeostasis, a number of features must be acquired. Importantly, in contrast to normal cells, which require exogenous growth signals (GS) to initiate proliferation by inducing the resting cells to leave the G_0 stage, many tumour cells are self-sufficient in GS. Continuous growth is further promoted by acquiring insensitivity to anti-GSs that normally limit the replicative power. Cell death is avoided by de-regulation of apoptosis-associated pathways. Furthermore, normal cells have a limited ability to go through mitosis, restricted by the length of the telomeres. On the contrary, many types of cancer cells acquire the ability to produce telomerase or use other mechanisms to prolong the telomeres and inhibit crisis. The tumour size is greatly restricted by the capacity to promote angiogenesis. Thus, together, the ability to both promote vascularisation and grow unsupported leaves the tumour cells with the great capability of exploring distant sites for establishing new colonies, and possibly forming metastases. Each of the mechanisms mentioned above will be discussed in greater detail below, firstly from a general perspective and further on, focusing on MCL tumours.

5.1 Escaping homeostasis – mechanisms involved in promoting cell growth and escaping cell death during tumorigenesis

5.1.1 Self-sufficiency in growth signals and insensitivity to anti-growth signals in malignant cells

Normal cells need exogenous GSs to leave the quiescent state and enter an active proliferative state. The cells receive these GSs through transmembrane receptors that bind signalling molecules of different classes, such as soluble growth factors, extracellular matrix components or cell-to-cell adhesion/interaction molecules (Hanahan *et al.*, 2000). In some malignant cells, autocrine loops are used to sustain their proliferation, rendering the tumour cells independent of exogenous GSs. This has been reported for Hodgkin

lymphoma, where the malignant Reed-Sternberg cells express both IL-13 and the corresponding receptor (Skinnider *et al.*, 2002). In breast cancer, several types of autocrine growth factors have been identified, such as the insulin-like growth factors and the epidermal growth factors (Serrero, 2003). In other tumour types, histamine has been suggested to function as an autocrine growth factor (Rivera *et al.*, 2000).

Another way for the tumour cells to achieve self-sufficiency in GSs is to over-express growth factor receptors. This may render the cells hypersensitive to normal levels of ligands, or the receptors may even cross-link in the absence of ligands, thereby activating downstream signalling pathways (Hanahan *et al.*, 2000). Several cancer treatments, which target different receptors, such as the epidermal growth factor receptor-2 (EGFR-2), EGFR-1 and the platelet-derived growth factor receptor, have already been developed (Surmacz, 2003).

Furthermore, interactions with the surrounding microenvironment are essential for the survival and proliferation of both normal and malignant cells. Integrin-mediated adhesion of adherent cell lines results in increased proliferation and cell cycle progression. The loss of cell anchorage may even induce apoptosis in some cells, a mechanism referred to as 'anoikis' (Hazlehurst *et al.*, 2003). Growth factors secreted by tumour-educated macrophages have been proposed to help the tumour cells overcome these apoptotic signals (Pollard, 2004). Thus, tumour cells that are not self-sufficent in GSs can be dependent on neighbouring cells that are stimulated to produce the necessary growth factors.

Both proliferative signals and most of the anti-proliferative signals are funnelled through the retinoblastoma protein (pRb) and are mediated by promoting or preventing phosphorylation of the protein. Phosphorylation of the pRb regulates the amount of free transcription factor, which further controls the transcription of genes necessary for the G1 to S phase cell cycle transition (Weinberg, 1995). TGFβ, which is the most well-documented anti-proliferative mediator, prevents phosphorylation of pRb. Cells may become insensitive to the effect of TGFβ by down-regulating the receptor or other components involved in TGFβ-associated pathways. However, other anti-growth factors are probably also effective in this pathway (Hanahan *et al.*, 2000).

5.1.2 Evading apoptosis

The apoptotic machinery is a complex process that proceeds through at least two pathways, the extrinsic and the intrinsic pathways. Basically, the extrinsic pathway uses cell surface receptors for induction of apoptosis, while the intrinsic pathway involves the mitochondria and the associated components, such as cytochrome c (Fridman *et al.*, 2003). p53 is a tumour suppressor with the ability to activate apoptosis and works mainly through the intrinsic pathway (Fridman *et al.*, 2003). Exposure of a cell to ionizing radiation, hypoxia or growth factor starvation induces a p53-dependent response that leads to arrest or apoptosis (Fisher, 2001). The inactivation of p53 is the most common loss of a tumour suppressor gene in human cancer, and virtually all human tumours show some de-regulation of p53 (Fisher, 2001; Ryan *et al.*, 2001). This makes the restoration of p53 function a major goal in cancer therapy.

NFκB, which is as key regulator of B cell survival during both differentiation and activation, is mainly involved in suppressing apoptosis – but may, in some cell types, also promote programmed cell death in response to certain death-inducing signals. NFκB protects activated B cells from apoptosis (Kucharczak *et al.*, 2003), and the inhibition of the constitutive NFκB activation in MCL has been shown to induce apoptosis (Pham *et al.*, 2003). In other types of cancer, suppression of NF-κB enhances the efficacy of treatment, as the activation of NF-κB is generally associated with chemo-resistance (Kucharczak *et al.*, 2003). Thus, although the effect of NF-κB-inhibition varies between different types of malignancies, specific NF-κB inhibitors would be of great interest in cancer therapy.

Even if cancer cells can in many cases de-regulate apoptotic pathways to inhibit apoptosis, tumour cells may also be dependent on the microenvironment and/or the surrounding cells to escape apoptosis (Hazlehurst *et al.*, 2003).

5.1.3 Limitless replicative potential

In most cells, the ends of the chromosomes, the telomeres, are shortened for each cell division, eventually leading to senescence or crisis, thus restricting the life-span of the cell (Cech, 2004). In 1985, it was discovered that an enzyme found in ciliates could extend DNA at chromosome telomeres (Greider *et al.*, 1985). However, it was not until the end of the 1990s that the telomerase protein was found and the human version, human Telomerase Reverse Transcriptases (hTERT), was identified as the catalytic subunit (Meyerson *et al.*, 1997). Most normal somatic cells lack telomerase activity – with the exception of certain stem cells, lymphocytes and germline cells (Mokbel, 2003). On the contrary, most human cancers show telomerase activity or use alternative lengthening of telomeres by exchanging sequences between telomeres (Dunham *et al.*, 2000). The targeting of hTERT for therapy is appealing, and phase I and II clinical trials evaluating indirect inhibitors of telomerase, such as hTERT immunotherapy, are already running (Mokbel, 2003). The idea is that cancer cells will be affected before telomerase-positive normal cells by the anti-telomerase therapy. Normal cells are also likely to recover rapidly after the completion of treatment (Mokbel, 2003). However, this type of therapy will probably be most effective in tumours that have short telomeres and less effective in for example malignant lymphomas that have been shown to have intermediate or long telomeres, even at relapse (Remes *et al.*, 2000). However, pre-GC-derived lymphomas have been shown to have shorter telomeres than GC-derived B cell malignancies, indicating that pre-GC-derived lymphomas may be more suitable for treatment with telomerase inhibitors (Ladetto *et al.*, 2004).

5.1.4 Sustained angiogenesis

Angiogenesis is the term used to describe the formation of new capillaries from a pre-existing vasculature (Hanahan *et al.*, 2000). This process is vital for a number of processes including embryonic development, wound healing and tumour growth. The need for oxygen and nutrients makes it mandatory for all cells in a tissue to reside within 100 μm of a capillary blood vessel (Hanahan *et al.*, 2000). However, hypoxia occurs frequently in

tumours due to the rapid proliferation of tumour cells which outgrow the capacity of the vasculature (Tonini *et al.*, 2003). Hypoxia is not always an inconvenience for the tumour, as low oxygen levels may also induce angiogenesis and metastasis, consistent with a poor prognosis for patients with tumours with low oxygenation (Tonini *et al.*, 2003).

The tumour microenvironment, which is ruled dominantly by inflammatory cells, is fundamentally important for the neoplastic process by fostering proliferation, survival and migration. Some types of tumours have adapted signalling molecules that are normally used by the innate immune system, and produce high levels of pro-inflammatory chemokines that may favour angiogenesis and neoplastic growth (Coussens *et al.*, 2002). In addition, the degradation of basement membrane and extra-cellular matrix, which is induced by the secretion of matrix metalloproteinases (MMPs) from tumour cells, promotes the release of pro-angiogenic factors from the matrix (Tonini *et al.*, 2003).

5.1.5 Tissue invasion and metastasis

Most types of cancer form metastases, which are colonies of cancer cells distant from the original tumour. These metastases are the cause of death for the majority of patients afflicted by cancer (Hanahan *et al.*, 2000). The critical steps involved in this process are not fully understood, but they are associated with cell-cell and cell-matrix adhesion, with the degradation of extracellular matrix and the initiation and maintenance of growth at the new site (Cairns *et al.*, 2003). Three major types of homing mechanisms have been shown to occur in experimental metastasis. Firstly, tumour cells extravasate ubiquitously but grow selectively in organs that have the appropriate growth factors. Secondly, adhesion and homing only occur to sites expressing corresponding receptor/ligands, enabling extravasation. Thirdly, chemotaxis of circulating tumour cells occurs selectively to the organs that produce soluble attraction factors (Bogenrieder *et al.*, 2003).

The existence of metastasis-suppressor genes has been controversial. The presence of such genes would indicate that primary tumours progress and form metastases as a result of losing the expression of one or several metastasis-suppressor genes. Others claim that primary and progressed metastatic tumours from an individual patient are identical and that clinical outcome can be predicted using gene-expression profiles of primary tumours at diagnosis (Bogenrieder *et al.*, 2003).

5.2 Escaping homeostasis in MCL

5.2.1 Self-sufficiency in growth signals and insensitivity to anti-growth signals in MCL

In MCL, few suggestions have been put forward concerning potential mechanisms for self-sufficiency in GSs. However, some studies have explored the over-expression of the IL-10R (Ek *et al.*, 2002; Martinez *et al.*, 2003) and the proliferation induced by IL-10, as discussed in greater detail below (Visser *et al.*, 2000). Another potential, autocrine stimulatory pathway was reported by Planken and colleagues, who showed that $CD5^{+}$, in contrast to $CD5^{-}$ malignant cells, responded to stimulation of CD72 by increased proliferation

(Planken *et al.*, 1998). MCL is positive for both the CD5 and CD72 antigens, possibly enabling an autocrine growth-promoting effect. Stimulation of MCLs with α-CD72 antibodies have been shown to lead to increased proliferation in some cases (Planken *et al.*, 1998). Also, the co-expression of CD27 and CD70 in various B-cell malignancies, including MCL, may contribute to progression (Lens *et al.*, 1999). Furthermore, infiltration of hemapoietic and follicular dendritic cells in MCL tumours may provide the tumour cells with necessary GSs.

De-regulation of cytokines and cytokine receptor expression that might affect cell growth in MCL. When comparing MCL cells with normal B cells using the Affymetrix GeneChip technology, a number of de-regulated cytokines and cytokine receptors were found (paper I). Among others, an increased expression of *IL-10Rα* was observed in MCL. Others have also reported an over-expression of *IL-10R* in MCL, but used a different microarray system (Martinez *et al.*, 2003). Furthermore, IL-10 has been shown to induce proliferation of MCL cells *in vitro*, in combination with stimuli through CD40 (Visser *et al.*, 2000). Interestingly, Mino, which is an MCL cell line, was originally established by stimulating the tumour cells with IL-10 (Lai *et al.*, 2002). The stimulation of primary MCL cells with IL-10 promoted proliferation, and initially saved the cells from cell death. However, after months of culture, the cells became independent of exogenous IL-10. This probably occurred as the cells started to produce endogenous IL-10, indicating an ongoing IL-10 dependency of the cell culture (Lai *et al.*, 2002). A proliferation-inducing effect of IL-10 has also been indicated for CLL, where the cytokine has been proposed to have an autocrine role in a mouse model of CLL, and the inhibition of IL-10, using the antisense technology, inhibited growth in the malignant cells (Peng *et al.*, 1995; Ramachandra *et al.*, 1996). Thus, signalling through IL10R may contribute to proliferation and survival in MCL, and the development of a blocking antibody targeting the receptor is of major interest for possible therapeutic applications.

While IL-10 has a potential role in cell proliferation in MCL, IL-4 has been shown to protect naïve B-cells from apoptosis, while GC and memory B cells were unaffected (Wagner *et al.*, 2000). The dependency on IL-4R signalling for the survival of naïve B cells correlates well with our observation that pre-GC B cells had significantly higher *IL-4R* gene expression than GC B cell populations (unpublished data). MCL cells showed a reduced expression of *IL-4R* compared to pre-GC B cells, indicating that stimulation by IL-4 is no longer necessary for the survival of the tumour cells (paper I). In fact, an inhibitory effect of IL-4 has been observed in MCL (Visser *et al.*, 2000).

