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Molecular Pathogenesis of MALT Lymphoma

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Declarations

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

I also declare that this thesis is not substantially the same as any that I submitted for a degree or diploma or other qualification at any other University, and that no part has already been, or is currently being, submitted for any degree, diploma, or other qualification.

This dissertation comprises a total of 264 pages. Excluding tables, figures, references and appendices, the number of pages corresponding to text is 195.

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Abbreviations

Some abbreviations used only once or a few times, particularly with gene names, may not be included in this list, but are explained in the main text. In general gene names and translocations are written in italics.

AP-1	Activator protein 1
CCR	Chemotactic cytokines receptor
cDNA	complementary DNA
CLL	Chronic lymphocytic leukaemia
cRNA	complementary RNA
DLBCL	Diffuse large B-cell lymphoma
DLR	Dual luciferase reporter assay
FL	Follicular lymphoma
gcRMA	GeneChip RMA
GO	Gene ontology
GSEA	Gene set enrichment analysis
H. pylori	Helicobactor pylori
IκB	Inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells
MALT	Mucosa associated lymphoid tissue
MAPK	Mitogen-activated protein kinases
MAS5	Affymetrix Microarray Suite 5
MCL	Mantle cell lymphoma
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin lymphoma
PBS	Phosphate buffered saline
qPCR	Quantitative PCR

qRT-PCR	Quantitative real time PCR
RMA	Robust Multiarray Averaging
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
SMZL	Splenic marginal zone lymphoma
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TLR	Toll-like receptor
AP-1	Activator protein 1
CCR	Chemotactic cytokines receptor
cDNA	complementary DNA
CLL	Chronic lymphocytic leukaemia
cRNA	complementary RNA
DLBCL	Diffuse large B-cell lymphoma
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MCL	Mantle cell lymphoma
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin lymphoma
PBS	Phosphate buffered saline
qPCR	Quantitative PCR
qRT-PCR	Quantitative real time PCR
RMA	Robust Multiarray Averaging

Abstract

Molecular Pathogenesis of MALT Lymphoma

By **Rifat Akram Hamoudi**, Churchill College, University of Cambridge

Mucosa associated lymphoid tissue (MALT) lymphoma is characterized by *t(11;18)(q21;q21)/API2-MALT1*, *t(1;14)(p22;q32)/BCL10-IGH* and *t(14;18)(q32;q21)/IGH-MALT1*, which commonly activate the NF-κB pathway. Gastric MALT lymphomas harbouring such translocation do not respond to *Helicobacter pylori* eradication, while those without translocation can be cured by antibiotics.

To understand the molecular mechanism of MALT lymphoma with and without chromosome translocation, 24 cases (15 translocation-positive and 9 translocation-negative) of MALT lymphomas together with 7 follicular lymphomas and 7 mantle cell lymphomas were analysed by Affymetrix gene expression microarray platform. Unsupervised clustering showed that cases of MALT lymphoma were clustered as a single branch. However, within the MALT lymphoma group, translocation-positive cases were intermingled with translocation-negative cases. Gene set enrichment analysis (GSEA) of the NF-κB target genes and 4394 additional gene sets covering various cellular pathways, biological processes and molecular functions showed that translocation-positive MALT lymphomas were characterized by an enhanced expression of NF-κB target genes, particularly *TLR6*, *CCR2*, *CD69* and *BCL2*, while translocation-negative cases were featured by active inflammatory and immune responses, such as *IL8*, *CD86*, *CD28* and *ICOS*. Separate analyses of the genes differentially expressed between translocation-positive and negative cases and measurement of gene ontology term in these differentially expressed genes by hypergeometric test reinforced the above findings by GSEA. The differential expression of these NF-κB target genes between MALT lymphoma with and without translocation was confirmed by quantitative RT-PCR and immunohistochemistry or Western blot.

Expression of TLR6, in the presence of TLR2, enhanced both API2-MALT1 and BCL10 mediated NF-κB activation *in vitro*. In addition, there was cooperation between expression of BCL10, MALT1 or API2-MALT1, and stimulation of the antigen receptor or CD40 or TLR in NF-κB activation as shown by both reporter assay and IκBα degradation. Interestingly, expression of BCL10 but not API2-MALT1 and MALT1, in the presence of LPS stimulation, also triggered IκBβ degradation, suggesting activation of different NF-κB dimers between these oncogenic products.

Study by co-immunoprecipitation showed that BCL10 directly interacts with MALT1. Sub-cellular localisation experiments in BJAB B-cells, showed that BCL10 localisation was affected by MALT1. When BCL10 was over-expressed, the protein was predominantly expressed in the nuclei, but when MALT1 was over-expressed, BCL10 was mainly localised in the cytoplasm. When both BCL10 and MALT1 were over-expressed, BCL10 was expressed in the cytoplasm in the early hours when the protein level was low, but in both the cytoplasm and nuclei after 9 hours when the protein level was high. Over-expression of API2-MALT1 did not shown any apparent effect on BCL10 sub-cellular localisation *in vitro*.

Finally, comparison of MALT lymphoma expression microarray with other lymphomas showed *lactoferrin* to be highly expressed in MALT lymphoma. This was confirmed by qRT-PCR, showing *lactoferrin* to be significantly over-expressed in MALT lymphoma compared to FL and MCL. Thus *lactoferrin* may be a potential marker for MALT lymphoma.

Publications

Arising from this thesis:

1) **Hamoudi RA**, Appert A, Ye H, Ruskone-Fourmestraux A, Streubel B, Chott A, Raderer M, Gong L, Wlodarska I, De Wolf-Peeters C, MacLennan KA, de Leval L, Isaacson PG, & Du MQ.
   Differential expression of NF-kappaB target genes in MALT lymphoma with and without chromosome translocation: insights into molecular mechanism.
   Leukemia. 2010 Aug;24(8):1487-1497

2) Ye H, Gong L, Liu H, **Hamoudi RA**, Shirali S, Ho L, Chott A, Streubel B, Siebert R, Gesk S, Martin-Subero JI, Radford JA, Banerjee S, Nicholson AG, Ranaldi R, Remstein ED, Gao Z, Zheng J, Isaacson PG, Dogan A & Du MQ.
   MALT lymphoma with t(14;18)(q32;q21)/IGH-MALT1 is characterized by strong cytoplasmic MALT1 and BCL10 expression.
   J Pathol. 2005 Feb;205(3):293-301.

Not arising from this thesis but related to MALT lymphoma:

1) Liu H, **Hamoudi RA**, Ye H, Ruskone-Fourmestraux A, Dogan A, Isaacson PG & Du MQ.
   t(11;18)(q21;q21) of mucosa-associated lymphoid tissue lymphoma results from illegitimate non-homologous end joining following double strand breaks.
   Br J Haematol. 2004 May;125(3):318-329.

2) Liu H, Ye H, Ruskone-Fourmestraux A, De Jong D, Pileri S, Thiede C, Lavergne A, Boot H, Caletti G, Wündisch T, Molina T, Taal BG, Elena S, Thomas T, Zinzani PL, Neubauer A, Stolte M, **Hamoudi RA**, Dogan A, Isaacson PG & Du MQ.
   T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to H. pylori eradication.
   Gastroenterology. 2002 May;122(5):1286-1294.

3) Liu H, Ye H, Dogan A, Ranaldi R, **Hamoudi RA**, Bearzi I, Isaacson PG & Du MQ.
   T(11;18)(q21;q21) is associated with advanced mucosa-associated lymphoid tissue lymphoma that expresses nuclear BCL10.
   Blood. 2001 Aug 15;98(4):1182-1187.

4) Liu H, Ruskon-Fourmestraux A, Lavergne-Slove A, Ye H, Molina T, Bouhnik Y, **Hamoudi RA**, Diss TC, Dogan A, Megraud F, Rambaud JC, Du MQ & Isaacson PG.
   Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to Helicobacter pylori eradication therapy.
   Lancet. 2001 Jan 6;357(9249):39-40.

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Finally, I would like to dedicate this thesis to all those who are about to give up on a difficult problem. This thesis is the proof that if you persevere long enough with a difficult problem, eventually a solution will be found.

CHAPTER 1 – General introduction

1.1 Brief overview of lymphomas

Lymphoma is defined as a neoplastic proliferation of lymphoid cells. 90% of all lymphoid malignancies are of B-cell lineage, whilst a minority are of T-cell (7%) or NK-cell lineage (<2%). In 1832 Thomas Hodgkin first described what became known as Hodgkin lymphoma. Hodgkin lymphoma (HL) accounts for approximately 30% of all lymphomas and comprises 2 distinct disease entities, the more frequent classical Hodgkin lymphoma (cHL) (95%) and the uncommon nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (5%). In cHL, the neoplastic cells are usually a minority population whose survival appears to be dependent on the majority of reactive or inflammatory cells (Aldinucci *et al.*, 2010). In the majority of cases the neoplastic (Reed-Sternberg (Reed, 1902)) cells appear to be of B-cell origin. NLPHL is characterised by lymphocytic and histocytic (L&H) or "popcorn" cells in that are distributed amidst abundant non-neoplastic inflammatory and accessory cells.

Approximately 70% of lymphomas do not have the clinical and pathological features of HL and have therefore been categorised historically as non-Hodgkin lymphomas (NHL). NHL are frequently disseminated and are all considered malignant or potentially malignant. Some are aggressive from the outset, while others are indolent for varying lengths of time, but may transform to more aggressive tumours. According to the World Health Organisation (WHO) 2008 classification (Swerdlow *et al.*, 2008), these lymphoid neoplasms which together account for the majority of lymphomas are classified as distinct disease entities under the broader categories of precursor lymphoid neoplasms, mature B-cell neoplasms, mature T and NK-cell neoplasms, immunodeficiency-associated lymphoproliferative disorders and histocytic and dendritic cell neoplasms.