In addition to cytokine receptors, several cytokines were de-regulated in the MCL tumours, possibly affecting proliferation (paper I). *IL-18* was over-expressed in MCL (paper I), while the corresponding receptor was heterogeneously expressed: 8 of 21 MCL samples expressed the gene (unpublished observation), which is consistent with more recent studies (Airoldi *et al.*, 2004). Controversially, IL-18 treatment has been suggested for immunotherapy, as anti-tumour activity has been shown to be induced by the cytokine in a mouse model (Golab, 2000). Furthermore, it remains to be investigated whether *IL-18* (if functionally translated in MCL) acts in an autocrine loop – by stimulating the tumour cells – or whether the target cells are the surrounding non-malignant cells.

Consistent with increased proliferation, down-regulation of *TGFβ*, encoding the major growth suppressing cytokine, was found in MCL compared to pre-GC B cells (paper I). TGFβ is not only the main growth inhibitor, but also influences various processes, such as migration, adhesion, differentiation and angiogenesis, depending on the type and state of the target (Siegel *et al.*, 2003). The growth inhibiting effect of TGFβ is de-regulated in many cancers. The receptor, TGFβR, can be down-regulated, or downstream targets can be mutated and lose their function (Hanahan *et al.*, 2000). We found that MCLs expressed *TGFβR* at a level comparable to normal B cells (unpublished observation). However, the tumour cells produced reduced amounts of *TGFβ* and *smad3* (paper I). Smad3 is critical for the signalling downstream of TGFβ (Roberts *et al.*, 2003), indicating that the TGFβ-induced pathway is impaired in MCL (paper I). Interestingly, TGFβ can be used as a tumour-progression factor by tumours, which have defective TGFβ signalling pathways (Siegel *et al.*, 2003). The secretion of TGFβ by the tumour produces a beneficial environment for tumour growth and metastasis, as surrounding, non-malignant cells are negatively affected by the increased levels of TGFβ (Siegel *et al.*, 2003).

Increased expression of CCL5, a potential activation-inducing chemokine, in MCL. *CCL5* was expressed in MCL, in contrast to the situation in normal B cells where expression was seen only in the memory B cell population (paper II). The expression of the corresponding protein was further confirmed in MCL cell lines (paper II). CCL5, which is produced by various cell populations such as $CD8^{+}$ T cells, platelets, epithelial cells and fibroblasts, attracts leukocytes to sites of inflammation (Payne *et al.*, 2002). *In vitro,* aggregates of CCL5 that form on the leukocyte cell surface have wide-ranging effects on activation, similar to that of mitogens (Appay *et al.*, 2001). It is noteworthy that this activation is independent of the corresponding chemokine receptors (CCR1 and CCR5). The relevance of this *in vitro* activation remains uncertain because the concentration required is non-physiological. Nevertheless, aggregation of CCL5 could take place *in vivo*, thereby inducing cross-linking of cell-surface signalling molecules and unwanted non-specific cell activation (Appay *et al.*, 2001). In B cells, CCL5 expression has only been detected in $CD20^{+}$ B cells and Reed-Sternberg cells, both in patients with Hodgkin's disease (Tedla *et al.*, 1999). Other types of tumours also express CCL5, as for example metastatic breast cancer (Tedla *et al.*, 1999) and melanoma, where CCL5 was shown to correlate to diminished anti-tumour response and tumour progression (Mellado *et al.*, 2001; Payne *et al.*, 2002). Thus, while no expression of CCL5 has been reported in normal B expression of CCL5 has been correlated to malignant behaviour in several types of tumours, indicating that CCL5 may have a role in MCL tumorigenesis.

Expression of 4-1BB-L, a FDC ligand, in MCL. As discussed previously, the interaction of tumour cells with the surrounding cells or matrix may influence survival and growth (Liu *et al.*, 1996). In MCL, the B cell follicles are partly defined by a prominent FDC meshwork, identified by staining for CD21, CD23 or CD35. Even in MCL with diffuse histological pattern, disordered arrays of FDCs intermixed with the neoplastic cells can be found (Kurtin, 1998). FDCs are stromal cells of unknown origin and are uniquely detected in primary and secondary B cell follicles. FDCs have the capacity to retain unprocessed antigen for long periods by binding antigen-antibody complexes to Ig-Fc

receptors expressed on the cell surface. The main feature of FDCs in the secondary B cell follicle is commonly believed to be rescue of B cells from apoptosis by providing survival signals to antigen-specific B cells (Liu *et al.*, 1996). The antigens are retained in immune complexes (ICs) bound to the surface of FDCs, and in contrast to the weak immunogenicity of free ICs, FDC-bound ICs are rapidly endocytosed and processed by B cells (Tew *et al.*, 2001). However, lately the absolute requirement of FDCs for affinity maturation have been questioned (Haberman *et al.*, 2003; Manser, 2004). In addition to potentially rescuing GC B cells from apoptosis (Tsunoda *et al.*, 2000), FDCs are involved in the survival of non-Hodgkin lymphomas (Freedman *et al.*, 1992; Petrasch *et al.*, 1992).

Recently, 4-1BB, which is a member of the TNF-receptor superfamily, was identified on the surface of FDCs (Pauly *et al.*, 2002; Lindstedt *et al.*, 2003) and it was demonstrated that 4-1BB could stimulate *in vitro*-activated B cells (Pauly *et al.*, 2002). The binding of 4-1BB to a variety of B cell populations had been shown previously, but the ligand was unknown at that time (Pollok *et al.*, 1994). A few reports have also identified the expression of soluble 4-1BB-L in sera of patients with haematological malignancies (Salih *et al.*, 2001) and on the surface of PMA/ionomycin-stimulated B cell lymphoma cell lines (Palma *et al.*, 2004). Recently, the co-expression of 4-1BB and 4-1BBL was reported in Raji cells, a B lymphoma cell line (Palma *et al.*, 2004). 4-1BB-L is also functionally expressed on carcinoma cells, and signalling through 4-1BB-L may potentially have survival- or proliferation-inducing effects on the tumour cells (Salih *et al.*, 2000). In paper II, we report one of the first sets of data on 4-1BB-L expression in a human lymphoma. The gene expression was detected in MCL patient samples and the expression of the 4-1BB-L protein was shown in MCL cell lines. Expression of the corresponding receptor, 4-1BB, was detected in the non-purified samples, possibly through being expressed by infiltrating FDCs. The lack of *4-1BB-L* expression in the different normal B-cell populations used in paper II (unpublished observations) was consistent with the need for *in vitro* activation for detection (Pauly *et al.*, 2002). In relation to previous reports, it is reasonable to assume that the possible interaction between 4-1BB on FDCs and 4-1BB-L on MCL cells can induce proliferation and contribute to resistance to apoptosis.

In summary, differential expression of cytokines, cytokine receptors (*IL-18*, *TGF*β, *CCL5*, *IL4R*, *IL-10R*α) and a member of the TNFR-family (*4-1BB-L*) in MCL compared to normal B cells may all contribute to an increased proliferation in MCL. Proliferation may possibly be sustained and/or promoted by infiltrating cells, producing IL-10 or providing stimulation through 4-1BB-L. Some of the potential survival mechanisms are summarised in Figure 5.1.

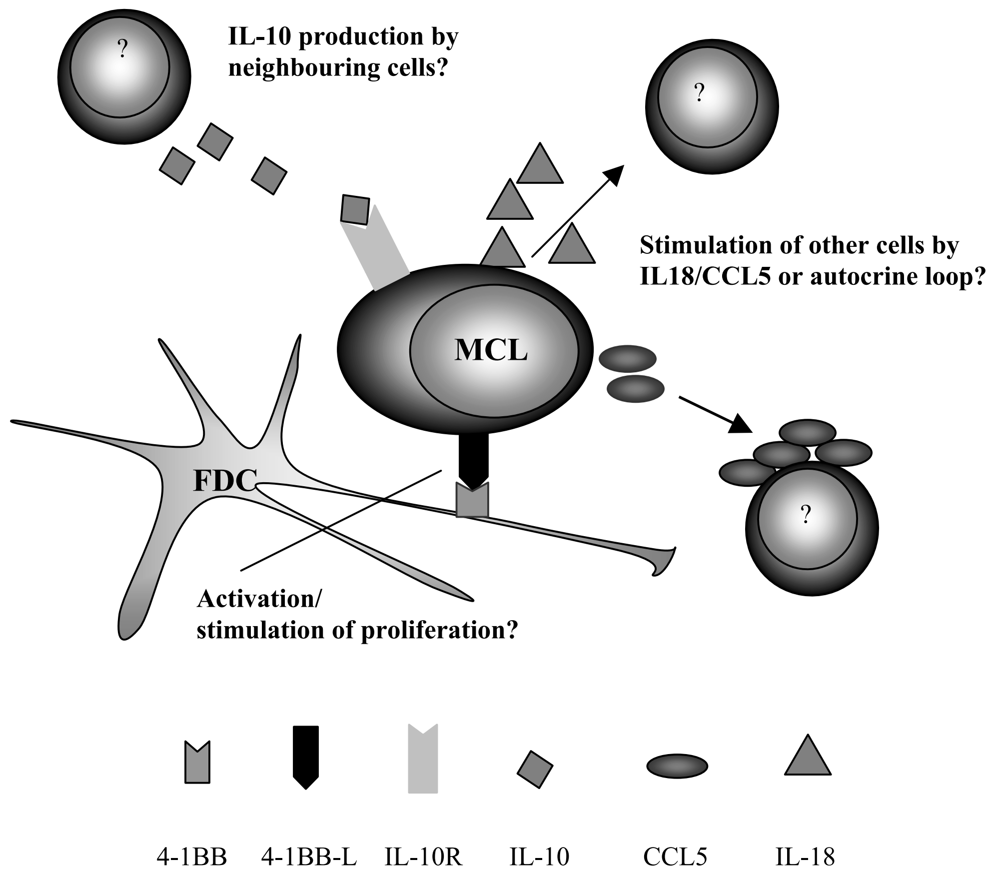


Figure 5.1 Potential immune evasion strategies of MCL cells through several different mechanisms. An increased expression of *IL10R*α, *IL-18*, *CCL5*, *4-1BB-L* were found in MCL compared to normal B cells.

5.2.2 Resistance to apoptosis in MCL cells

Several apoptosis-associated mechanisms have been reported to be dysfunctional in MCL. Among others, c-Myc, which is involved in both proliferation and apoptosis, has been shown to be over-expressed in MCL and is an indicator of poor prognosis (Nagy *et al.*, 2003). In normal cells, over-expression of c-Myc induces apoptosis by blocking Bcl-2 and Bcl-X_L. However, these mechanisms are bypassed in tumours that over-express c-Myc (Nilsson *et al.*, 2003). Furthermore, survivin, a member of the inhibitor of apoptosis (IAP) gene family (Altieri, 2003), is associated with cell proliferation and survival in MCL (Martinez *et al.*, 2004). Survivin has been shown to inhibit apoptosis in several studies, and the most intriguing feature of the protein is the sharp differential expression in cancer as compared to normal tissue (Altieri, 2003). Deletion or mutation of ATM, which is characteristic of the initial events in MCL (Schaffner *et al.*, 2000; Fang *et al.*, 2003), has also been associated with a defective response to DNA damage in CLL (Stankovi *et al.*, 2002) and may be one of the mechanisms leading to unsatisfactory response to treatment in MCL patients.

De-regulation of genes involved in apoptosis in MCL compared to normal B cells. We and others (Hofmann *et al.*, 2001) have shown that Bcl-2 is over-expressed in MCL (paper I). Bcl-2 is differentially expressed in normal B-cells, depending on activation stage, but no differences were seen in expression when comparing MCL with mutated vs. unmutated V_H genes (paper II). Bcl-2 blocks the release of pro-apoptotic factor, in particular cytochrome c, from the mitochondria into the cytosol (Hengartner, 2000). In combination with Apaf-1 (apoptotic protease activating factor-1), cytochrome c is necessary for the activation of caspase-9 in the cytosol. Caspase-9 and Apaf-1 form the apoptosome, a large protein complex that activates caspase-3, one of the main apoptotic effector proteins (Hengartner, 2000). The mechanism of the over-expression of Bcl-2 in MCL is not known; this is in contrast to CLL, where Bcl-2 is most likely over-expressed due to complete demethylation of the *Bcl-2* gene (Meinhardt *et al.*, 1999).

TNFR1, which at the protein level may induce activation of the NF-κB or apoptotic pathway, was found to be over-expressed in MCL compared to normal B cells (paper I). *DR3* (APO-3), which is highly homologous to TNFR1, was also over-expressed (paper I) and probably signals to the same pathways, using the same set of receptor-associated proteins as TNFR1 (Wajant, 2003). Interestingly, when NF-κB activity is lost, the susceptibility to TNF-induced apoptosis increases (Chen *et al.*, 2002), thereby indicating that MCL with a constitutively activated NF-κB pathway may be resistant to TNF-induced apoptosis. Thus, the overexpression of TNFR1 is more likely to contribute to activation of NF-κB than apoptotic pathways in MCL. In support of this, *IEX-1L*, which has a key role in the cellular resistance to TNF-induced apoptosis (Wu *et al.*, 1998), was also found to be over-express in MCL (paper I). Furthermore, *FLAME*, which was up-regulated in MCL (paper I), can interact specifically with FADD and abrogate FAS/TNFR-induced apoptosis, possibly by acting as a dominant-negative inhibitor (Srinivasula *et al.*, 1997). The down-regulation of DRAK-2 (paper I), which has been associated with induction of apoptosis, was also supportive of a negative regulation of the apoptotic pathways (Sanjo *et al.*, 1998).

Thus, the de-regulation of several genes (*Bcl-2*, *TNFR1*, *IEX-1L*, *FLAME*, *DRAK2*, *DR-3*) indicated an increased resistance to apoptosis in MCL (paper I). However, the expression of several other apoptosis-associated genes was not as easy to understand, again pointing to the complex regulation of apoptosis.

5.2.3 Unlimited replicative potential

The telomere length in a number of B cell lymphomas, including MCL, has been assessed by others and it was shown that MCLs have shorter telomeres than GC-derived B cell lymphomas (Ladetto *et al.*, 2004). This is in agreement with an increased expression of telomerase in normal GC B cells. The association between telomerase activity and proliferation index has been accessed in a study of aggressive mature B cell lymphoma, which included MCLs (Chiu *et al.*, 2003). Even if no statistically valid correlation was found between Ki-67 and telomerase activity, an increased mean telomerase activity was found when comparing MCL with cases of reactive follicular hyperplasia (Chiu *et al.*, 2003).

5.2.4 Metastasis and angiogenesis in MCL

Patients with MCL often show disseminated disease at the time of diagnosis. The sites of involvement are concentrated to the lymphoid compartments, such as lymph nodes, bone marrow and spleen (Swerdlow *et al.*, 2002). The infrequent spreading outside the lymphoid system is probably due to a limited ability to sustain growth, indicative of the importance of the tumour microenvironment in MCL. However, infiltration of the CNS occurs, although rarely, and is potentially associated with blastoid transformation (Raty *et al.*, 2003). Thus, as MCL progress the tumour cells may acquire self-sufficiency in GS, enabling spreading outside the lymphoid system.

Tumour growth and spreading are both dependent on mechanisms promoting angiogenesis, to make room for the tumour and secure a continuous blood supply. When comparing MCLs with normal B-cells, up-regulation of *MMP-9* and down-regulation of *RECK* was found (paper I). MMP-9, also known as gelatinase B, is involved in the destruction of extra-cellular matrix (ECM) and is probably secreted by both tumour cells and surrounding stromal cells (Fridman *et al.*, 2003). MMP-9 is a key enzyme in tumour progression, due to its role in invasion and angiogenesis (Fridman *et al.*, 2003). RECK, which is a membrane receptor and regulator of extra-cellular matrix remodelling, has been shown to inhibit activation of MMP-9 from secreted pro-MMP-9. The absence of *RECK* in MCL (paper I) is likely to lead to excessive activation of MMPs and promotion of malignant progression, as shown in other studies (Noda *et al.*, 2003). *RECK* expression has been shown to be inhibited by several oncogenes, as reviewed elsewhere (Sasahara *et al.*, 2002).

Although MCL mainly keeps to the lymphoid system for a proper microenvironment to sustain tumour growth, spread though the blood system and promotion of angiogenesis may be induced by the de-regulation of *MMP-9* and *RECK*.

Chapter 6

Tumour progression and relapse in mantle cell lymphoma

Malignant transformation typically induces increased proliferation in the target cell. This is achieved by a number of mechanisms, as discussed in the previous chapter. Similarly, morphological and biological progression, which occurs in many types of lymphoma, is sometimes also associated with an increase in proliferation and often with acceleration of clinical symptoms, as will be discussed below (Muller-Hermelink *et al.*, 2001). Recurrent tumours, on the other hand, are not necessarily associated with increased proliferation compared to primary tumours, but are in most cases a clonal evolution of a tumour cell, providing additional survival and/or growth advantages (Wlodarska *et al.*, 1999; Naoe *et al.*, 2000). The differences between primary and relapsed MCL will also be discussed further below.

However, malignant transformation, tumour progression and relapse are all related events, characterised by a sequential selection and survival of the fittest tumour cells.

6.1 Progression of different types of lymphoma

Some lymphomas progress from indolent to more aggressive variants over time. This is the case for CLL, FL, mucosa-associated lymphoid tissue (MALT) lymphomas and MCL (as reviewed by Matolcsy 1999 and Muller-Hermelink *et al.* 2001).

Confusingly, the term progression is used for several different types of changes in a tumour and/or patient, such as clinical, morphological and biological progression (Muller-Hermelink *et al.*, 2001). Clinical progression is defined by an increased tumour burden and clinical symptoms. Morphological progression, on the other hand, describes the transition of a lymphoma entity into another, morphologically distinct, entity. This progression often involves the transition from one histological variant (low or intermediate grade) to another (intermediate or high grade) (Grossbard, 2002). In contrast to low-grade lymphomas, which are characterised by an accumulation of malignant cells due to de-regulated apoptotic pathways, high-grade lymphomas show high proliferation index and often have alterations in signalling pathways funnelled through Rb and/or p53 (Sanchez-Beato *et al.*, 2003). Lastly, biological progression describes the additional genetic alterations that may

accumulate in the tumour cells. However, in most cases the three types of progression are intimately associated, as clinical and morphological progression are due in many cases to biological progression.

6.1.1 Progression of CLL

CLL is a low-grade, clinically indolent lymphoma that is usually stable over several years (Matolcsy, 1999). In rare events, morphological progression to Reed-Sternberg-like cells, normally found in Hodgkin's disease, is seen – an event possibly associated with Epstein Barr virus infection (Petrella *et al.*, 1997). More frequent (5%) is the morphological progression of CLL to DLBCL; this process is referred to as Richter's syndrome (Matolcsy, 1999; Muller-Hermelink *et al.*, 2001). Richter's syndrome is associated with increased genetic alterations, specifically losses of 8p and chromosome 9 (Bea *et al.*, 2002). Furthermore, loss of TGF-β-induced growth inhibition has been shown after DLBCL transformation (Nowell *et al.*, 1994).

The morphological progression of CLL to DLBCL does not occur in a single homogeneous manner. In some cases, the transformed tumour cells express identical Ig CDR3 sequences, indicating that morphological progression to DLBCL occurs through clonal evolution of a single CLL cell (Matolcsy *et al.*, 1999). In the remaining cases, different CDR3 sequences suggest that CLL and DLBCL develop as transformations of different B cell clones in the same patient, representing clonally unrelated secondary *de novo* lymphomas (Matolcsy, 1999; Muller-Hermelink *et al.*, 2001). Of note, morphological progression to large-cell variants has been detected in relation to some types of therapy, but this association must to be confirmed using a larger patient material (Cohen *et al.*, 2002).

6.1.2 Progression of FL

FL is characterised by a relatively indolent clinical course and long survival. However, morphological progression into more aggressive types of lymphoma, such as DLBCL, occurs in 10–60% of FL patients (Lossos *et al.*, 2003). The morphological progression is associated with an increasing proportion of large cells, a diffuse growth pattern in the lymph node and clinical acceleration of the disease (Matolcsy, 1999). Like CLL, FL may also progress into Hodgkin's lymphoma, although this is rarely reported (Muller-Hermelink *et al.*, 2001). The most commonly identified genetic alterations associated with transformation of FL are p53 mutation and deregulation of the c-Myc gene (Lossos *et al.*, 2003). In addition, *BCL6* translocation or deletion is detected more frequently in FL that later progress morphologically, than in FLs that do not (Akasaka *et al.*, 2003). Gene expression profiling studies have been used to define the alterations in gene expression associated with morphological progression of FL to DLBCL, and a number of de-regulated genes have been found (de Vos *et al.*, 2003; Martinez-Climent *et al.*, 2003).

Somatic hypermutation is ongoing in FL and somatically mutated variants of the original clone have been shown to progress to DLBCL (Matolcsy, 1999; Muller-Hermelink *et al.*, 2001). In the majority of cases, the FL and the DLBCL cells had the same t(14;18) breakpoint, indicating clonal evolution from a single FL cell (Matolcsy *et al.*, 1999).

However, some patients had several distinct t(14;18) translocation junctions, detected in both unprogressed (8/44) and progressed (1/6) FL (Matolcsy, 1999). Thus, such studies suggest that FL and DLBCL occurring in the same patient may either be clonally related or unrelated (Muller-Hermelink *et al.*, 2001). Furthermore, unprogressed, biclonal FL may be more common than previously recognised.

6.1.3 Progression of MALT lymphomas

The MALT lymphomas are B cell tumours that can show a spectrum of histological variants, ranging from low-grade to high-grade (Matolcsy, 1999). Infection by *Helicobacter felis* in a mouse model of MALT lymphoma has been shown to cause morphological progression of low-grade MALT lymphoma toward intermediate and high-grade disease, indicating that persistent antigenic stimulation by *Helicobacter felis* may be the cause of tumour progression (Matolcsy, 1999). Other studies have shown that loss of $\alpha4\beta7$ integrin and L-selectin is associated with progression (Liu *et al.*, 2001). Furthermore, partial inactivation of p53 may promote the development of low-grade MALT lymphomas, whereas the complete inactivation may be associated with high-grade morphological progression (Du *et al.*, 1995). Like other types of lymphomas, both distinct and clonally identical DLBCL have been detected in patients with MALT lymphomas (Matolcsy, 1999; Muller-Hermelink *et al.*, 2001).

6.1.4 Progression of MCL

See Chapter 3.3.2.

6.2 Differential expression of cell cycle-associated genes in MCLs with different proliferative index

The expression of Ki-67, a nuclear protein, is strictly associated with cell proliferation and therefore routinely used to measure the fraction of proliferating cells in tumours (Schluter *et al.*, 1993). Silencing of the Ki-67 gene in a multiple myeloma cell line, using anti-sense oligonucleotides, inhibited the proliferation, indicating that the Ki-67 antigen may be an absolute requirement for maintaining cell proliferation (Schluter *et al.*, 1993). As discussed previously, morphological and clinical progression of MCL is associated with increased proliferation. Analysis of MCLs with differential Ki-67 expression is therefore an indirect method of identifying genes that are potentially involved in blastoid transformation and associated with decreased survival time in MCL.

6.2.1 Identification of a proliferation-associated signature in MCL

To identify the mechanisms involved in the increased proliferation of blastoid MCL, we analysed MCLs with different proliferative indices, using gene expression profiling (Affymetrix system) (paper III). The proliferative indices for the MCL samples were determined by immunohistochemical staining for Ki-67. A genetic signature of 32 genes was identified, separating high-proliferative MCLs from low. The significance of the

signature was confirmed by successfully assigning new MCLs with > 20% Ki-67^{+} cells to the group of MCLs with high-proliferative index, using hierarchical clustering (paper III). The increased expression of *Ki*-67 mRNA in the tumours with high proliferative index further validated the classification of the tumours, which was based on Ki-67 protein expression, into different groups.

The majority of the genes in the signature have a documented effect on proliferation, or are necessary for mechanisms associated with cell cycling. Of note, *cyclin D1*, the hallmark of MCL, was more up-regulated in the high-proliferative MCLs (paper III). Cyclin D1 promotes the transition from G_1 to S phase in the cell cycle (Donnellan *et al.*, 1998; Stacey, 2003). Furthermore, *cyclin F*, which is involved in a later phase of the cell cycle, the G_2-M phase transition (Bai *et al.*, 1994; Fung *et al.*, 2002), was also significantly over-expressed in MCLs with high-proliferative index (paper III). The differential expression of the cyclin F protein was further confirmed in MCL paraffin sections from tumours with different proliferative indices. However, even if the majority of the genes in the "Ki-67 signature" supported increased cell cycling of the high-proliferative MCLs, no genes were the obvious initiators of this progression (paper III). As indicated previously, blastoid transformation is potentially associated with involvement of the CNS (Raty *et al.*, 2003). The spreading of the blastoid variant of MCL outside the lymphoid compartments indicates that the tumour cells have transformed and are able to grow independently of the microenvironment and GSs provided by the lymphoid organs. However, no up-regulated genes were identified that could promote independent growth, such as growth factor receptors (paper III).