Most NHLs are mature B-cell neoplasms corresponding to a clonal proliferation of B cells at various stages of differentiation. They can be categorised into pre-germinal, germinal and post-germinal centre origin according to their differentiation stage using the germinal centre reaction as a reference. The most common type of NHL is diffuse large B-cell lymphoma (DLBCL) which represents 37% of all NHL and consists of a heterogeneous group of large B-cell tumours (Swerdlow *et al.*, 2008), followed by follicular lymphoma (FL) which represents 29% of all NHL and is characterised by clonal expansion of neoplastic follicle centre-type cells. The third most frequent subtype of B-cell NHL is chronic lymphocytic leukaemia/small lymphocytic lymphoma (12%), followed closely by extranodal marginal zone lymphoma of mucosa associated lymphoid tissue (MALT lymphoma) which accounts for 9% of all B-cell lymphomas. Both MALT and follicular lymphomas may transform to a high grade lymphoma, most frequently diffuse large B-cell lymphoma. Nodal marginal zone (MGZ) lymphoma and splenic MGZ lymphomas are separate rare entities that involve lymph nodes (nodal MGZ lymphoma) and spleen usually with bone marrow and blood involvement (splenic MGZ lymphoma) respectively (Swerdlow *et al.*, 2008).

MALT lymphoma can occur in a wide range of organs (Swerdlow *et al.*, 2008). Amongst the various extranodal sites, gastric MALT lymphoma is the most common and thus the best characterised form of this disease (Du *et al.*, 2002). Nodal MZL may be seen as an apparently primary disease as a result of either splenic MZL or extranodal MZL.

1.2 Overview of MALT lymphoma

MALT (Mucosa associated lymphoid tissue) lymphoma is an indolent neoplasm where tumours tend to stay localised at their site of origin until the late phase of the disease. It occurs at various extra-nodal sites. The most common site is the gastrointestinal (GI) tract, comprising 50% of all cases, and within the GI tract, the stomach is the most common location accounting for 85% of GI MALT lymphomas. The small intestine is typically involved in patients with immunoproliferative small intestinal disease (IPSID). Other frequent sites include salivary gland, lung, ocular adnexa, skin, thyroid and breast (Swerdlow *et al.*, 2008). Apart from the small intestine, these anatomical sites are normally devoid of organised lymphoid tissues, thus MALT lymphoma appears to arise from acquired MALT, commonly due to chronic immunological stimulation, resulting either from pathogen infection or autoimmune disorders (Du, 2007). Patients generally present with MALT lymphoma at stages I or II. The average age of disease onset is 61 and the 5-year survival rate of patients has been as high as 93% in some studies. The median time before progression of the disease is approximately 5 years although it is significantly longer for cases with a gastrointestinal origin compared to those from other sites (Thieblemont *et al.*, 1997). 2-20% of patients have bone marrow involvement and up to 10% have lymphoma in multiple extra-nodal sites (Isaacson *et al.*, 1987; Wotherspoon *et al.*, 1993), but they generally respond well to therapy and have a good overall prognosis (Fischbach *et al.*, 2007).

1.2.1 Aetiology of MALT lymphoma

As mentioned in section 1.2, pathogen infection and autoimmune disorders play a major role in the development of MALT lymphoma. In this section, the aetiological role of pathogens such as *Helicobacter pylori, Campylobacter jejuni*, *Borrelia burgdorferi* and *Chlamydia*

psittaci and autoimmune disorders such as Sjögren's syndrome and Hashimoto's thyroiditis will be discussed.

1.2.1.1 Chronic infection

It has been shown that infection with certain microorganisms leads to the acquisition of lymphoid tissue at extra nodal sites that are normally devoid of any organised lymphoid tissues and hence plays a role in MALT lymphoma development (Banks, 2007).

Helicobacter pylori

In 1984 Marshall and Warren isolated a newly recognised bacterium, *Helicobacter pylori*, from patients with chronic gastritis and gastric ulcer (Marshall *et al.*, 1984). *H. pylori*, originally called *Campylobacter pylori*, is a unipolar, multiflagellate spiral shaped, microaerophilic, gram negative bacterium that lives in the luminal surface of the stomach and duodenum (Bolin *et al.*, 1995). *H. pylori* is widespread and has been implicated in several gastrointestinal diseases, such as chronic gastritis and peptic ulcer, (Howden *et al.*, 1998) gastric adenocarcinoma, (Asaka *et al.*, 1997) and MALT lymphoma (Hussell *et al.*, 1993b).

In 1987, Smith *et al.* showed monoclonal rearrangements of the Ig-heavy chains in IPSID, including cases that responded to antibiotics (Smith *et al.*, 1987), and suggested the possible involvement of bacteria-driven antigen stimulation in the development of the lymphoma. Subsequent studies established that, in the stomach, MALT is acquired as a result of colonisation of the gastric mucosa by *H. pylori* (Stolte *et al.*, 2002; Wotherspoon *et al.*, 1991). In 1991, Wotherspoon *et al.* demonstrated the presence of *H. pylori* in 101 of 110 (92%) patients with gastric MALT lymphoma (Wotherspoon *et al.*, 1991) and suggested for the first time that gastric MALT lymphoma may develop from the MALT acquired in response to *H. pylori* infection.

At least 80-90% of patients with gastric MALT lymphoma are infected with *H. pylori*, which is much higher than the frequency of infection in the rest of the population (Wotherspoon *et al.*, 1991). The gastric mucosa is a hostile environment to most organisms due to its acidic environment. However, *H. pylori* secretes urease that raises the local pH so it is able to survive and colonise the gastric mucosa. The incidence of gastric MALT lymphoma was found to be high in North Eastern Italy where *H. pylori* infection is prevalent (Doglioni *et al.*, 1992).

Campylobacter jejuni and *Borrelia burgdorferi*

These were shown to be associated with a proportion of primary cutaneous MALT lymphoma and IPSID respectively (Cerroni *et al.*, 1997; Lecuit *et al.*, 2004).

Chlamydia psittaci

Chlamydia psittaci (*C. psittaci*) infection was recently shown to be associated with the development of ocular adnexal MALT lymphoma. In Italian studies, *Chlamydia psittaci* was detected in 80% of these lymphomas (Ferreri *et al.*, 2004). However, the association between *Chlamydia psittaci* and ocular adnexal MALT lymphoma was not reproduced by several other studies from the USA (Vargas *et al.*, 2006), Japan (Daibata *et al.*, 2006) and the Netherlands (Mulder *et al.*, 2006). Subsequently, Chanudet and co-workers confirmed geographical variation in the association between *Chlamydia psittaci* and ocular adnexal MALT lymphoma (Chanudet *et al.*, 2006).

Further investigations are needed to confirm or refute the causal association between the organisms described above and extranodal marginal zone lymphoma at various sites.

1.2.1.2 Autoimmune disease

In addition to microbial infections discussed in section 1.2.1.1, autoimmune disease plays a role in MALT lymphomagenesis. For example, thyroid MALT lymphoma is associated with Hashimoto's thyroiditis and salivary gland MALT lymphoma is associated with lymphoepithelial sialadenitis (Kassan *et al.*, 1978; Kato *et al.*, 1985). Patients with Sjögren's syndrome, and a number of other autoimmune disorders such as rheumatoid arthritis show an increased risk of lymphoma development (Smedby *et al.*, 2006). These autoimmune diseases result in chronic immune responses and the formation of acquired MALT. Patients with these diseases are approximately 40 times more likely to develop lymphoma, and 85% of the lymphomas developed are MALT lymphoma (Kassan *et al.*, 1978; Talal *et al.*, 1967). In both Hashimoto thyroiditis and Sjögren's syndrome, lymphomagenesis is thought to be mediated by sustained T-cell dependent antigenic stimulation, similarly to that in *H. pylori*-driven gastric MALT lymphoma (Yamamoto, 2003).

1.2.2 Histopathology of MALT lymphoma

Since the development of MALT lymphoma relies on the acquisition of organised MALT, it is best to describe MALT followed by MALT lymphoma.

1.2.2.1 Mucosa associated lymphoid tissue

The mucosa-associated lymphoid tissue is situated within mucosal tissues. The main function of MALT is to prevent foreign antigen invasion from the mucosal sites. In response to antigen specific T-cell activation, naïve B cells undergo clonal expansion, differentiation and become effector cells secreting immunoglobulins, thus conveying the mucosal immune response. Native MALT is found in the gastrointestinal tract where it is abundantly present in the Peyer's patches of terminal ileum (Figure 1.1a). Typically, MALT in the Peyer's patches consists of germinal centres where B cells encounter antigens and undergo a series of mutations to enhance their antigen specificity. Germinal centres contain activated B cells, named centroblasts, as well as their differentiated counterpart expressing immunoglobulins called centrocytes, macrophages and follicular dendritic cells essential to germinal centre reactions. The germinal centre is surrounded by a follicular mantle, formed by naïve B cells not yet exposed to antigen. Outer to the mantle zone is a marginal zone, where memory B cells reside. A distinct marginal zone, although a feature of Peyer's patches is not present in human tonsillar tissue, and therefore not universally present in all MALT. However, there is evidence to show that intra-epithelial B-cells in tonsillar tissue could represent the equivalent of marginal zone B cells (Morente *et al.*, 1992).