As the increased proliferation is associated with the blastoid variant and decreased survival, genes over-expressed in the high-proliferative MCLs are of interest as therapeutic targets. Interestingly, *thymidylate synthetase* (*TYMS*), which was overexpressed in the high proliferative MCLs (paper III), is a potential target for chemo-therapeutic agents as the gene-product is involved in translational repression of p53 and the c-Myc family of transcription factors (Liu *et al.*, 2002). HMGA1 may also be a potential target, as the protein is necessary for modulation of chromosome structure and expression of the gene is associated with various types of tumours (Reeves *et al.*, 2003). The majority of the genes found to differ between high- and low-proliferative MCLs are, as indicated above, known to be involved in processes necessary for proliferation (paper III). Thus, most over-expressed genes are potential targets but the consequences of therapy directed against any of the genes or gene-products are difficult to survey as the genes are expressed in other cell types as well. To investigate how the expression of the genes in the "Ki-67 signature" correlated with proliferation of normal human tonsil B cells, the mean expression of the genes was compared in low- and high-proliferative MCLs together with pre-GC and GC B cells (Figure 6.1). A potential therapy would most likely target an over-expressed gene. Exclusion of the genes that are also associated with cell cycling in normal B cells leaves five over-expressed genes as potential targets: *HSPA6*, a member of the heat shock HSP70 family of molecular chaperones (Leung *et al.*, 1992); *HCK*, a hemopoietic protein tyrosine cell kinase (Hauses *et al.*, 1998); *HMGA1*, involved in cellular processes such as regulating inducible gene transcription and metastatic progression of cancer cells (Reeves *et al.*, 2003);

CCND1 (cyclin D1), involved in the G1 to S phase transition of the cell cycle (Bai *et al.*, 1994; Fung *et al.*, 2002), and *TREX2*, an exonuclease involved in DNA replication and repair (Shevelev *et al.*, 2002). Thus, although the majority of the genes in the Ki-67 signature were associated with proliferation, only a few are suitable targets for therapy, as most of the genes associated with proliferation also show increased expression in highly proliferating normal cells.

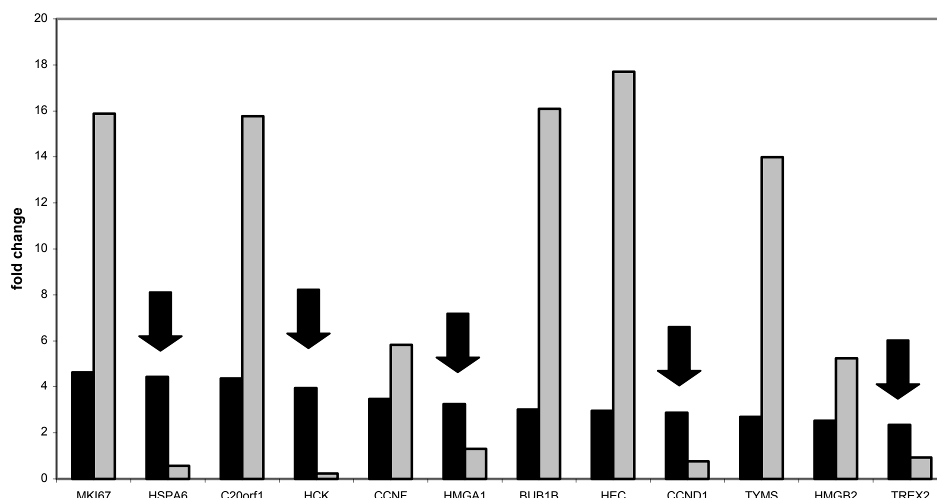


Figure 6.1. The presented genes are overexpressed in MCLs with a high proliferative index compared to MCL with a low proliferative index (paper III). The fold change in the MCL samples was calculated as ratios between highly proliferative MCL (n=2) and low proliferative MCL (n=7). The fold change for the normal B cells was calculated as the ratio between GC B cells (n=4) and pre-GC B cells (n=5). The arrows indicate which genes that are specifically overexpressed in MCL with high vs. low proliferation but not in GC vs. non GC B cells. Black bars represent MCL, grey bars represent normal B cells.

6.3 Analysis of recurrent and primary MCL

Although MCL patients respond to treatment, they soon relapse with recurrent tumours (Swerdlow *et al.*, 2002). Thus, in most cases MCL is an incurable malignancy (Bertoni *et al.*, 2002). Studies of relapses in leukaemia have served as a model of clonal progression, as reviewed by Naoe *et al.* (Naoe *et al.*, 2000). Generally, recurrent leukaemia cells are clonally selected and frequently resistant to chemotherapy. The observed changes in phenotype and karyotype are indicative of a continuous genetic evolution. Similarly, recurrent FLs are also clonally selected, as relapsed tumours show clonal identity, in contrast to the primary FLs that often show clonal heterogeneity (Zhu *et al.*, 1994).

6.3.1 Comparative analysis of recurrent and primary MCL

In an attempt to determine the genetic differences between recurrent and primary tumours, we compared patient samples at diagnosis and relapse, using gene expression profiling (paper III). To our knowledge, most antigens found to differentiate recurrent and primary MCL were not previously reported to be associated with relapse. However, *STAT1*, which was over-expressed in the recurrent MCL samples, has recently been reported to be expressed at increased levels after fludarabine treatment of chronic lymphocytic leukaemia (CLL) (Friedberg *et al.*, 2004). Thus, expression of *STAT1* may potentially mediate an important survival advantage for the lymphoma cells, as chemotherapy seems to select for high expression of *STAT1* (Friedberg *et al.*, 2004). Furthermore, an increased usage of the transferrin receptor was detected at relapse (paper III). Increased iron uptake is essential for proliferating cells, which thus highly express the transferrin receptor (Tusenius *et al.*, 1992; Bjerner *et al.*, 2002). To eliminate tumour cells efficiently, treatment strategies must strike at a wide range of mechanisms. Malignant cells often have numerous genetic alterations, which provide the cells with an exceptional ability to resist different therapies. Thus, a single therapy-resistant cell may give rise to a new, clonally selected, tumour with an adapted ability to survive.

Chapter 7

Cell lines as *in vitro* models for mantle cell lymphoma

7.1 Establishment and culture of cell lines

Since the beginning of the last century, temporary cell cultures have been used to perform experiments *ex vivo* (Drexler *et al.*, 2000). However, continuous cultures (i.e. cell lines) have obvious advantages since the supply of primary human cells is restricted. A cell culture is generally defined as immortalised, and thus continuous, after one year of uninterrupted growth (Drexler *et al.*, 2000). The process of establishing cell lines is hard to control and success seems to be random. The greatest success rates in immortalising lymphoma/leukaemia cell lines are achieved when using peripheral blood from patients at relapse; these cell lines are established with a success rate of ~50% (Drexler, 2001). The common method for establishing cell lines is simply to seed the tumour cells into cell culture media, keeping them at 37°C in a 5% CO_2 atmosphere. Since success rates are low, other methods have been developed to improve the number of established cell lines, as discussed below.

7.1.1 Use and effect of Epstein Barr virus (EBV) for immortalisation of cell cultures

One method frequently used for establishing cell cultures is immortalisation using EBV. EBV is associated with the concurrency of both lymphoid and epithelial tumours, and is commonly detected in for example Burkitt's lymphoma and Hodgkin's disease (Young *et al.*, 2003). When using EBV to immortalise tumour cells, the success rates are improved. However, it is essential to authenticate these cell lines, since normal cells are also immortalised by EBV and may acquire malignant properties, thus resembling tumour cells (Drexler *et al.*, 2000). One of the mechanisms involved in the increased success rate of establishing EBV-immortalised cell lines is the increased resistance to apoptosis (Fagard *et al.*, 2002). The sister cell lines SP53 and SP50B, which were established from the same MCL patient, are negative and positive for EBV, respectively (Daibata *et al.*, 1996). These cell lines have been studied to determine the effect of EBV infection in human

malignancies (Daibata *et al.*, 1996). In contrast to SP53, SP50B showed malignant features, such as the ability to grow in low serum concentrations, colony formation in soft agarose and tumorigenicity in nude mice, (Daibata *et al.*, 1996).

7.1.2 General problems associated with the use of cell lines

To be suitable as continuous models, cell lines must be stable, i.e. display the same phenotype and preserve genetic alterations, over time. Frequent problems with cross-contamination, mycoplasma infections and genetic instability may completely change the characteristics of cell lines, making them useless for most applications.

Cross-contamination is a common problem when handling cell lines, as reviewed by Drexler et al. (Drexler *et al.*, 1999). One of the most famous examples of cross-contamination is the cell line J-111, which was established as the first human hematopoietic cell line in 1955 (Osgood *et al.*, 1955). J-111 was later proved to be the HeLa cell line, the first solid tumour-derived cell line that was established in 1951 (Drexler *et al.*, 2000).

The second major problem is the common infections with mycoplasma. In fact, 30% of the cell lines in the German collection of micro-organisms and cell cultures (DSMZ) examined between 1989 and 2000 were positive for mycoplasma (Drexler *et al.*, 2000). Problems with cross-contamination and mycoplasma infections seem to be related, as 50% of cross-contaminated cell lines have been shown to be mycoplasma-positive, whereas the corresponding number for authenticated cell lines is 20% (Drexler *et al.*, 2002). This indicates that inferior handling of cell lines is the cause of both cross-contamination and mycoplasma infections.

A third general concern when using cell lines is their genetic stability. Although some report that genetic stability is characteristic of leukaemia/lymphoma cell lines (Drexler *et al.*, 2000), other frequently show that sub-clones of the original cell lines develop different characteristics over time (Zhang *et al.*, 2001).

7.2 Lymphoma/leukaemia cell lines

Cell lines have been used as in vitro models of lymphomas since 1963, when Raji was established from a patient with Burkitt's lymphoma (Pulvertaft, 1964; Drexler *et al.*, 2000). There are more than 250 established B cell lymphoma/leukaemia cell lines, but only approximately 100 of them have been characterised with regard to clinical, cell culture, immunophenotypic, genetic and functional features (Drexler *et al.*, 2000).

7.2.1 Usefulness of lymphoma/leukaemia cell lines

Lymphoma/leukaemia cell lines have valuable distinctive features. For example, the cells are monoclonal, they are arrested in a specific differentiation stage and they are autonomous in GSs (Drexler *et al.*, 2000). The usefulness of lymphoma/leukaemia cell lines is vast and examples of possible applications are screening of monoclonal antibodies, pharmaceutical

drugs and hormones, and selection of sub-clones based on specific features, such as drug resistance or additional chromosomal or molecular aberrations (Drexler *et al.*, 1998).

Cell lines may also be used to study cellular mechanisms, such as somatic hypermutation. Ramos, a Burkitt's lymphoma cell line, has for example been used to study somatic hypermutation, as both stable and continuously mutating sub-clones have been isolated from the cell line (Zhang *et al.*, 2001).

Further applications are the use of cell lines for studying functional characteristics. An example is the study of cell lines derived from DLBCL by Davis and colleagues (Davis *et al.*, 2001). As discussed in the chapter on microarray analysis, two sub-types of DLBCL with different characteristics have been identified (Alizadeh *et al.*, 2000). Cell lines resembling either the activated B cell line (ABC) DLBCL or the GC-derived DLBCL have been used to study the differences between these two distinct types of DLBCL (Davis *et al.*, 2001).

7.2.2 History of MCL cell lines

Establishment of MCL cell lines has been difficult and only a small number of cell lines are available. A recent review has summarised the majority of MCL-derived cell lines and their main features (Drexler *et al.*, 2002). In total, cell lines from 8 patients diagnosed with MCL have been published as either MCL-derived or possibly MCL-derived (Drexler *et al.*, 2002; Amin *et al.*, 2003). The MCL-derived cell lines are Granta 519 (Jadayel *et al.*, 1997), HF-4a/-4b, JeKo-1 (Jeon *et al.*, 1998), Mino (Lai *et al.*, 2002) and Rec-1, and the possibly MCL-derived cell lines are HBL-2, NCEB-1 (Saltman *et al.*, 1988), and SP49/-50B/-53 (Daibata *et al.*, 1989; Jadayel *et al.*, 1997). Further characterisation of these cell lines is necessary to determine their usefulness as *in vitro* models for MCL tumours. The few published functional studies have explored the adenoviral transduction efficiency (Amin *et al.*, 2003), or the effect of inhibiting NFκB activation (Bogner *et al.*, 2003; Pham *et al.*, 2003). A mouse model of MCL has been created using the cell line UPN1 (M'Kacher *et al.*, 2003), and may hopefully be used as a complement to the cell line-based *in vitro* models.

7.2.3 MCL cell lines as *in vitro* models for MCL tumours

Analysing MCL using expression profiling was a first step to identify potential targets for therapy (paper I) (see Chapter 5). To evaluate the function of the genes and the corresponding gene products that we found to be up-regulated in MCL as compared to normal B cells, well-characterised and suitable *in vitro* models are of central importance. Since *in vitro* studies require continuous access to cells, and since MCL tumours are scarce, it was important to evaluate the genotypic and phenotypic properties of cell lines that could be used as models for MCL (paper IV). To our knowledge, MCL cell lines have not previously been used in microarray studies and compared to normal B cells or primary MCL.

We attempted to collect the majority of the MCL-derived, or possible MCL-derived cell lines, but due to problems with availability, we performed our study using just three cell lines (SP53, Granta 519 and NCEB1).