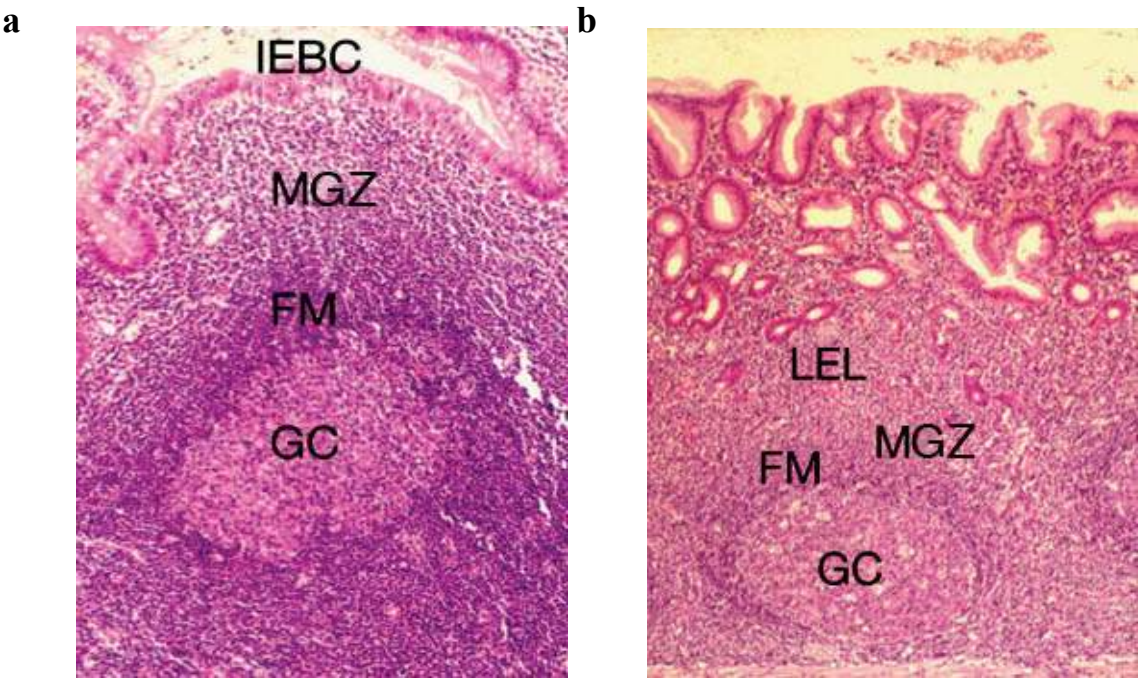


Figure 1.1 - Morphology of MALT and gastric MALT lymphoma from the gastrointestinal tract.
a. The Peyer's patches are characterised histologically by the presence of a germinal centre (GC) surrounded by a follicular mantle (FM) and a marginal-zone (MGZ). Intraepithelial marginal-zone B cells (IEBC) are observed within the epithelium covering the Peyer's patch, forming the lymphoepithelium characteristic of MALT.
b. The morphology of gastric MALT lymphoma. The germinal centre (GC), where B cells proliferate and mature following antigen stimulation, is surrounded by a follicular mantle (FM), which comprises naive B cells. The reactive B-cell follicle is surrounded by neoplastic marginal-zone (MGZ) B cells that infiltrate the neighbouring epithelium forming characteristic lymphoepithelial lesions (LEL).

Figure adapted from Isaacson *et al.* (2004). Nature Review Cancer, 4, 644-653.

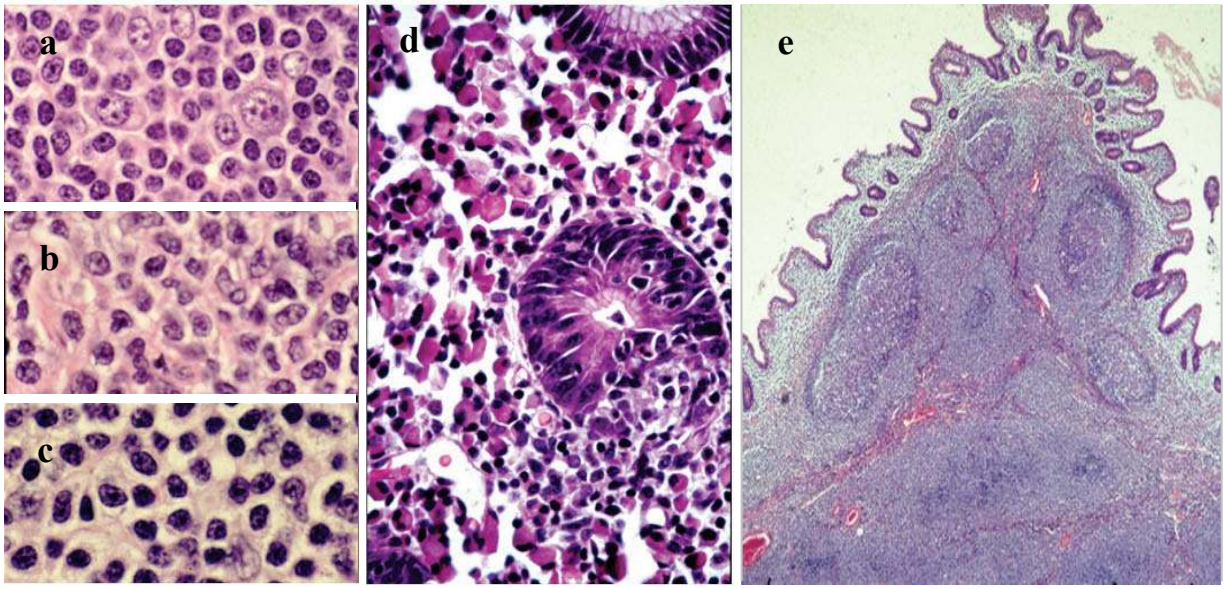


Figure 1.2 - Cytological and histological features of MALT lymphoma.
a-c. Cytology: neoplastic cells can resemble small lymphocytes (a), or have the appearance of centrocytes but with more abundant cytoplasm (b), or rather have the features of monocytoid cells with abundant pale cytoplasm and well-defined borders (c). Scattered transformed centroblasts or immunoblast-like cells can also be observed (a). **d-e**. Histological features: MALT lymphoma can show prominent plasma cell differentiation (d). The neoplastic cells can infiltrate the surrounding epithelium, forming lymphoepithelial lesions (d), or invade B-cell reactive follicles (follicular colonisation) (e).

Figure adapted from Isaacson *et al.* (2005). Journal of Pathology, 205, 255-274.

1.2.2.2 Extranodal marginal zone B-cell lymphoma of MALT

Histologically, MALT lymphoma mimics the features of normal MALT (Figure 1.1b). The striking histological resemblance between some low grade B-cell lymphomas of the stomach, IPSID and the morphology of the Peyer's patches which led to the proposition of the MALT lymphoma entity by Isaacson and Wright in 1983 (Isaacson *et al.*, 1983). Similar to the structure of Peyer's patches, MALT lymphoma consists of neoplastic B-cell infiltrates that are primarily located in the marginal zone (which lies outer to the mantle zone that surrounds reactive germinal centres) and can extend into the interfollicular region (Figure 1.1b). An important feature of MALT lymphoma is the presence of aggregates of neoplastic cells

infiltrating individual mucosal glands or other epithelial structures. Such aggregates, referred to as lymphoepithelial lesions (Figure 1.1b, Figure 1.2d), resemble lymphoepithelium in Peyer's patches (Figure 1.1b) (Isaacson *et al.*, 2004).

Cytologically, MALT lymphoma cells are variable in their appearance, resembling centrocyte-like cells, sometimes with round nuclei and pale staining cytoplasm, monocytoid B cells or small lymphocytes (Figure 1.2 a-c). Lymphoma cells may also show prominent plasma cell differentiation particularly in the sub-epithelial lamina propria (Figure 1.2d). In some cases the lymphoma cells may invade B-cell reactive follicles (Figure 1.2e). This so-called follicular colonisation can lead to morphological resemblance to FL.

1.2.3 Immunophenotype

The typical immunophenotype of MALT lymphoma is detailed in Table 1.1. MALT lymphoma cells mostly share the immunophenotype of non-neoplastic MGZ cells (Isaacson *et al.*, 1987). Currently there is no available marker specific to MALT lymphoma, nevertheless immunophenotyping is helpful for the differential diagnosis, notably from other small B-cell lymphomas (Table 1.1).

Table 1.1 - Typical immunophenotyping of MALT lymphoma cells.

Cellular marker	MALT lymphoma	FL	MCL	SLL	DLBCL
CD20	+	+	+	+	+
CD79a	+	+	+	+	+
BCL2	+/-	+	+	+	+/-
CD43	+/-	-	+	+	
CD5	-	-	+	+	+/-
CD10	-	+	-	-	+/-
CD23	-	+/-	-	+/-	
Cyclin D1	-	-	+	-	
BCL6	-	+	-	-	+/-
Ig heavy chain	M>>A>G	G>M+D	M+D	M	Variable
CD21 and FDC meshwork	+	+	+/-	-	-

Abbreviations: MALT, mucosa-associated lymphoid tissue; FL, follicular lymphoma; MCL, mantle-cell lymphoma; SLL, small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; CD, cluster of differentiation; FDC, follicular dendritic cell; + positive; - negative.