SP53 was originally established from peripheral blood from a 58-year-old woman (Daibata *et al.*, 1989) afflicted with MCL, but the cell line has not been authenticated (Drexler *et al.*, 2002). Authentication is normally performed using DNA fingerprinting or cytogenetic analysis to determine that the given cell line was derived from the presumed patients, as cross-contamination has been a major problem with human cell lines (Drexler *et al.*, 1999; Drexler *et al.*, 2002). Granta 519 and NCEB1, which have been authenticated, were established from peripheral blood and were EBV-transformed to enable continuous growth (Saltman *et al.*, 1988; Jadayel *et al.*, 1997).

To determine the characteristics of the cell lines and their usefulness as *in vitro* models for MCL, a combination of gene expression profiling and mutational analysis, phenotypic characterisation and functional studies was performed (paper IV). In addition to MCL cell lines, an FL cell line (RL), a Burkitt lymphoma cell line (Raji), primary tumours (MCL (n=2) and FL (n=5)), and five human B-cell populations were used for the microarray analysis.

The similarity of the MCL-derived cell lines to the primary MCLs was determined by using an MCL-specific gene list. This gene list was created by comparing primary MCL samples (n=19) with B cells from five separate differentiation stages (n=11), isolated from tonsils from healthy individuals. Genes significantly up-regulated in MCL (n=55) were chosen for the analysis, as products of up-regulated genes are more likely to be targets for therapy than products of down-regulated genes. Using hierarchical clustering, the MCL cell lines were shown to be associated with the primary MCL tumours, although the association of NCEB1 to the primary unmutated MCL tumours was not statistically significant ($p > 0.05$). However, all of the MCL cell lines were distinctly separated from the FL and Burkitt's lymphoma cell lines and primary FL tumours (paper IV). It is noteworthy that the primary MCL used unmutated V_H genes and, as discussed further below, NCEB1 used mutated V_H genes.

MCL harbours a sub-group with mutated V_H genes (Pittaluga *et al.*, 1998; Laszlo *et al.*, 2000; Welzel *et al.*, 2001; Thorselius *et al.*, 2002; Camacho *et al.*, 2003; Kienle *et al.*, 2003). To determine whether any of the MCL cell lines showed mutated V_H genes, the frequency of somatic mutations in the different lymphoma cell lines was determined (paper IV). Moreover, a restricted V_H usage has been reported for MCL, as discussed previously (Chapter 3) (Thorselius *et al.*, 2002; Camacho *et al.*, 2003). Thus, the V_H-gene family usage was determined and, interestingly, SP53 was shown to use V_H 4-34 – which is one of the most commonly used V_H genes in MCL – while Granta 519 and NCEB1 used random V_H genes (paper IV).

The frequency of somatic mutations in the cell lines was determined by comparing the V_H gene sequences with germline sequences for different V_H gene families. As expected, RL and Raji displayed mutated V_H genes, 11.8% and 15.3% respectively, characteristic of a GC or post-GC origin. The MCL cell lines showed differences in frequency of somatic mutations, ranging from zero (Granta 519) to 2.1% (NCEB1), but had distinctly less

mutations than RL and Raji. According to normal standards, a V_H gene with less than 98% similarity to the germline sequence is considered to correspond to a somatically mutated V_H gene (Hamblin *et al.*, 1999) (discussed in Chapter 4). Thus, NCEB1 was considered to be somatically mutated, while the other MCL cell lines were considered to be unmutated. The differences in mutation frequency and the associated changes in phenotype may have implications for how the cell lines respond to stimuli, and the mutational status should thus be considered when using the cell lines for *in vitro* studies. The increased number of somatic mutations in NCEB1 correlated with the down-regulation of several differentiation-associated antigens, such as CCR7, CCR6, CD44 and CD31 (paper IV). However, up-regulation of typical GC markers, such as CD10 and CD77, was only detected in the GC-derived cell lines (RL and Raji) (paper IV).

In summary, gene expression profiling is a unique tool when attempting to determine the relationship between different samples. Genetic signatures can be studied and compared, as a complement to the use of discrete phenotypic or genetic markers. Using gene expression profiling, SP53 and Granta 519 showed a high similarity to the primary MCL, based on the expression of 55 genes, constituting an MCL-specific gene signature (paper IV). NCEB1 was also separated from the other lymphoma cell lines and primary tumours, but the association was not statistically significant. NCEB1, which showed 2.1% deviation from the germline sequence, may potentially be a better model for somatically mutated than for unmutated MCL tumours, although this remains to be determined.

Chapter 8

Concluding remarks

This thesis is based upon four original papers investigating the characteristics of MCL tumours and cell lines in comparison with different populations of normal B cells. The studies were performed using the microarray technology, which has developed as an important tool in characterisation of different types of malignancies during recent years. The technique provides information about the gene expression of thousands of genes in parallel and may be used to identify gene signatures associated with a sample or groups of samples. Thus, a global view of the transcriptome is achieved, enabling, for example, identification of the normal counterparts of different malignancies or measurement of the similarities between different samples.

The main finding presented in paper I was that the expression of several genes indicated that MCL has an antigen-activated origin, which is in contrast to the current dogma. De-regulation of apoptosis and proliferation-associated pathways were further identified and possibly therapeutic targets were recognised. Additional evidence for an antigen-activated origin of MCL and different survival strategies were presented in paper II. The origin of the sub-group of MCL with mutated V_H genes was also discussed. In an attempt to define the differences between high and low proliferative MCL, a proliferation-associated signature was established in paper III. Since the genes constituting the proliferative signature are associated with proliferation, which is related with decreased survival time in MCL patients, they are interesting targets for therapy.

In order to study the effect of individual genes on cellular events as proliferation and apoptosis, continuous access to MCL cells are needed. In paper IV, MCL cell lines (SP53, Granta 519, NCEB1) were studied to determine their value as *in vitro* models for MCL. Hierarchical clustering, using a MCL-specific gene list, showed that the cell lines had preserved the expression of MCL-associated genes, as they were readily separated from other lymphoma cell lines, primary FL samples and normal B cells. However, one of the MCL-derived cell lines (NCEB1) lacked statistical significance in the clustering to the primary unmutated MCL samples. This cell line further carried a mutated V_H gene, in contrast to the other MCL cell lines, and may potentially be more suitable as an *in vitro* model for MCL with mutated than unmutated V_H genes.

One of the main goals of this thesis has been to characterise the MCL tumours in relation to normal B cell differentiation. It is important to investigate and establish which the normal counterpart is, in order to understand the potential interactions with the microenvironment, neighbouring cells and soluble factors, which may influence the behaviour of the tumour cell. The identification of possible therapeutic targets has further been emphasised, since improved therapy is needed to prolong survival. The papers, which the thesis is based upon, mainly focus on characterised genes, or their gene-products, as potential targets for therapy. The functional effect of these genes can be studied, using different techniques to silence gene expression in *in vitro* models, to determine whether they are involved in for example proliferation or resistance to apoptosis. However, information about uncharacterised genes, such as cDNA clones and expressed sequence tags (ESTs), has also been gathered. In fact, antibodies against 50–100 uncharacterised gene-products, which are overexpressed in MCL, are being raised and will be used to identify the potentially corresponding proteins, which may be future targets for new treatment strategies of MCL. Thus, the focus of the project will, in the near future, move further from characterised genes to uncharacterised proteins! So I finally conclude, "Let's move beyond gene listing and get down to the biology" (adapted from (Liang *et al.*, 2003).

Populärvetenskaplig sammanfattning

Immunförsvaret har till uppgift att skydda kroppen mot angrepp i form av virus, bakterier eller andra sjukdomsalstrande organismer eller ämne, sk patogener. Flera olika typer av celler och molekylära mekanismer, med unika funktioner, är involverade i detta försvar. De celler som jag har intresserat mig för är B-celler som i immunförsvaret har till sin främsta uppgift att producera antikroppar som kan binda till och oskadliggöra olika patogener. Ironiskt nog kan de celler som är till för att skydda oss också ge upphov till många olika typer av cancer. Vi har studerat Mantel cells lymfom (MCL), en typ av B-cells cancer som ger upphov till tumörer i lymfnoder och som också kan sprida sig till mjälte, mag-tarm systemet och benmärgen.

Genom att använda en teknik som kan ge information om genuttrycket, dvs om genen är vilande eller om genen används, har vi samlat information om mer än 12 000 gener i MCL prover från 21 patienter. Denna information jämförs med hur samma gener är uttryckta i B-celler från friska individer. De flesta generna används inte eller skiljer sig inte åt i uttryck mellan cancer- och normala celler, men ett hundratal gener var signifikant förändrade. Vi har analyserat dessa gener och försökt förstå vad det förändrade genmönstret har för betydelse för t ex tumörens förmåga att växa och undvika att bli dödad av immunförsvaret. I manuskript III diskuteras hur vi har identifierat ett antal gener som är viktiga för ökningen av tillväxthastighet som sker i de flesta MCL tumörer och som är associerade med en kortare överlevnad. Ett annat syfte med studien har varit att hitta gener som eventuellt skulle kunna användas som mål för en ny terapi av MCL. De gener eller genprodukter (proteiner) som är viktiga för tumörens snabba tillväxt och/eller överlevnadsförmåga är lämpliga mål för t ex en antikroppsbaserad terapi. Antikropparna binder specifikt in till målet och kan t ex blockera upptag av nödvändiga tillväxtämnen till cellen. Dock är det viktigt att dessa gener eller genprodukter inte också är nödvändiga för andra celler i kroppen eftersom de friska cellerna då skulle påverkas av behandlingen dvs urskiljningsförmågan (specificiteten) är viktig för att minska graden av biverkningar. De gener som är involverade i ökningen av tillväxthastigheten, som diskuteras ovan, är exempel på gener som eventuellt skulle vara intressanta mål för behandling av MCL.

Det område som jag har fokuserat på i den här avhandlingen (Manuskript I-II) är när, i vilket normalt utvecklingsstadie, B-cellen blir en tumörcell. Detta är viktigt eftersom man då skulle kunna förstå vilka normala cell-mekanismer som är ur funktion. De resultat vi ser i analysen av MCL i jämförelse med normala B-celler visar att tumörcellerna har ett mer aktiverat ursprung än vad som tidigare beskrivs i litteraturen. Våra slutsatser baserar vi på att tumörcellerna använder gener som i normala fall bara används av celler som har blivit stimulerade eller att gener som normalt används i ostimulerade B-celler saknas. Andra forskargrupper har också nyligen publicerat resultat som visar att en stor andel av MCL verkar producera samma typ av antikroppar, vilket också tyder på att cellerna har blivit utvalda och aktiverade.

För att kunna studera MCL krävs att man under lång tid har möjlighet att studera cellerna. Eftersom tumörceller snabbt dör utanför kroppen är det viktigt att använda celler som på olika sätt har behandlats så att de kan växa i en näringslösning under vissa betingelser (37°C, 5% CO_2 tryck), sk cellinjer. Det finns ett antal cellinjer som är etablerade från MCL celler och som ska kunna fungera som modeller för denna tumörtyp. Dock är informationen om likheten jämfört med de ursprungliga cellerna otillfredsställande. Vi har genom att använda den tidigare beskrivna tekniken, där tusentals gener kan analyseras, jämfört tre MCL cellinjer med MCL från patienter och även med andra typer av cellinjer och patientprover (Manuskript IV). Vi kom fram till att de MCL cellinjerna som vi har studerat (SP53, Granta 519 och NCEB1) mycket väl kan användas som modeller för MCL eftersom genanvändningen var mycket lik den i MCL från patienterna.

Arbetet kring MCL är inte avslutat och nästa fas i projektet, som inte ryms inom den här avhandlingen, kommer bestå i att utvärdera de antikroppar som är framtagna (samarbete med KTH, Kungliga Tekniska Högskolan) mot 50-100 av genprodukterna som används i MCL men inte i normala B-celler. Dessutom kommer funktionen av vissa gener att testas genom att blockera uttrycket av dem i MCL cellinjer och studera hur cellerna klarar sig utan genen.

“Education is an admirable thing, but it is well to remember that nothing that is worth knowing can be taught.”
Oscar Wilde

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References

Agematsu, K. (2000). "Memory B cells and CD27." Histol Histopathol **15**(2): 573-6.

Agematsu, K., H. Nagumo, et al. (1997). "B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production." Eur J Immunol **27**(8): 2073-9.

Ager, A. (1997). "Regulation of lymphocyte migration into lymph nodes by high endothelial venules." Biochem Soc Trans **25**(2): 421-8.

Airoldi, I., L. Raffaghello, et al. (2004). "Heterogeneous expression of interleukin-18 and its receptor in B-cell lymphoproliferative disorders deriving from naive, germinal center, and memory B lymphocytes." Clin Cancer Res **10**(1 Pt 1): 144-54.

Akasaka, T., I. S. Lossos, et al. (2003). "BCL6 gene translocation in follicular lymphoma: a harbinger of eventual transformation to diffuse aggressive lymphoma." Blood **102**(4): 1443-8.

Alizadeh, A. A., M. B. Eisen, et al. (2000). "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling." Nature **403**(6769): 503-11.

Alizadeh, A. A., D. T. Ross, et al. (2001). "Towards a novel classification of human malignancies based on gene expression patterns." J Pathol **195**(1): 41-52.

Altieri, D. C. (2003). "Survivin, versatile modulation of cell division and apoptosis in cancer." Oncogene **22**(53): 8581-9.

Amin, H. M., T. J. McDonnell, et al. (2003). "Characterization of 4 mantle cell lymphoma cell lines." Arch Pathol Lab Med **127**(4): 424-31.

Appay, V. and S. L. Rowland-Jones (2001). "RANTES: a versatile and controversial chemokine." Trends Immunol **22**(2): 83-7.

Babbage, G., R. Garand, et al. (2004). "Mantle cell lymphoma with t(11;14) and unmutated or mutated VH genes expresses AID and undergoes isotype switch events." Blood **103**(7): 2795-8.

Baekkevold, E. S., T. Yamanaka, et al. (2001). "The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment." J Exp Med **193**(9): 1105-12.

Bahler, D. W. and S. H. Swerdlow (1998). "Clonal salivary gland infiltrates associated with myoepithelial sialadenitis (Sjogren's syndrome) begin as nonmalignant antigen-selected expansions." Blood **91**(6): 1864-72.

Bai, C., R. Richman, et al. (1994). "Human cyclin F." Embo J **13**(24): 6087-98.

Banks, P. M., J. Chan, et al. (1992). "Mantle cell lymphoma. A proposal for unification of morphologic, immunologic, and molecular data." Am J Surg Pathol **16**(7): 637-40.

Bayerdorffer, E., A. Neubauer, et al. (1995). "Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of Helicobacter pylori infection. MALT Lymphoma Study Group." Lancet **345**(8965): 1591-4.

Bea, S., A. Lopez-Guillermo, et al. (2002). "Genetic imbalances in progressed B-cell chronic lymphocytic leukemia and transformed large-cell lymphoma (Richter's syndrome)." Am J Pathol **161**(3): 957-68.

Bea, S., M. Ribas, et al. (1999). "Increased number of chromosomal imbalances and high-level DNA amplifications in mantle cell lymphoma are associated with blastoid variants." Blood **93**(12): 4365-74.
Bertoni, F., M. Ghielmini, et al. (2002). "Mantle cell lymphoma: new treatments targeted to the biology." Clin Lymphoma **3**(2): 90-6.
Bertoni, F., E. Zucca, et al. (2004). "Molecular basis of mantle cell lymphoma." Br J Haematol **124**(2): 130-40.
Bjerner, J., L. M. Amlie, et al. (2002). "Serum levels of soluble transferrin receptor correlate with severity of disease but not with iron stores in patients with malignant lymphomas." Tumour Biol **23**(3): 146-53.
Bogenrieder, T. and M. Herlyn (2003). "Axis of evil: molecular mechanisms of cancer metastasis." Oncogene **22**(42): 6524-36.
Bogner, C., I. Ringshausen, et al. (2003). "Inhibition of the proteasome induces cell cycle arrest and apoptosis in mantle cell lymphoma cells." Br J Haematol **122**(2): 260-8.
Boye, J., T. Elter, et al. (2003). "An overview of the current clinical use of the anti-CD20 monoclonal antibody rituximab." Ann Oncol **14**(4): 520-35.
Bystry, R. S., V. Aluvihare, et al. (2001). "B cells and professional APCs recruit regulatory T cells via CCL4." Nat Immunol **2**(12): 1126-32.
Cairns, R. A., R. Khokha, et al. (2003). "Molecular mechanisms of tumor invasion and metastasis: an integrated view." Curr Mol Med **3**(7): 659-71.
Camacho, F. I., P. Algara, et al. (2003). "Molecular heterogeneity in MCL, defined by the use of specific VH genes and the frequency of somatic mutations." Blood.
Casamayor-Palleja, M., P. Mondiere, et al. (2002). "BCR ligation reprograms B cells for migration to the T zone and B-cell follicle sequentially." Blood **99**(6): 1913-21.
Cech, T. R. (2004). "Beginning to understand the end of the chromosome." Cell **116**(2): 273-9.
Chee, M., R. Yang, et al. (1996). "Accessing genetic information with high-density DNA arrays." Science **274**(5287): 610-4.
Chen, G. and D. V. Goeddel (2002). "TNF-R1 signaling: a beautiful pathway." Science **296**(5573): 1634-5.
Chiu, K. C., M. Fine, et al. (2003). "Telomerase activity and proliferation index in aggressive mature B-cell lymphoma: comparison to germinal center phenotypic markers." Hum Pathol **34**(12): 1259-64.
Cohen, Y., N. Da'as, et al. (2002). "Large-cell transformation of chronic lymphocytic leukemia and follicular lymphoma during or soon after treatment with fludarabine-rituximab-containing regimens: natural history- or therapy-related complication?" Eur J Haematol **68**(2): 80-3.
Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**(6917): 860-7.
Daibata, M., I. Kubonishi, et al. (1989). "The establishment of Epstein-Barr virus nuclear antigen-positive (SP-50B) and Epstein-Barr virus nuclear antigen-negative (SP-53) cell lines with t(11;14)(q13;q32) chromosome abnormality from an intermediate lymphocytic lymphoma." Cancer **64**(6): 1248-53.

Daibata, M., I. Kubonishi, et al. (1996). "Differential tumorigenicity between Epstein-Barr virus genome-positive and genome-negative cell lines with t(11;14)(q13;q32) derived from mantle cell lymphoma." J Virol **70**(12): 9003-7.
Davis, R. E., K. D. Brown, et al. (2001). "Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells." J Exp Med **194**(12): 1861-74.
Davis, R. E. and L. M. Staudt (2002). "Molecular diagnosis of lymphoid malignancies by gene expression profiling." Curr Opin Hematol **9**(4): 333-8.
de Vos, S., W. K. Hofmann, et al. (2003). "Gene expression profile of serial samples of transformed B-cell lymphomas." Lab Invest **83**(2): 271-85.
De Vos, S., U. Krug, et al. (2003). "Cell Cycle Alterations in the Blastoid Variant of Mantle Cell Lymphoma (MCL-BV) as Detected by Gene Expression Profiling of Mantle Cell Lymphoma (MCL) and MCL-BV." Diagn Mol Pathol **12**(1): 35-43.
Dong, H. Y., A. Shahsafaei, et al. (2002). "CD148 and CD27 are expressed in B cell lymphomas derived from both memory and naive B cells." Leuk Lymphoma **43**(9): 1855-8.
Donnellan, R. and R. Chetty (1998). "Cyclin D1 and human neoplasia." Mol Pathol **51**(1): 1-7.
Dono, M., V. L. Burgio, et al. (1996). "Subepithelial B cells in the human palatine tonsil. I. Morphologic, cytochemical and phenotypic characterization." Eur J Immunol **26**(9): 2035-42.
Dono, M., S. Zupo, et al. (1996). "Subepithelial B cells in the human palatine tonsil. II. Functional characterization." Eur J Immunol **26**(9): 2043-9.
Dono, M., S. Zupo, et al. (2003). "The human marginal zone B cell." Ann N Y Acad Sci **987**: 117-24.
Dono, M., S. Zupo, et al. (2000). "Heterogeneity of tonsillar subepithelial B lymphocytes, the splenic marginal zone equivalents." J Immunol **164**(11): 5596-604.
Drexler, H. G. (2001). The Leukemia-Lymphoma Cell line Facts Book, Academic Press.
Drexler, H. G., W. G. Dirks, et al. (1999). "False human hematopoietic cell lines: cross-contaminations and misinterpretations." Leukemia **13**(10): 1601-7.
Drexler, H. G. and R. A. MacLeod (2002). "Malignant hematopoietic cell lines: in vitro models for the study of mantle cell lymphoma." Leuk Res **26**(9): 781-7.
Drexler, H. G., A. Y. Matsuo, et al. (2000). "Continuous hematopoietic cell lines as model systems for leukemia-lymphoma research." Leuk Res **24**(11): 881-911.
Drexler, H. G. and J. Minowada (1998). "History and classification of human leukemia-lymphoma cell lines." Leuk Lymphoma **31**(3-4): 305-16.
Drexler, H. G., C. C. Uphoff, et al. (2002). "Mix-ups and mycoplasma: the enemies within." Leuk Res **26**(4): 329-33.
Du, M., H. Peng, et al. (1995). "The accumulation of p53 abnormalities is associated with progression of mucosa-associated lymphoid tissue lymphoma." Blood **86**(12): 4587-93.
Dunham, M. A., A. A. Neumann, et al. (2000). "Telomere maintenance by recombination in human cells." Nat Genet **26**(4): 447-50.

Ek, S., C. M. Hogerkorp, et al. (2002). "Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells." Cancer Res **62**(15): 4398-405.

Fagard, R., H. Mouas, et al. (2002). "Resistance to fludarabine-induced apoptosis in Epstein-Barr virus infected B cells." Oncogene **21**(29): 4473-80.

Fang, N. Y., T. C. Greiner, et al. (2003). "Oligonucleotide microarrays demonstrate the highest frequency of ATM mutations in the mantle cell subtype of lymphoma." Proc Natl Acad Sci U S A **100**(9): 5372-7.

Fisher, D. E. (2001). "The p53 tumor suppressor: critical regulator of life & death in cancer." Apoptosis **6**(1-2): 7-15.

Freedman, A. S., J. M. Munro, et al. (1992). "Follicular non-Hodgkin's lymphoma cell adhesion to normal germinal centers and neoplastic follicles involves very late antigen-4 and vascular cell adhesion molecule-1." Blood **79**(1): 206-12.

Fridman, J. S. and S. W. Lowe (2003). "Control of apoptosis by p53." Oncogene **22**(56): 9030-40.

Fridman, R., M. Toth, et al. (2003). "Cell surface association of matrix metalloproteinase-9 (gelatinase B)." Cancer Metastasis Rev **22**(2-3): 153-66.

Friedberg, J. W., D. A. Dong, et al. (2004). "Oral fludarabine has significant activity in patients with previously untreated chronic lymphocytic leukemia, and leads to increased STAT1 levels in vivo." Leuk Res **28**(2): 139-47.

Fung, T. K., W. Y. Siu, et al. (2002). "Cyclin F is degraded during G2-M by mechanisms fundamentally different from other cyclins." J Biol Chem **277**(38): 35140-9.

Golab, J. (2000). "Interleukin 18--interferon gamma inducing factor--a novel player in tumour immunotherapy?" Cytokine **12**(4): 332-8.

Golub, T. R., D. K. Slonim, et al. (1999). "Molecular classification of cancer: class discovery and class prediction by gene expression monitoring." Science **286**(5439): 531-7.

Greider, C. W. and E. H. Blackburn (1985). "Identification of a specific telomere terminal transferase activity in Tetrahymena extracts." Cell **43**(2 Pt 1): 405-13.

Grossbard, M. L. (2002). Malignant lymphomas. London, BC Decker Inc.

Gurrieri, C., P. McGuire, et al. (2002). "Chronic lymphocytic leukemia B cells can undergo somatic hypermutation and intraclonal immunoglobulin V(H)DJ(H) gene diversification." J Exp Med **196**(5): 629-39.

Haberman, A. M. and M. J. Shlomchik (2003). "Reassessing the function of immune-complex retention by follicular dendritic cells." Nat Rev Immunol **3**(9): 757-64.

Hamblin, T. J., Z. Davis, et al. (1999). "Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia." Blood **94**(6): 1848-54.

Hampton, G. M. and H. F. Frierson (2003). "Classifying human cancer by analysis of gene expression." Trends Mol Med **9**(1): 5-10.

Han, S., S. R. Dillon, et al. (1997). "V(D)J recombinase activity in a subset of germinal center B lymphocytes." Science **278**(5336): 301-5.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hardy, R. R. (2003). B-Lymphocyte Development and Biology. Fundamental Immunology. W. E. Paul. Philadelphia, Lippincott Williams & Wilkins.

Harris, N. L., E. S. Jaffe, et al. (2000). "The World Health Organization classification of hematological malignancies report of the Clinical Advisory Committee Meeting, Airlie House, Virginia, November 1997." Mod Pathol **13**(2): 193-207.

Hauses, M., R. R. Tonjes, et al. (1998). "The transcription factor Sp1 regulates the myeloid-specific expression of the human hematopoietic cell kinase (HCK) gene through binding to two adjacent GC boxes within the HCK promoter-proximal region." J Biol Chem **273**(48): 31844-52.

Hazlehurst, L. A., T. H. Landowski, et al. (2003). "Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death." Oncogene **22**(47): 7396-402.

He, X., J. J. Goronzy, et al. (1995). "VH3-21 B cells escape from a state of tolerance in rheumatoid arthritis and secrete rheumatoid factor." Mol Med **1**(7): 768-80.

Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature **407**(6805): 770-6.

Hiddemann, W. and M. Dreyling (2003). "Mantle cell lymphoma: therapeutic strategies are different from CLL." Curr Treat Options Oncol **4**(3): 219-26.