The neoplastic cells of MALT lymphoma share the cytological features and immunophenotype ($CD20^+$, IgM^+, IgD^-) of marginal zone B cells (Spencer *et al.*, 1985). MALT lymphoma cells are typically negative for CD10 and BCL6, which are characteristically positive in FL. Unlike mantle cell lymphoma (MCL) and small lymphocytic lymphoma, MALT lymphoma is only infrequently positive for CD5. It is negative for cyclin D1, a feature which helps to distinguish it from MCL (Table 1.1). The most important immunophenotypic feature favouring MALT lymphoma diagnosis is the presence of a diffuse infiltrate of $CD20^+$, IgM^+, IgD^- B cells outside the mantle zone of reactive follicles. Once the marginal zone phenotype is established, light chain restriction in this marginal zone population, or if present, within the plasma cells confirms the diagnosis. However, having a marker specific for MALT lymphoma would lead to more accurate MALT lymphoma diagnosis.

1.2.4 Pathogenesis of MALT lymphoma

The development of MALT lymphoma is a multistage process which is best understood in the gastric disease (Isaacson *et al.*, 1994; Isaacson *et al.*, 1995; Isaacson, 1995). Both gastric MALT lymphoma and pre-lymphomatous lesions are aetiologically related to *H. pylori* infection. Understanding the role of *H. pylori* has provided insights into the pathogenesis of MALT lymphoma.

1.2.4.1 H. pylori and its role in the development of gastric MALT lymphoma

There is now strong evidence that *H. pylori* is causally linked to gastric MALT lymphoma and this fulfils all criteria of Koch's postulates set in 1884 (Koch, 1884); Firstly, *H. pylori* was found in the majority of gastric MALT lymphomas (Eidt *et al.*, 1994). Secondly, *H. pylori* could be isolated from gastric MALT lymphoma, and grown in culture. Thirdly, infecting the stomach of pathogen-free mice with *H. pylori* could induce the development of MALT lymphoma (Enno *et al.*, 1995).

Additionally, early functional and clinical studies provided evidence of the crucial role of *H. pylori* in the development of gastric MALT lymphoma. Hussell and colleagues demonstrated that lymphoma cell growth *in vitro* was dependent on *H. pylori* specific T cells (Hussell *et al.*, 1996). Furthermore, Wotherspoon and colleagues first showed that *H. pylori* eradication by antibiotic treatment led to lymphoma regression in *H. pylori*–associated gastric MALT lymphomas (Wotherspoon *et al.*, 1991). Taken together, these data established that *H. pylori* infection could cause gastric MALT lymphoma.

It is now known that the inflammatory process triggered by *H. pylori* infection is directly responsible not only for the acquisition of MALT in the gastric mucosa, but also for subsequent malignant transformation and the development of gastric MALT lymphoma

(Isaacson *et al.*, 2004). As in other bacterial diseases, the development of *H. pylori*-associated MALT lymphoma is hypothesised to be associated with the host response and bacterial status. However, the precise role of *H. pylori* in MALT lymphomas is not clear.

1.2.4.2 Immunological stimulation

Several histological features of MALT lymphoma including the presence of plasma-cell differentiation, blasts, follicular colonisation and proliferation, suggest that MALT lymphoma cells preserve B-cell properties and that their growth may be partially driven by antigenic stimulation via antigen receptors (Isaacson *et al.*, 2004) and T-cell and B-cell interaction. Recent studies indicate that both direct and indirect antigen stimulation mechanisms are involved.

1.2.4.2.1 Direct antigen stimulation

MALT lymphomas invariably express surface immunoglobulin. The anti-idiotype antibody has been shown to stimulate MALT lymphoma cell proliferation and synergise with mitogen stimulation (Hussell *et al.*, 1993b). The data also showed that the tumour-derived immunoglobulin does not recognise *H. pylori,* but recognises various autoantigens (Hussell *et al.*, 1993a). Antibodies to gastric epithelial cells are commonly present in serum samples from patients with *H. pylori* gastritis (Negrini *et al.*, 1996). An anti-idiotype antibody to immunoglobulin of a gastric MALT lymphoma cross-reacts specifically with reactive B cells in *H. pylori*-associated gastritis (Greiner *et al.*, 1997). These findings suggest that gastric MALT lymphoma cells are transformed from autoreactive B cells, which are induced after *H. pylori* infection.

Sequence analysis of the rearranged *immunoglobulin* of MALT lymphoma also reveals evidence that MALT lymphoma cells respond to direct antigen stimulation, indicating the

tumour clone has undergone antigen selection (Bertoni *et al.*, 1997; Du *et al.*, 1996b). During the evolution of gastric MALT lymphoma, particularly in the early stage, the rearranged tumour immunoglobulin gene frequently showed further somatic mutations, commonly referred to as ongoing mutations (Bertoni *et al.*, 1997; Du *et al.*, 1996a). Since somatic mutations occurs in the rearranged immunoglobulin gene only during the germinal centre reaction, and depends on antigen and T cells, the finding of ongoing immunoglobulin mutations in MALT lymphoma suggests that tumour-cell growth is partially driven by direct antigen stimulation.

1.2.4.2.2 Indirect antigen stimulation

The close association of *H. pylori* infection with gastric MALT lymphoma development prompted research into the immunological responses of the tumour cells to *H. pylori*. By co-culturing tumour cells with 13 clinical strains of heat-killed *H. pylori*, Hussell and co-workers demonstrated that *H. pylori* induced tumour cells to proliferate (Hussell *et al.*, 1993b). The effect was strain-specific but was T-cell mediated and not due to specificity of lymphoma cells for *H. pylori* antigens. This effect was associated with expression of interleukin-2 (IL-2) receptors and secretion of immunoglobulin by tumour cells. Removal of tumour-infiltrating T cells before the experiment abolished all the effects of *H. pylori* on tumour cells. Furthermore, these authors confirmed that *H. pylori* did not directly stimulate tumour cells but did so via specifically activated tumour infiltrating T cells (Hussell *et al.*, 1996). Furthermore, the stimulating effect of *H. pylori* on tumour B cells can be completely blocked by an antibody to CD40L. Thus, *H. pylori* stimulates lymphoma B cells through tumour-infiltrating T cells, involving CD40 and CD40L. It is possible that CD80/CD86 costimulatory molecules promote T-cell-mediated neoplastic B-cell growth.

The active role of tumour-infiltrating T cells in the growth of tumour B cells is further supported by a study of T-cell clones isolated from gastric MALT lymphoma. T-cell clones responding to *H. pylori* stimulation were CD4-positive helper cells rather than CD8-positive cytotoxic cells. These specific T-cell clones activated tumour B cells in a dose dependent manner (D'Elios *et al.*, 1997).

Additionally, complete regression of gastric MALT lymphoma following *H. pylori* eradication strongly indicates the role of *H. pylori* stimulation in the survival and growth of gastric MALT lymphoma (Begum *et al.*, 2000; Wotherspoon, 1996). Unlike low-grade tumour cells, high-grade tumour cells do not show any growth response to *H. pylor*i mediated T-cell stimulation *in vitro* (Hussell *et al.*, 1993b). High grade lymphomas tend to be resistant to *H. pylor*i eradication therapy (Bayerdorffer *et al.*, 1995; Thiede *et al.*, 1997), though recently published reports show a complete remission rate of 62.5% with a median follow-up of over 30 months in gastric DLBCLs (Chen *et al.*, 2001; Morgner *et al.*, 2001).

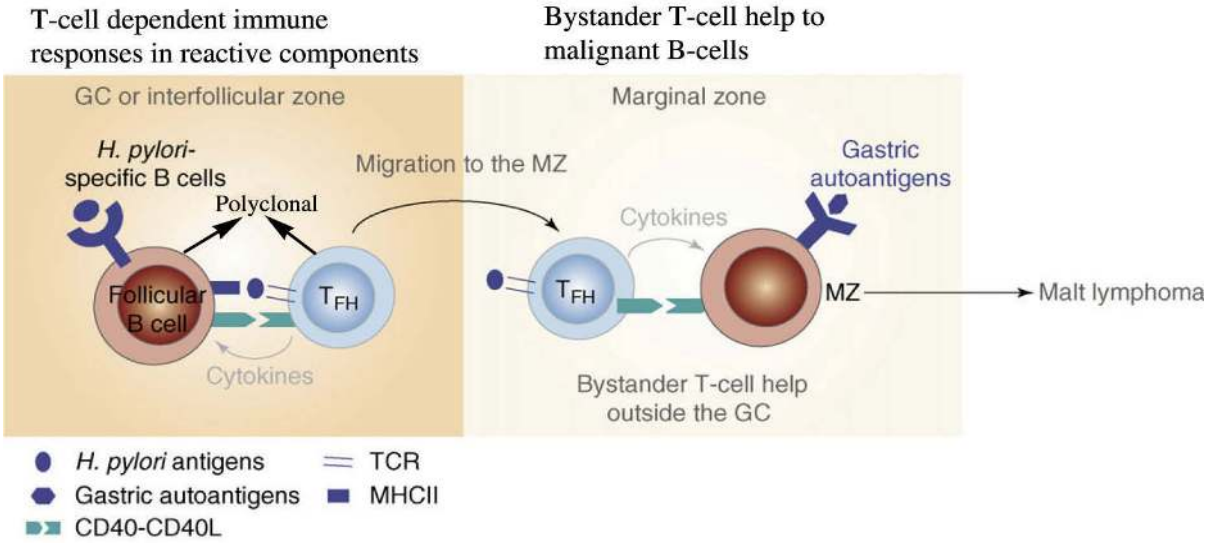


Figure 1.3 - The role of T- and B- cell interaction in the development of MALT lymphoma.
FH: Follicular Helper cells, TCR: T-cell receptor, MZ: marginal zone.
Figure adapted from Roulland *et al.* (2008). Trends in Immunology, 29, 25-33.