Hofmann, W. K., S. de Vos, et al. (2001). "Altered apoptosis pathways in mantle cell lymphoma detected by oligonucleotide microarray." Blood **98**(3): 787-94.

Jadayel, D. M., J. Lukas, et al. (1997). "Potential role for concurrent abnormalities of the cyclin D1, p16CDKN2 and p15CDKN2B genes in certain B cell non-Hodgkin's lymphomas. Functional studies in a cell line (Granta 519)." Leukemia **11**(1): 64-72.

Jeon, H. J., C. W. Kim, et al. (1998). "Establishment and characterization of a mantle cell lymphoma cell line." Br J Haematol **102**(5): 1323-6.

Kienle, D., A. Krober, et al. (2003). "VH mutation status and VDJ rearrangement structure in mantle cell lymphoma: correlation with genomic aberrations, clinical characteristics, and outcome." Blood **102**(8): 3003-9.

Kikutani, H., M. Suemura, et al. (1986). "Fc epsilon receptor, a specific differentiation marker transiently expressed on mature B cells before isotype switching." J Exp Med **164**(5): 1455-69.

Klein, U., K. Rajewsky, et al. (1998). "Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells." J Exp Med **188**(9): 1679-89.

Klein, U., Y. Tu, et al. (2003). "Gene expression dynamics during germinal center transit in B cells." Ann N Y Acad Sci **987**: 166-72.

Kosmas, C., K. Stamatopoulos, et al. (2000). "Origin and diversification of the clonogenic cell in multiple myeloma: lessons from the immunoglobulin repertoire." Leukemia **14**(10): 1718-26.

Krzysiek, R., E. A. Lefevre, et al. (2000). "Regulation of CCR6 chemokine receptor expression and responsiveness to macrophage inflammatory protein-3alpha/CCL20 in human B cells." Blood **96**(7): 2338-45.

Krzysiek, R., E. A. Lefevre, et al. (1999). "Antigen receptor engagement selectively induces macrophage inflammatory protein-1 alpha (MIP-1 alpha) and MIP-1 beta chemokine production in human B cells." J Immunol **162**(8): 4455-63.

Kucharczak, J., M. J. Simmons, et al. (2003). "To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis." Oncogene **22**(56): 8961-82.

Kuroki, M., H. Shibaguchi, et al. (2003). "Immunotherapy and gene therapy of cancer using antibodies or their genes against tumor-associated antigens." Anticancer Res **23**(6a): 4377-81.

Kurtin, P. J. (1998). "Mantle cell lymphoma." Adv Anat Pathol **5**(6): 376-98.

Ladetto, M., M. Compagno, et al. (2004). "Telomere length correlates with histopathogenesis according to the germinal center in mature B-cell lymphoproliferative disorders." Blood.

Lai, R., T. J. McDonnell, et al. (2002). "Establishment and characterization of a new mantle cell lymphoma cell line, Mino." Leuk Res **26**(9): 849-55.

Lam, K. P., R. Kuhn, et al. (1997). "In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death." Cell **90**(6): 1073-83.

Lardelli, P., M. A. Bookman, et al. (1990). "Lymphocytic lymphoma of intermediate differentiation. Morphologic and immunophenotypic spectrum and clinical correlations." Am J Surg Pathol **14**(8): 752-63.

Laszlo, T., M. Nagy, et al. (2000). "Immunoglobulin V(H) gene mutational analysis suggests that blastic variant of mantle cell lymphoma derives from different stages of B-cell maturation." Leuk Res **24**(1): 27-31.

Lennert, K., H. Stein, et al. (1975). "Cytological and functional criteria for the classification of malignant lymphomata." Br J Cancer **31 SUPPL 2**: 29-43.

Lens, S. M., P. Drillenburg, et al. (1999). "Aberrant expression and reverse signalling of CD70 on malignant B cells." Br J Haematol **106**(2): 491-503.

Leung, T. K., C. Hall, et al. (1992). "The human heat-shock genes HSPA6 and HSPA7 are both expressed and localize to chromosome 1." Genomics **12**(1): 74-9.

Leung, Y. F. and D. Cavalieri (2003). "Fundamentals of cDNA microarray data analysis." Trends Genet **19**(11): 649-59.

Liang, P. and A. B. Pardee (2003). "Analysing differential gene expression in cancer." Nat Rev Cancer **3**(11): 869-76.

Liao, F., A. K. Shirakawa, et al. (2002). "Human B cells become highly responsive to macrophage-inflammatory protein-3 alpha/CC chemokine ligand-20 after cellular activation without changes in CCR6 expression or ligand binding." J Immunol **168**(10): 4871-80.

Lindstedt, M., B. Johansson-Lindbom, et al. (2003). "Expression of CD137 (4-1BB) on human follicular dendritic cells." Scand J Immunol **57**(4): 305-10.

Liu, G., A. E. Loraine, et al. (2003). "NetAffx: Affymetrix probesets and annotations." Nucleic Acids Res **31**(1): 82-6.

Liu, J., J. C. Schmitz, et al. (2002). "Thymidylate synthase as a translational regulator of cellular gene expression." Biochim Biophys Acta **1587**(2-3): 174-82.

Liu, Y. J., G. Grouard, et al. (1996). "Follicular dendritic cells and germinal centers." Int Rev Cytol **166**: 139-79.
Liu, Y. X., T. Yoshino, et al. (2001). "Loss of expression of alpha4beta7 integrin and L-selectin is associated with high-grade progression of low-grade MALT lymphoma." Mod Pathol **14**(8): 798-805.
Lossos, I. S. and R. Levy (2003). "Higher grade transformation of follicular lymphoma: phenotypic tumor progression associated with diverse genetic lesions." Semin Cancer Biol **13**(3): 191-202.
Lovec, H., A. Grzeschiczek, et al. (1994). "Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice." Embo J **13**(15): 3487-95.
Lu, J., M. Honczarenko, et al. (2003). "Independent expression of the two paralogous CCL4 genes in monocytes and B lymphocytes." Immunogenetics.
Ma, C. and L. M. Staudt (2001). "Molecular definition of the germinal centre stage of B-cell differentiation." Philos Trans R Soc Lond B Biol Sci **356**(1405): 83-9.
Manser, T. (2004). "Textbook germinal centers?" J Immunol **172**(6): 3369-75.
Martinez, A., B. Bellosillo, et al. (2004). "Nuclear survivin expression in mantle cell lymphoma is associated with cell proliferation and survival." Am J Pathol **164**(2): 501-10.
Martinez, N., F. I. Camacho, et al. (2003). "The molecular signature of mantle cell lymphoma reveals multiple signals favoring cell survival." Cancer Res **63**(23): 8226-32.
Martinez-Climent, J. A., A. A. Alizadeh, et al. (2003). "Transformation of follicular lymphoma to diffuse large cell lymphoma is associated with a heterogeneous set of DNA copy number and gene expression alterations." Blood **101**(8): 3109-17.
Matolcsy, A. (1999). "High-grade transformation of low-grade non-Hodgkin's lymphomas: mechanisms of tumor progression." Leuk Lymphoma **34**(3-4): 251-9.
Matolcsy, A., E. J. Schattner, et al. (1999). "Clonal evolution of B cells in transformation from low- to high-grade lymphoma." Eur J Immunol **29**(4): 1253-64.
Matsuda, F., E. K. Shin, et al. (1993). "Structure and physical map of 64 variable segments in the 3'0.8-megabase region of the human immunoglobulin heavy-chain locus." Nat Genet **3**(1): 88-94.
Matsumoto, M., S. F. Lo, et al. (1996). "Affinity maturation without germinal centres in lymphotoxin-alpha-deficient mice." Nature **382**(6590): 462-6.
Meinhardt, G., C. M. Wendtner, et al. (1999). "Molecular pathogenesis of chronic lymphocytic leukemia: factors and signaling pathways regulating cell growth and survival." J Mol Med **77**(2): 282-93.
Mellado, M., A. M. de Ana, et al. (2001). "A potential immune escape mechanism by melanoma cells through the activation of chemokine-induced T cell death." Curr Biol **11**(9): 691-6.
Meyerson, M., C. M. Counter, et al. (1997). "hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization." Cell **90**(4): 785-95.

M'Kacher, R., F. Farace, et al. (2003). "Blastoid mantle cell lymphoma: evidence for nonrandom cytogenetic abnormalities additional to t(11;14) and generation of a mouse model." Cancer Genet Cytogenet **143**(1): 32-8.

Mokbel, K. (2003). "The evolving role of telomerase inhibitors in the treatment of cancer." Curr Med Res Opin **19**(6): 470-2.

Moser, B. and P. Loetscher (2001). "Lymphocyte traffic control by chemokines." Nat Immunol **2**(2): 123-8.

Muller, G., U. E. Hopken, et al. (2003). "The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity." Immunol Rev **195**: 117-35.

Muller-Hermelink, H. K., A. Zettl, et al. (2001). "Pathology of lymphoma progression." Histopathology **38**(4): 285-306.

Nagy, B., T. Lundan, et al. (2003). "Abnormal expression of apoptosis-related genes in haematological malignancies: overexpression of MYC is poor prognostic sign in mantle cell lymphoma." Br J Haematol **120**(3): 434-41.

Naoe, T., Y. Nakano, et al. (2000). "Leukemia relapse reconsidered from the molecular aspect." Leuk Lymphoma **37**(5-6): 527-34.

Nicholson, I. C., M. J. Brisco, et al. (1995). "Memory B lymphocytes in human tonsil do not express surface IgD." J Immunol **154**(3): 1105-13.

Nilsson, J. A. and J. L. Cleveland (2003). "Myc pathways provoking cell suicide and cancer." Oncogene **22**(56): 9007-21.

Noda, M., J. Oh, et al. (2003). "RECK: a novel suppressor of malignancy linking oncogenic signaling to extracellular matrix remodeling." Cancer Metastasis Rev **22**(2-3): 167-75.

Norton, A. J., J. Matthews, et al. (1995). "Mantle cell lymphoma: natural history defined in a serially biopsied population over a 20-year period." Ann Oncol **6**(3): 249-56.

Nowell, P. C. and D. A. Hungerford (1960). "Chromosome studies on normal and leukemic human leukocytes." J Natl Cancer Inst **25**: 85-109.

Nowell, P. C., J. S. Moore, et al. (1994). "Richter's syndrome associated with loss of response to transforming growth factor-beta." Leuk Res **18**(2): 85-9.

Okada, T., V. N. Ngo, et al. (2002). "Chemokine requirements for B cell entry to lymph nodes and Peyer's patches." J Exp Med **196**(1): 65-75.

Osgood, E. E. and J. H. Brooke (1955). "Continuous tissue culture of leukocytes from human leukemic bloods by application of gradient principles." Blood **10**(10): 1010-22.

Palma, C., M. Binaschi, et al. (2004). "CD137 and CD137 ligand constitutively coexpressed on human T and B leukemia cells signal proliferation and survival." Int J Cancer **108**(3): 390-8.

Pascual, V., Y. J. Liu, et al. (1994). "Analysis of somatic mutation in five B cell subsets of human tonsil." J Exp Med **180**(1): 329-39.

Pauly, S., K. Broll, et al. (2002). "CD137 is expressed by follicular dendritic cells and costimulates B lymphocyte activation in germinal centers." J Leukoc Biol **72**(1): 35-42.

Payne, A. S. and L. A. Cornelius (2002). "The role of chemokines in melanoma tumor growth and metastasis." J Invest Dermatol **118**(6): 915-22.

Peng, B., N. H. Mehta, et al. (1995). "Growth inhibition of malignant CD5+B (B-1) cells by antisense IL-10 oligonucleotide." Leuk Res **19**(3): 159-67.

Petrasch, S., M. Kosco, et al. (1992). "Follicular dendritic cells in non-Hodgkin-lymphoma express adhesion molecules complementary to ligands on neoplastic B-cells." Br J Haematol **82**(4): 695-700.

Petrella, T., N. Yaziji, et al. (1997). "Implication of the Epstein-Barr virus in the progression of chronic lymphocytic leukaemia/small lymphocytic lymphoma to Hodgkin-like lymphomas." Anticancer Res **17**(5B): 3907-13.

Pham, L. V., A. T. Tamayo, et al. (2003). "Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis." J Immunol **171**(1): 88-95.

Pillai, S. (1999). "The chosen few? Positive selection and the generation of naive B lymphocytes." Immunity **10**(5): 493-502.

Pittaluga, S., A. Tierens, et al. (1998). "Blastic variant of mantle cell lymphoma shows a heterogenous pattern of somatic mutations of the rearranged immunoglobulin heavy chain variable genes." Br J Haematol **102**(5): 1301-6.

Planken, E. V., H. Van de Velde, et al. (1998). "Selective response of CD5+ B cell malignancies to activation of the CD72 antigen." Clin Immunol Immunopathol **87**(1): 42-9.