Therefore, it can be hypothesised that in MALT lymphomas, cells are autoreactive and can be stimulated at low levels by autoantigens, but additional stimuli are required for their enhanced proliferation and ultimate malignant transformation. In gastric MALT lymphoma, *H. pylori* infection may cause two main events. Firstly, T-cell dependent immune responses in reactive components lead to the generation of a sustained pool of polyclonal *H. pylori*-specific T cells. Secondly, these *H. pylori* specific T cells in the marginal zone containing tumour cells provide non-cognate bystander T-cell help to the autoreactive MALT lymphoma cells (Figure 1.3). This working hypothesis explains how polyclonal *H. pylori* specific T cells can be critical for the growth and survival of monoclonal neoplastic B cells, which recognise autoantigens rather than *H. pylori.*

1.2.4.3 H. pylori virulent factors and host genetics in the development of gastric MALT lymphoma

The fact that the a majority of patients infected with *H. pylori* are asymptomatic and only a small proportion of them develop gastric MALT lymphoma, indicates a role of bacterial virulence factors and host genetic susceptibility. The interaction between the bacterium and the host immune reaction may determine the risk of MALT lymphoma development.

1.2.4.3.1 Host factors

A number of gene polymorphisms are associated with susceptibility to autoimmune disorders (Lettre *et al.*, 2008) and some have been shown to be associated with MALT lymphoma development. Polymorphisms in immune regulators such as cytokines are commonly involved in auto-reactive conditions (Hajeer *et al.*, 2000). A recent large case control study on 1172 patients with NHL and 982 population-based controls demonstrated a significantly higher susceptibility to NHL among patients with an autoimmune disorder and *TNFA*

(tumour necrosis factor A) G308A variant, or *interleukin 10* (*IL10*) T3575A variant (Wang *et al.*, 2007). *TNFA* G308A polymorphism associates with higher levels of TNFA expression which enhances chronic inflammatory responses (Bayley *et al.*, 2004). Among NHLs, *TNFA* G308A polymorphism particularly increases the risk of several lymphoma subtypes, including MALT lymphoma and DLBCLs (Morton *et al.*, 2008).

A number of other genetic polymorphisms have been investigated, particularly in genes encoding molecules involved in immune or inflammatory responses. For example, gene polymorphisms in *toll-like receptors* (*TLR*) have been shown to be associated with an increased risk of lymphoma, including MALT lymphoma (Nieters *et al.*, 2006). The *TLR4* Asp299Gly variant increases susceptibility to gram-negative bacteria infection, thus potentially representing one of the host factors affecting the risk of lymphoma among *H. pylori* infected individuals (Nieters *et al.*, 2006). Likewise, polymorphisms in the T-cell receptor cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene, encoding a negative regulator of T-cell activation, increase the risk of gastric MALT lymphoma, especially in patients with *H. pylori* infection (Cheng *et al.*, 2006).

1.2.4.3.2 Bacterial virulence factors

In addition to host genetic susceptibility, virulence factors associated with infectious agents also play a critical role in the outcome. The risk of developing gastric carcinoma has been shown to depend on the combined effect of pro-inflammatory cytokine gene polymorphisms in the host and virulence-associated genes in *H. pylori* (Figueiredo *et al.*, 2002). Several *H. pylori* virulence factors are associated with an increased risk of gastric ulcer and gastric carcinoma (Maeda *et al.*, 2007), especially the cytotoxin-associated antigen (CagA), vacuolating toxin (VacA) and outer membrane adhesion (BabA) virulence factors. CagA

antigens are known to induce a strong inflammatory response. CagA-positive strains contain a long pathogenicity island including over 30 genes (Prinz *et al.*, 2003). Such pathogenesis-associated genes are usually absent in *H. pylori* strains isolated from asymptomatic hosts (Baldwin *et al.*, 2007). t(11;18)(q21;q21) associated gastric MALT lymphoma cases are significantly associated with infection by CagA strains of *H. pylori* (Ye *et al.*, 2003). CagA-positive strains of *H. pylori* are also associated with a strong response mediated by neutrophils. This is thought to increase the release of ROS (reactive oxygen species) within the environment of inflammation, which may not only promote cell growth via activation of kinases (Cerutti *et al.*, 1991), but may also contribute to the genomic instability already inherent in B cells. *H. pylori* are strong inducers of interleukin-8, a potent chemokine for neutrophil activation. Activated neutrophils are known to release reactive oxygen species, which can cause a wide range of DNA damage, including double-strand breaks (Wiseman *et al.*, 1996). *H. pylori* VacA strains can alter intracellular vesicular trafficking, ultimately inducing the formation of large intracellular vacuoles and mediating mucosal damage (Smoot, 1997). BabA strains express the BabA adherence factor that can bind to Lewis blood sugar molecules present on the membrane of gastric epithelial cells. This results in enhanced adhesion and higher colonisation capacity. Nonetheless, no differences in the above *H. pylori* virulent factors have yet been observed between patients with *H. pylori*-associated MALT lymphoma and those with *H. pylori*-induced gastritis (Lehours *et al.*, 2004). Virulence factors associated with gastric MALT lymphoma remain largely unknown.

Similarly, *C. jejuni* has been shown to produce a toxin that can directly cause DNA damage by inducing double-strand DNA breaks. This may be responsible for the truncated Ig observed in IPSID (Al Saleem *et al.*, 2005).

1.2.4.4 Role of anti-microbial and other therapies in the treatment of MALT lymphoma

The role of *H. pylori* infection in the development of gastric MALT lymphomas has led to the successful use of antibiotic treatment in the cases localised to the stomach and had a profound implication in the clinical management of MALT lymphoma. Evidence indicates that eradication of *H. pylori* with antibiotics such as metronidazole, tetracycline, clarithromycin and amoxicillin can be effectively employed as the first line therapy of localised gastric MALT lymphoma. *H. pylori* eradication is the primary therapy for stage I gastric MALT lymphoma. Multiple trials carried out in independent reference centres on large cohorts of patients with long follow-up, have demonstrated that complete histological remission can be obtained in roughly 80% of early stage gastric MALT lymphomas following *H. pylori* eradication (Morgner *et al.*, 2007). Such a response is durable, as illustrated by the low incidence of lymphoma relapse (3%) at 75 months follow-up after *H. pylori* eradication (Wundisch *et al.*, 2005). When they occur, relapses are often due to *H. pylori* re-infection (Fischbach *et al.*, 2004; Montalban *et al.*, 2006). Presence of histological residual disease or clonal neoplastic cells is seen in gastric biopsies after *H. pylori* eradication. The cells are detected by PCR in a proportion of antibiotic-responsive cases, but are not associated with lymphoma progression, nor with transformation into DLBCL (Fischbach *et al.*, 2002; Fischbach *et al.*, 2007), supporting a "watch and wait" strategy with regular monitoring instead of a systematic second line treatment.

Overall, about 30% of gastric MALT lymphomas do not respond to *H. pylori* eradication, which suggests the acquisition of an antigen-independent autonomous growth. The majority of the non-responsive cases are at stage II or higher (Ruskone-Fourmestraux *et al.*, 2001), while a high proportion of stage I cases show complete remission following *H. pylori* eradication (Stolte *et al.*, 1989). Resistance to *H. pylori* treatment is mainly accompanied by

the presence of t(11;18)(q21;q21) (Liu *et al.*, 2001a; Liu *et al.*, 2002b) and infrequently to t(1;14)(p22;q32) (Ye *et al.*, 2006). The role of *H. pylori* eradication in the treatment of transformed MALT lymphoma is controversial. Although gastric MALT lymphomas of stage II or higher are less likely to respond to *H. pylori* eradication (Ruskone-Fourmestraux *et al.*, 2001; Sackmann *et al.*, 1997), antibiotic treatment showed noticeable success in some *H. pylori*-positive cases, particularly those with limited dissemination. If the presence of the bacterium is confirmed, concurrent eradication of *H. pylori* is recommended, along with other treatment approaches, in order to limit the risk of lymphoma relapse (Morgner *et al.*, 2007). This strategy also applies to transformed MALT lymphoma. A significant proportion of stage I *H. pylori* positive transformed MALT lymphomas achieve complete lymphoma regression after *H. pylori* eradication (Morgner *et al.*, 2007).

MALT lymphoma that is non-responsive to antibiotic therapy can be treated with surgical resection alone or in combination with radiotherapy or chemotherapy. This treatment results in 90-100% 5-year survival for cases at stage I_E and 82% survival at stage II_E (Du *et al.*, 2002; Schechter *et al.*, 2000). Unfortunately, partial resection cannot remove all lymphoma cells, as they disseminate widely in the gastric mucosa. To completely remove the lymphoma, a total gastrectomy needs to be carried out which significantly reduces the patient's quality of life (Zucca *et al.*, 2000). Low-dose localised radiotherapy can be used alone to treat gastric MALT lymphoma. In a small study, patients with gastric MALT lymphoma with no evidence of *H. pylori* infection or with resistance to antibiotic therapy were treated with radiotherapy alone. 100% event-free survival was achieved by this treatment with a median follow-up time of 27 months (Parveen *et al.*, 1993). Other treatments include the use of therapeutic monoclonal antibody drugs such as rituximab. The improvement of some autoimmune conditions following the administration of rituximab leads to new therapeutic strategies in the

treatment of autoimmune-related MALT lymphomas. A phase II clinical trial including patients with salivary gland MALT lymphoma associated with Sjögren's syndrome showed that rituximab could lead to complete remission in 3 out of 7 patients (Pijpe *et al.*, 2005). Further investigations are needed, especially as other B-cell targeting therapies are emerging.

The therapeutic model of *H. pylori*-associated gastric MALT lymphoma may be extended to MALT lymphomas of other anatomical locations similarly associated with infectious agents. For example, eradication of *Borrelia burgdorferi* and *Campylobacter jejuni* infections has been shown to result in complete regression of some cases of cutaneous MALT lymphoma and IPSID respectively (Kutting *et al.*, 1997; Lecuit *et al.*, 2004). Ferreri and colleagues similarly demonstrated lymphoma regression in 13 out of 27 ocular adnexal MALT lymphomas (6 complete and 7 partial regressions) associated with *Chlamydia psittaci* infection, following eradicating antibiotic therapy with doxycycline (Ferreri *et al.*, 2006).

1.3 Genetics of MALT lymphoma

1.3.1 Chromosomal translocations

In addition to chronic immune stimulation, which plays an important role in the development of MALT lymphoma particularly at the early stages, the acquisition of genetic abnormalities contributes to both the genesis and the progression of the lymphoma. Four recurrent balanced chromosomal translocations have been described in MALT lymphoma (Du, 2007). Three of the four translocations involve *MALT1* or *BCL10* genes.

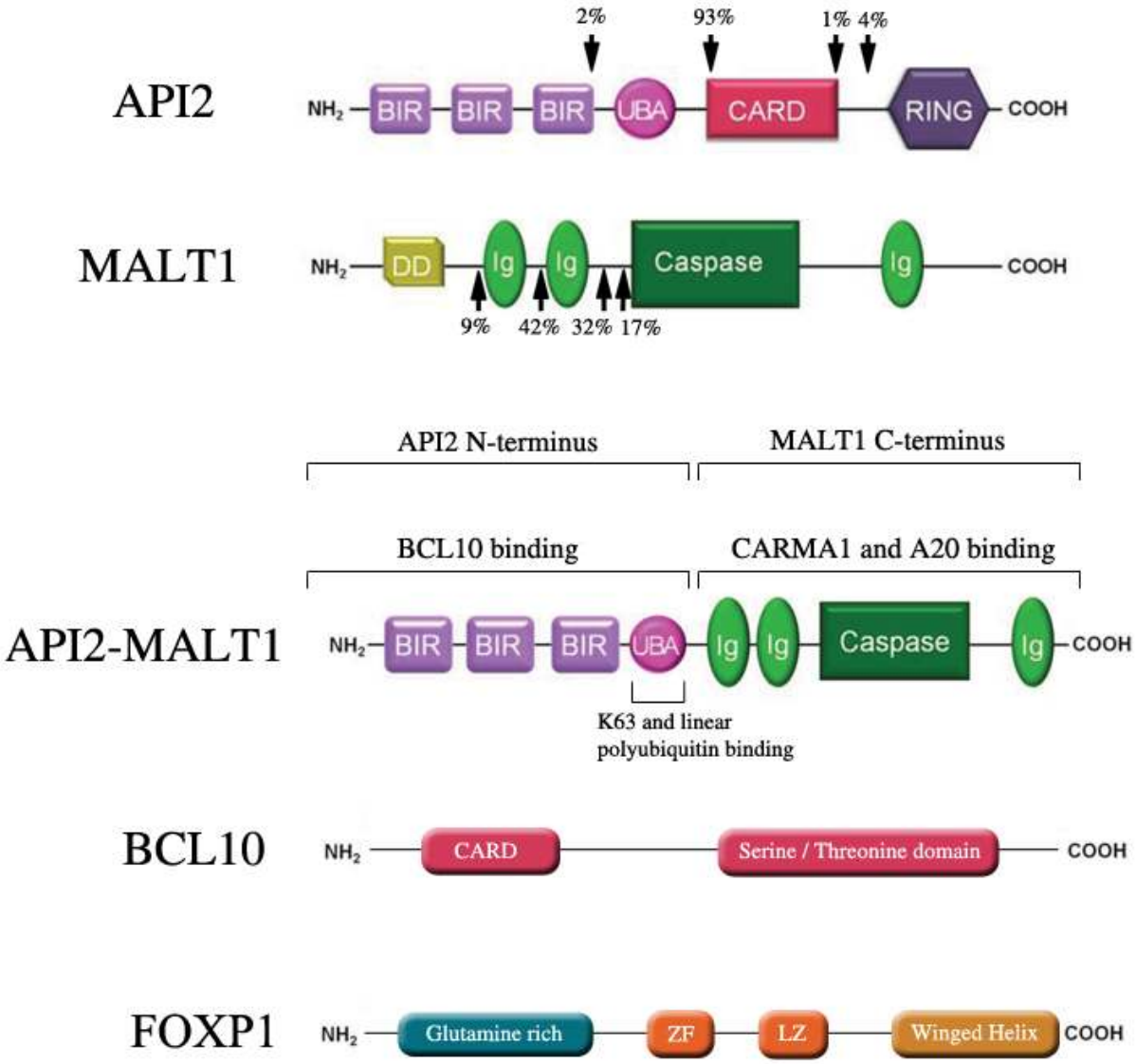


Figure 1.4. Structural domains of the oncogenic products resulting from MALT lymphoma associated chromosomal translocations.

BIR = baculovirus IAP repeats; UBA = ubiquitin associated domain; CARD = caspase associated recruitment domain; RING = really interesting new gene domain; Ig = immunoglobulin-like domain; ZF = zinc finger domain; LZ = leucine zipper domain. Arrows indicate the distribution of sites of fusion between *API2* and *MALT1* in individual MALT lymphomas.

API2 belongs to the inhibitor of apoptosis family and mediates apoptosis suppression by binding caspases via its caspase recruitment domain (CARD). API2 baculovirus inhibitor of apoptosis protein repeats (BIR) domains can mediate oligomerisation of BIR-domain containing proteins. MALT1 is a paracaspase. In addition to a caspase-like domain, MALT1 contains a death domain (DD) and 2 immunoglobulin-like domains (Ig). Although various breakpoints have been reported (Du, 2007), especially within the MALT1 gene, API2-MALT1 fusion transcript always contains the 3 intact BIR domains of API2 and the intact caspase-like domain of MALT1, suggesting a role for these domains in oncogenesis. BCL10 is an intracellular protein with a CARD domain and a serine/threonine-rich domain of unknown function. FOXP1 is a member of the FOX family of transcription factor containing both DNA-binding domains (zinc finger domains ZF; leucine zipper domain, LZ; winged helix) and protein-protein binding glutamine-rich domains.

1.3.1.1 t(11;18)(q21;q21)/API2-MALT1

t(11;18)(q21;q21) fuses in frame the amino-terminal sequence of the baculovirus inhibitor of apoptosis protein repeats (BIR) containing 3 (*BIRC3*, also known as *API2*) gene (11q21), to the carboxyl-terminal caspase-like domain of the MALT lymphoma translocation gene 1 (*MALT1*) (18q21) (Figure 1.4). API2 suppresses apoptosis by inhibiting specific caspases via its BIR domains (Roy *et al.*, 1997). *MALT1* is an essential component in the antigen-receptor-mediated activation of the master transcription factor nuclear factor κB (NF-κB) (Ho *et al.*, 2005; Lin *et al.*, 2004). t(11;18)(q21;q21) generates a functional fusion protein API2-MALT1 capable of activating NF-κB (Ho *et al.*, 2005; Uren *et al.*, 2000). An intact UBA (Ubiquitin Associated Domain) is present in roughly 98% of API2-MALT1 fusions and a functional UBA is required for efficient API2-MALT1 mediated NF-κB activation, although the precise molecular mechanism has yet to be established (Gyrd-Hansen *et al.*, 2008).

Incidence. t(11;18)(q21;q21) is the most common and specific structural chromosomal abnormality in MALT lymphoma. It has not been described in other B-cell lymphoma subtypes, including nodal and splenic MGZ lymphoma, nor in MALT lymphoma associated inflammatory conditions such as *H. pylori* associated gastritis, lymphoepithelial sialadenitis or Hashimoto's thyroiditis (Du, 2007). t(11;18)(q21;q21) occurs frequently in MALT lymphomas of the lung (40%), stomach (25%) and ocular adnexa (~10%), but not in those of the salivary gland and thyroid (Isaacson *et al.*, 2004; Ye *et al.*, 2003) (Figure 1.5).

Clinical impact. In gastric MALT lymphoma, t(11;18)(q21;q21) is associated with advanced stages, but not transformation into DLBCL (Huang *et al.*, 2003; Liu *et al.*, 2001a). t(11;18)(q21;q21) is associated with gastric MALT lymphomas that do not respond to *H. pylori* eradication, irrespective of the clinical stage of the lymphoma (Liu *et al.*, 2001b; Liu *et*

al., 2002a), and that are resistant to oral alkylating agents (Levy *et al.*, 2005). The detection of t(11;18)(q21;q21) is thus critical for the clinical management of gastric MALT lymphoma.

Genetic correlations. t(11;18)(q21;q21) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations (Du, 2007; Liu *et al.*, 2004a). t(11;18) positive cases rarely show aneuploidies frequently associated with translocation negative MALT lymphoma, such as trisomies 3, 12 and 18 (Remstein *et al.*, 2002; Starostik *et al.*, 2002).