Pollard, J. W. (2004). "Tumour-educated macrophages promote tumour progression and metastasis." Nat Rev Cancer **4**(1): 71-8.

Pollok, K. E., Y. J. Kim, et al. (1994). "4-1BB T-cell antigen binds to mature B cells and macrophages, and costimulates anti-mu-primed splenic B cells." Eur J Immunol **24**(2): 367-74.

Porcu, P. and C. R. Nichols (1998). "Evaluation and management of the "new" lymphoma entities: mantle cell lymphoma, lymphoma of mucosa-associated lymphoid tissue, anaplastic large-cell lymphoma, and primary mediastinal B-cell lymphoma." Curr Probl Cancer **22**(5): 283-368.

Pulvertaft, J. V. (1964). "Cytology of Burkitt's Tumour (African Lymphoma)." Lancet **39**: 238-40.

Ramachandra, S., R. A. Metcalf, et al. (1996). "Requirement for increased IL-10 in the development of B-1 lymphoproliferative disease in a murine model of CLL." J Clin Invest **98**(8): 1788-93.

Raty, R., K. Franssila, et al. (2003). "Predictive factors for blastoid transformation in the common variant of mantle cell lymphoma." Eur J Cancer **39**(3): 321-9.

Raty, R., K. Franssila, et al. (2002). "Ki-67 expression level, histological subtype, and the International Prognostic Index as outcome predictors in mantle cell lymphoma." Eur J Haematol **69**(1): 11-20.

Reeves, R. and L. M. Beckerbauer (2003). "HMGA proteins as therapeutic drug targets." Prog Cell Cycle Res **5**: 279-86.

Reif, K., E. H. Ekland, et al. (2002). "Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position." Nature **416**(6876): 94-9.

Remes, K., K. F. Norrback, et al. (2000). "Telomere length and telomerase activity in malignant lymphomas at diagnosis and relapse." Br J Cancer **82**(3): 601-7.

Renan, M. J. (1993). "How many mutations are required for tumorigenesis? Implications from human cancer data." Mol Carcinog **7**(3): 139-46.

Rivera, E. S., G. P. Cricco, et al. (2000). "Histamine as an autocrine growth factor: an unusual role for a widespread mediator." Semin Cancer Biol **10**(1): 15-23.

Roberts, A. B., A. Russo, et al. (2003). "Smad3: a key player in pathogenetic mechanisms dependent on TGF-beta." Ann N Y Acad Sci **995**: 1-10.

Rosenwald, A., G. Wright, et al. (2003). "The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma." Cancer Cell **3**(2): 185-97.

Roy, M. P., C. H. Kim, et al. (2002). "Cytokine control of memory B cell homing machinery." J Immunol **169**(4): 1676-82.

Ryan, K. M., A. C. Phillips, et al. (2001). "Regulation and function of the p53 tumor suppressor protein." Curr Opin Cell Biol **13**(3): 332-7.

Salih, H. R., S. G. Kosowski, et al. (2000). "Constitutive expression of functional 4-1BB (CD137) ligand on carcinoma cells." J Immunol **165**(5): 2903-10.

Salih, H. R., H. M. Schmetzer, et al. (2001). "Soluble CD137 (4-1BB) ligand is released following leukocyte activation and is found in sera of patients with hematological malignancies." J Immunol **167**(7): 4059-66.

Saltman, D. L., P. G. Cachia, et al. (1988). "Characterization of a new non-Hodgkin's lymphoma cell line (NCEB-1) with a chromosomal (11:14) translocation [t(11:14)(q13;q32)]." Blood **72**(6): 2026-30.

Sanchez-Beato, M., A. Sanchez-Aguilera, et al. (2003). "Cell cycle deregulation in B-cell lymphomas." Blood **101**(4): 1220-35.

Sanjo, H., T. Kawai, et al. (1998). "DRAKs, novel serine/threonine kinases related to death-associated protein kinase that trigger apoptosis." J Biol Chem **273**(44): 29066-71.

Sargent, T. D. (1987). "Isolation of differentially expressed genes." Methods Enzymol **152**: 423-32.

Sasahara, R. M., S. M. Brochado, et al. (2002). "Transcriptional control of the RECK metastasis/angiogenesis suppressor gene." Cancer Detect Prev **26**(6): 435-43.

Schaffner, C., I. Idler, et al. (2000). "Mantle cell lymphoma is characterized by inactivation of the ATM gene." Proc Natl Acad Sci U S A **97**(6): 2773-8.

Schena, M., D. Shalon, et al. (1995). "Quantitative monitoring of gene expression patterns with a complementary DNA microarray." Science **270**(5235): 467-70.

Schluter, C., M. Duchrow, et al. (1993). "The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins." J Cell Biol **123**(3): 513-22.

Schmidt, U. and C. G. Begley (2003). "Cancer diagnosis and microarrays." Int J Biochem Cell Biol **35**(2): 119-24.
Seidl, S., H. Kaufmann, et al. (2003). "New insights into the pathophysiology of multiple myeloma." Lancet Oncol **4**(9): 557-64.
Serrero, G. (2003). "Autocrine growth factor revisited: PC-cell-derived growth factor (progranulin), a critical player in breast cancer tumorigenesis." Biochem Biophys Res Commun **308**(3): 409-13.
Shaffer, A. L., A. Rosenwald, et al. (2002). "Lymphoid malignancies: the dark side of B-cell differentiation." Nat Rev Immunol **2**(12): 920-32.
Shevelev, I. V. and U. Hubscher (2002). "The 3' 5' exonucleases." Nat Rev Mol Cell Biol **3**(5): 364-76.
Siegel, P. M. and J. Massague (2003). "Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer." Nat Rev Cancer **3**(11): 807-21.
Skinnider, B. F., U. Kapp, et al. (2002). "The role of interleukin 13 in classical Hodgkin lymphoma." Leuk Lymphoma **43**(6): 1203-10.
Socialstyrelsen (2003). "Cancer Incidence in Sweden 2002." Statistics -Health and Diseases: 1-117.
Sprent, D. F. T. a. J. (2003). Immunological Memory. Fundamental Immunology. W. E. Paul. Philadelphia, Lippincott Williams & Wilkins.
Srinivasula, S. M., M. Ahmad, et al. (1997). "FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR1-induced apoptosis." J Biol Chem **272**(30): 18542-5.
Stacey, D. W. (2003). "Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells." Curr Opin Cell Biol **15**(2): 158-63.
Stankovi, T., G. S. Stewart, et al. (2002). "ATM mutations in sporadic lymphoid tumours." Leuk Lymphoma **43**(8): 1563-71.
Starczynski, J., W. Simmons, et al. (2003). "Variations in ATM protein expression during normal lymphoid differentiation and among B-cell-derived neoplasias." Am J Pathol **163**(2): 423-32.
Staudt, L. M. (2002). "Gene expression profiling of lymphoid malignancies." Annu Rev Med **53**: 303-18.
Surmacz, E. (2003). "Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor." Oncogene **22**(42): 6589-97.
Swerdlow, S. H., J. A. Habeshaw, et al. (1983). "Centrocytic lymphoma: a distinct clinicopathologic and immunologic entity. A multiparameter study of 18 cases at diagnosis and relapse." Am J Pathol **113**(2): 181-97.
Swerdlow, S. H. and M. E. Williams (2002). "From centrocytic to mantle cell lymphoma: a clinicopathologic and molecular review of 3 decades." Hum Pathol **33**(1): 7-20.
Tarlinton, D. (1998). "Germinal centers: form and function." Curr Opin Immunol **10**(3): 245-51.
Tedla, N., P. Palladinetti, et al. (1999). "Abundant expression of chemokines in malignant and infective human lymphadenopathies." Cytokine **11**(7): 531-40.

Tew, J. G., J. Wu, et al. (2001). "Follicular dendritic cells: beyond the necessity of T-cell help." Trends Immunol **22**(7): 361-7.

Thorselius, M., S. Walsh, et al. (2002). "Somatic hypermutation and V(H) gene usage in mantle cell lymphoma." Eur J Haematol **68**(4): 217-24.

Thunberg, U., A. Johnson, et al. (2001). "CD38 expression is a poor predictor for VH gene mutational status and prognosis in chronic lymphocytic leukemia." Blood **97**(6): 1892-4.

Till, K. J., K. Lin, et al. (2002). "The chemokine receptor CCR7 and alpha4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes." Blood **99**(8): 2977-84.

Tobin, G., U. Thunberg, et al. (2003). "Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vlambda2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope." Blood **101**(12): 4952-7.

Tolksdorf, G., H. Stein, et al. (1980). "Morphological and immunological definition of a malignant lymphoma derived from germinal-centre cells with cleaved nuclei (centrocytes)." Br J Cancer **41**(2): 168-82.

Tonini, T., F. Rossi, et al. (2003). "Molecular basis of angiogenesis and cancer." Oncogene **22**(42): 6549-56.

Tsujimoto, Y., J. Yunis, et al. (1984). "Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation." Science **224**(4656): 1403-6.

Tsunoda, R., E. Heinen, et al. (2000). "Follicular dendritic cells in vitro modulate the expression of Fas and Bcl-2 on germinal center B cells." Cell Tissue Res **299**(3): 395-402.

Tusenius, K. J., P. J. Bakker, et al. (1992). "Measurement of proliferation indices in non Hodgkin's lymphoma--is it useful?" Leuk Lymphoma **7**(3): 181-7.

Vilpo, J., G. Tobin, et al. (2003). "Surface antigen expression and correlation with variable heavy-chain gene mutation status in chronic lymphocytic leukemia." Eur J Haematol **70**(1): 53-9.

Visser, H. P., M. Tewis, et al. (2000). "Mantle cell lymphoma proliferates upon IL-10 in the CD40 system." Leukemia **14**(8): 1483-9.

Wagner, E. F., N. Hanna, et al. (2000). "Novel diversity in IL-4-mediated responses in resting human naive B cells versus germinal center/memory B cells." J Immunol **165**(10): 5573-9.

Wajant, H. (2003). "Death receptors." Essays Biochem **39**: 53-71.

Walsh, S. H., M. Thorselius, et al. (2003). "Mutated VH genes and preferential VH3-21 use define new subsets of mantle cell lymphoma." Blood **101**(10): 4047-54.

Weinberg, R. A. (1995). "The retinoblastoma protein and cell cycle control." Cell **81**(3): 323-30.

Weisenburger, D. D., J. M. Vose, et al. (2000). "Mantle cell lymphoma. A clinicopathologic study of 68 cases from the Nebraska Lymphoma Study Group." Am J Hematol **64**(3): 190-6.

Weller, S., A. Faili, et al. (2003). "Hypermutation in human B cells in vivo and in vitro." Ann N Y Acad Sci **987**: 158-65.
Weller, S., A. Faili, et al. (2001). "CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans." Proc Natl Acad Sci U S A **98**(3): 1166-70.
Welzel, N., T. Le, et al. (2001). "Templated nucleotide addition and immunoglobulin JH-gene utilization in t(11;14) junctions: implications for the mechanism of translocation and the origin of mantle cell lymphoma." Cancer Res **61**(4): 1629-36.
Willis, T. G. and M. J. Dyer (2000). "The role of immunoglobulin translocations in the pathogenesis of B-cell malignancies." Blood **96**(3): 808-22.
Wlodarska, I., S. Pittaluga, et al. (1999). "Secondary chromosome changes in mantle cell lymphoma." Haematologica **84**(7): 594-9.
Wotherspoon, A. C., C. Doglioni, et al. (1993). "Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori." Lancet **342**(8871): 575-7.
Wu, M. X., Z. Ao, et al. (1998). "IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival." Science **281**(5379): 998-1001.
Yatabe, Y., R. Suzuki, et al. (2000). "Significance of cyclin D1 overexpression for the diagnosis of mantle cell lymphoma: a clinicopathologic comparison of cyclin D1-positive MCL and cyclin D1-negative MCL-like B-cell lymphoma." Blood **95**(7): 2253-61.
Young, L. S. and P. G. Murray (2003). "Epstein-Barr virus and oncogenesis: from latent genes to tumours." Oncogene **22**(33): 5108-21.
Zelenetz, A. D., T. T. Chen, et al. (1992). "Clonal expansion in follicular lymphoma occurs subsequent to antigenic selection." J Exp Med **176**(4): 1137-48.
Zhang, W., P. D. Bardwell, et al. (2001). "Clonal instability of V region hypermutation in the Ramos Burkitt's lymphoma cell line." Int Immunol **13**(9): 1175-84.
Zhu, D., R. E. Hawkins, et al. (1994). "Clonal history of a human follicular lymphoma as revealed in the immunoglobulin variable region genes." Br J Haematol **86**(3): 505-12.
Zimmermann, C. R., W. C. Orr, et al. (1980). "Molecular cloning and selection of genes regulated in Aspergillus development." Cell **21**(3): 709-15.
Zucca, E., H. Stein, et al. (1994). "European Lymphoma Task Force (ELTF): Report of the workshop on Mantle Cell Lymphoma (MCL)." Ann Oncol **5**(6): 507-11.