Function. The biological importance of the API2–MALT1 fusion is indicated by the analysis of breakpoints at both the genomic and transcript levels. Although some genomic breakpoints were found to cause frameshifts, the API2–MALT1 fusion transcripts are always in frame, because of splicing out of the exon that causes the frameshift (Mestecky *et al.*, 1999). The API2 gene contains three amino-terminal baculovirus IAP repeats, a central caspase recruitment domain (CARD) and a carboxy-terminal zinc-binding RING finger domain. The MALT1 protein comprises an N-terminal death domain, followed by two Ig-like domains and a caspase-like domain. Within the API2 gene, all breakpoints occur downstream of the third BIR domain, upstream of the C-terminal RING domain, with 91% occurring just before the CARD domain. In the *MALT1* gene, the breakpoints occur in four different introns upstream of the caspase-like domain (Liu *et al.*, 2004a). Therefore, the resulting API2–MALT fusion transcripts always comprise the N-terminal region of API2 with three intact BIR domains and the C-terminal MALT1 region containing an intact caspase-like domain. The specific selection of these domains of the API2 and MALT1 gene to form a fusion product strongly indicates that they are required for the oncogenic activities of the fusion product. API2-MALT1 fusion product is capable of activating the NF-κB pathway in the absence of any surface receptor stimulation, although neither API2 nor MALT1 alone are able to activate NF-κB (Hu *et al.*, 2006b).

1.3.1.2 t(1;14)(p22;q32)/BCL10-IGH

t(1;14)(p22;q32) translocates the entire B-cell leukaemia/lymphoma 10 (*BCL10)* gene on chromosome 1p22 under the control of the immunoglobulin heavy chain locus (*IGH*) gene enhancer on chromosome 14q32 (Willis *et al.*, 1999; Zhang *et al.*, 1999). This translocation thus results in the over-expression of BCL10 which contains an N-terminal CARD, followed by a serine/threonine rich C-terminal.

The CARD motif of BCL10 can interact with the Ig-Like domains of MALT1 (Figure 1.4). *In vivo* studies showed that BCL10 and MALT1 link antigen receptor signalling and NF-κB activation pathway (Ruland *et al.*, 2001; Xue *et al.*, 2003).

Incidence. t(1;14)(p22;q32) is specifically associated with MALT lymphoma, though relatively infrequently. It is mostly seen in MALT lymphoma of the lung (7%) and stomach (4%) but not in those of other sites (Figure 1.5).

Clinical impact. Strong BCL10 nuclear localisation, characteristic of t(1;14)(p22;q32)-positive MALT lymphoma, correlates with gastric MALT lymphomas of advanced stages and those not responsive to *H. pylori* eradication (Ye *et al.*, 2006). Also, nuclear BCL10 expression is associated with advanced MALT lymphoma (Liu *et al.*, 2001b), and associated with shorter treatment failure-free survival in ocular adnexal MALT lymphomas (Franco *et al.*, 2006).

Genetic correlations. t(1;14)(p22;q32) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations. Translocation positive cases commonly harbour trisomies 3, 12 and or 18 (Streubel *et al.*, 2006).

Function. BCL10-knockout mice showed that BCL10 is essential for both the development and function of mature B and T cells, linking antigen-receptor signalling to the NF-κB pathway (Ruland *et al.*, 2001; Xue *et al.*, 2003). Over-expression of BCL10 activates the IκB

kinase (IKK) complex through CARD domain-mediated oligomerisation, resulting in NF-κB activation (Hofmann *et al.*, 1997). In line with these findings, a recent *in vitro* study indicated that BCL10 could prevent an immature B-cell line from antigen receptor induced apoptosis (Tian *et al.*, 2005b).

1.3.1.3 t(14;18)(q32;q21)/IGH-MALT1

t(14;18)(q32;q21) brings the entire *MALT1* gene (18q21) under the regulatory control of *IGH* (14q32), leading to the over-expression of *MALT1* (Figure 1.4) (Streubel *et al.*, 2003). The MALT1 gene is 5Mb away from the B-cell CLL/lymphoma 2 (*BCL2*) gene, targeted by t(14;18)(q32;q21) *IGH/BCL2* characterising follicular lymphoma (Sagaert *et al.*, 2007).

Incidence. t(14;18)(q32;q21)/*IGH-MALT1* is a rare translocation, mainly described in MALT lymphomas of the lung (6%) and ocular adnexa (7%) (Remstein *et al.*, 2006; Ye *et al.*, 2005) (Figure 1.5). It is also seen in some DLBCLs (Du, 2007).

Clinical impact. As t(14;18)(q32;q21) occurs exclusively in extra-gastric MALT lymphoma, for which a specific therapy has not yet been established, the potential impact of this translocation in the clinical management of MALT lymphoma remains unknown.

Genetic correlations. t(14;18)(q32;q21) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations. Translocation positive cases commonly harbour trisomies 3 and/or 18 (Streubel *et al.*, 2003).

Function. Initial studies of *MALT1* demonstrated that the wild-type protein has no independent ability to activate NF-κB and shows only modest enhancement of *BCL10*-mediated NF-κB activation, despite direct physical association with BCL10 (Uren *et al.*, 2000). MALT1 over-expression led to increased constitutive NF-κB activity and enhanced IκB kinase (IKK) activation induced by CD40 stimulation (Ho *et al.*, 2005).

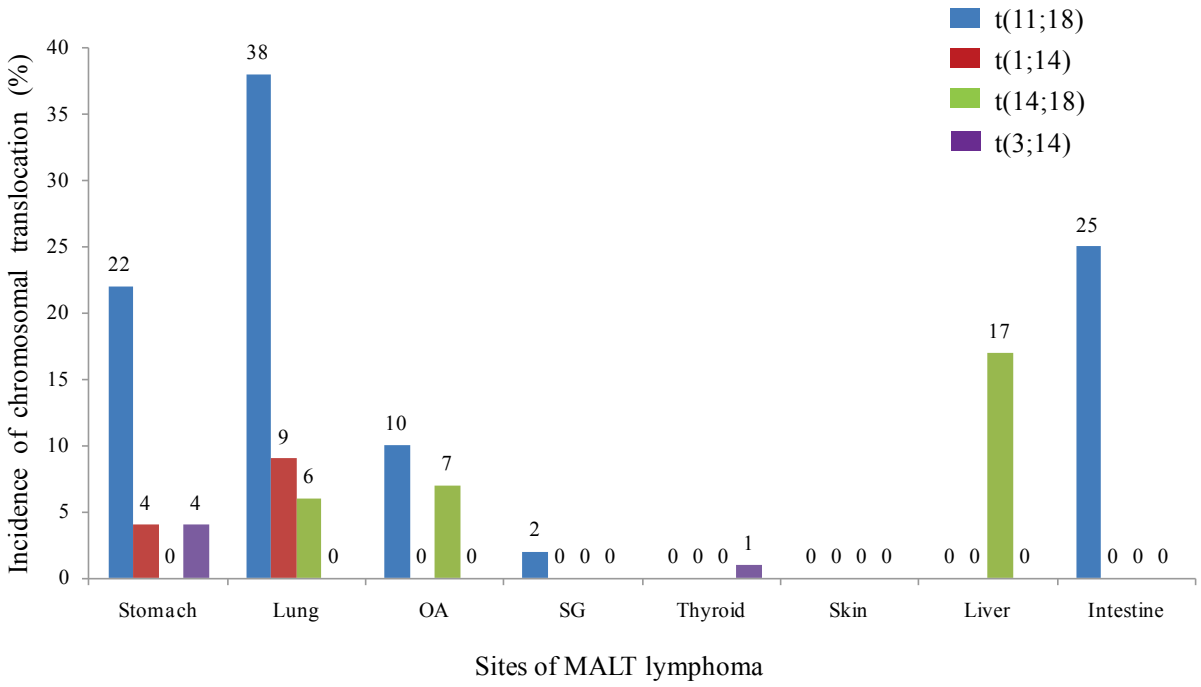


Figure 1.5 - Incidence of MALT lymphoma translocations in various sites

Figure adapted from Ye *et al.* (2005). Journal of Pathology, 205, 293-301.

1.3.1.4 BCL10 and MALT1 expression patterns in MALT lymphoma with different translocation status.

MALT1 and BCL10 proteins are essential components of NF-κB activation signalling pathway (Thome, 2004). In normal MGZ B cells, both MALT1 and BCL10 are weakly expressed, predominantly in the cytoplasm (Ye *et al.*, 2005). In MALT lymphoma, tumour cells with different translocations show distinct BCL10 and MALT1 expression patterns (Ye *et al.*, 2005). MALT lymphoma cells with t(11;18)(q21;q21)/*API2-MALT1* display moderate BCL10 nuclear expression and weak MALT1 cytoplasmic expression (Figure 1.6). Tumour cells with t(1;14)(p22;q32)/*BCL10-IGH* show strong BCL10 nuclear expression and weak/negative cytoplasmic expression of MALT1. Tumour cells with t(14;18)(q32;q21)/*IGH-MALT1* show strong homogeneous cytoplasmic expression of both BCL10 and MALT1 (Figure 1.6). Around 20% of MALT lymphoma cases without

translocation show BCL10 nuclear expression and around 6% show high BCL10 nuclear expression similar to that seen in t(1;14) positive MALT lymphomas (Liu *et al.*, 2001b).

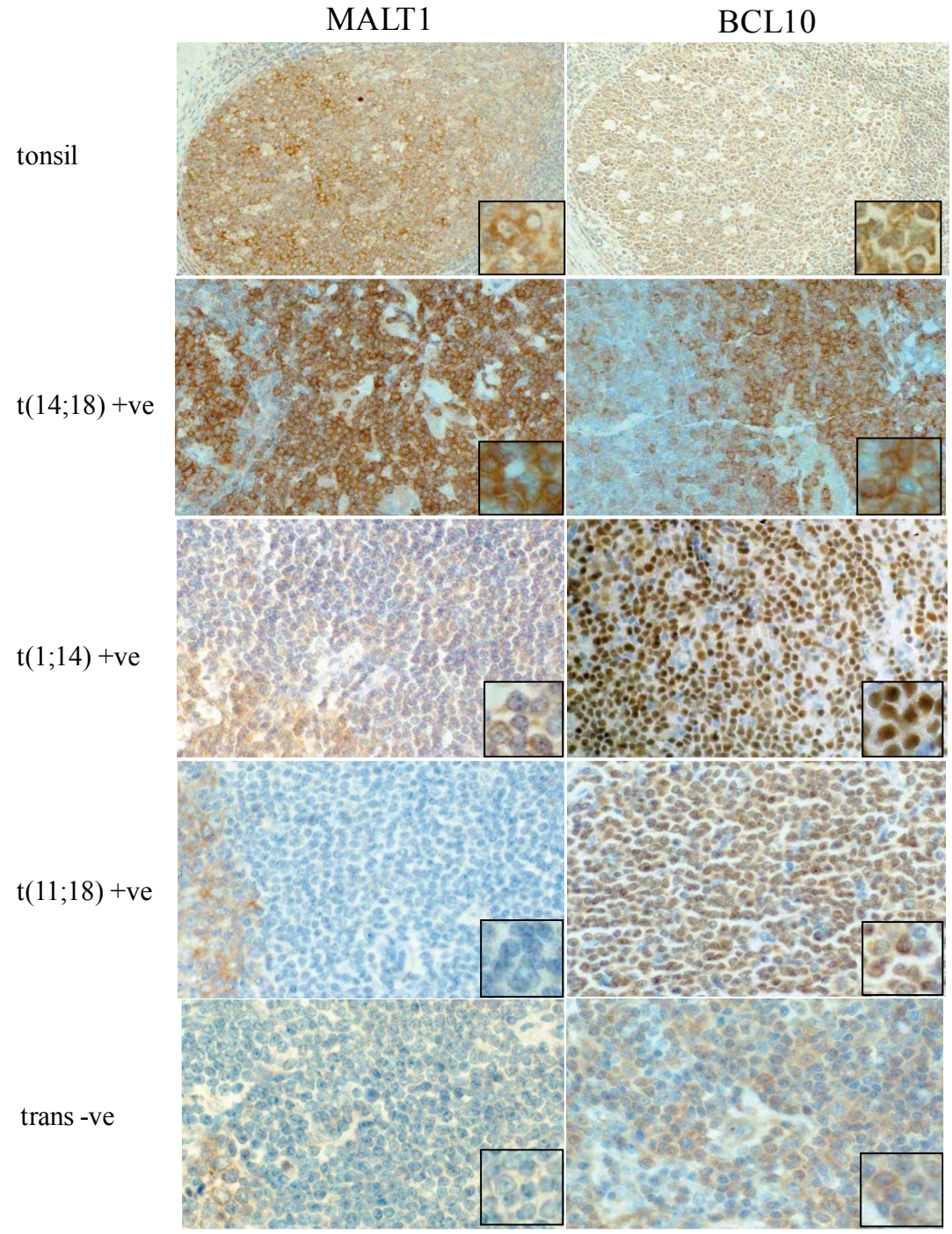


Figure 1.6 - BCL10 and MALT1 staining patterns in MALT lymphoma translocations.
BCL10 and MALT1 immunohistochemistry on FFPE translocation positive and negative MALT lymphoma tissue.

Figure adapted from Ye *et al.* (2005). Journal of Pathology, 205, 293-301.

Nakagawa and colleagues demonstrated that MALT1, but not its fusion counterpart API2-MALT1, was involved in BCL10 export from the nucleus to the cytoplasm, providing an explanation for the various BCL10 expression patterns observed in MALT lymphoma with different chromosomal translocations (Nakagawa *et al.*, 2005). In cells with t(11;18)(q21;q21)/*API2-MALT1*, MALT1 expression is reduced by half due to the API2-MALT1 fusion, hence an expected reduced efficiency of BCL10 nuclear export. In cells with t(1;14)(p22;q32)/*BCL10-IGH*, MALT1 normal expression may not be sufficient for the export of the over-expressed BCL10, resulting in BCL10 nuclear retention. Finally, in cells with t(14;18)(q32;q21)/*IGH-MALT1*, the over-expression of MALT1 results in an increased export of BCL10 from the nucleus to the cytoplasm, where both proteins are thus strongly expressed in the cytoplasm (Ye *et al.*, 2005). The temporal interplay between API2-MALT1, MALT1 and BCL10 in cellular localisation *in vivo* remains to be investigated.

1.3.1.5 t(3;14)(p13;q32)/FOXP1-IGH

t(3;14)(p13;q32) is a newly described MALT lymphoma- associated translocation involving *IGH* (Streubel *et al.*, 2005). It deregulates the forkhead box protein P1 (*FOXP1*) gene (3p13), a member of the FOX transcription factor family, which includes numerous proteins involved in a variety of functions such as cellular differentiation and immune regulation. Foxp1 has been recently shown to be an essential regulator of early B-cell development (Hu *et al.*, 2006a), but the molecular mechanism underlying its oncogenic activity in lymphoma remains to be investigated.

Incidence. Streubel *et al.* (Streubel *et al.*, 2005) initially showed that FOXP1 was involved in t(3;14)(p13;q32) in MALT lymphoma which was present in 10% of MALT lymphomas, in those from the ocular adnexa (20%), thyroid (50%) and skin (10%), but not in those from the

salivary gland, stomach and lung (Streubel *et al.*, 2005). However, the translocation was not found in 122 extranodal MALT lymphomas from various sites (9 cutaneous, 13 salivary gland, 50 pulmonary, 36 ocular adnexa, 8 thyroid gland and 6 gastric tumors) (Haralambieva *et al.*, 2006b). The t(3;14)(p13;q32) was subsequently found in one case of MALT lymphoma of the stomach (Wlodarska *et al.*, 2005), seven cases of diffuse large B-cell lymphoma of the stomach, thyroid and lymph nodes and also in two cases of B-cell non-Hodgkin lymphoma unclassified (Haralambieva *et al.*, 2006b; Wlodarska *et al.*, 2005). On a larger series of 321 MALT lymphomas, Goatly *et al.* showed that t(3;14)(p13;q32) is rare in MALT lymphoma, and primarily found in gastric cases (4%) (Goatly *et al.*, 2008; Haralambieva *et al.*, 2006a) (Figure 1.5).

Clinical impact. No correlation has yet been established between the presence of t(3;14)(p13;q32) and response to *H. pylori* eradication in gastric MALT lymphoma. In DLBCL, strong expression of FOXP1 identifies cases with poor prognosis (Barrans *et al.*, 2004). Similarly, FOXP1 over-expression is associated with MALT lymphomas with poor clinical outcome (Sagaert *et al.*, 2006a). MALT lymphomas with concurrent over-expression of FOXP1 and trisomies 3 and 18 may be at risk of transformation into DLBCL (Sagaert *et al.*, 2006a).

Genetic correlations. t(3;14)(p13;q32) is mutually exclusive from other MALT lymphoma-associated chromosomal translocations. Cases with t(3;14)(p13;q32) often harbour additional genetic abnormalities, especially trisomy 3 (Streubel *et al.*, 2005).

Function. Activation of the NF-κB pathway by isoforms of FOXP1 has been investigated (unpublished data in Professor Ming Du's laboratory). The full length FOXP1 protein and 2 short isoforms were able to activate NF-κB both alone and synergistically with cell surface stimulation of B cells by LPS and T cells by CD3 and CD28. The mechanism of NF-κB

activation is unknown but there is evidence that it is not through the classical or alternative pathways, in keeping with the nuclear localisation of FOXP1.

1.3.2 Other chromosomal translocations

In addition to the oncogenes mentioned in sections (1.3.1.1 – 1.3.1.4), novel translocations were identified using cytogenetics and long distance inverse PCR. Those include rearrangements of t(6;7)(q25;q11), t(1;14)(p22;q32)/CNN3-IGH, t(5;14)(q34;q32)/ODZ2-IGH and t(9;14)(p24;q32)/JMJD2C-IGH (Vinatzer *et al.*, 2008), t(X;14)(p11;q32)/GPR34-IGH (Novak *et al.*, 2008; Wlodarska *et al.*, 2009) and t(3;13)(q24;p11) (Aamot *et al.*, 2005). It has been shown that over-expression of GPR34 activated NF-κB *in vivo* (Novak *et al.*, 2008). However no functional characterisation for any of the translocations listed above has been performed. Nonetheless, the translocations above illustrate the heterogeneous nature of genetic alterations in MALT lymphoma.

1.3.3 Genetics of translocation negative MALT lymphoma

Trisomies are frequently associated with MALT lymphoma, particularly those without t(11;18)(q21;q21) (Brynes *et al.*, 1996; Streubel *et al.*, 2004). Trisomies 3 and 18 are found in about 35% and 25% of cases respectively. However, beyond those numerical aberrations, the molecular genetics of MALT lymphoma, especially those without chromosomal translocation, is poorly understood (Isaacson *et al.*, 2004). The chromosomal gains and losses in translocation negative MALT lymphomas of the stomach and salivary glands were investigated using metaphase comparative genomic hybridisation (CGH) and recurrent chromosomal gains involving the whole or major part of chromosomes 3, 12 and 18, as well as recurrent discrete gains at 9q34 and 11q11-13 were found (Zhou *et al.*, 2006; Zhou *et al.*, 2007). Array comparative genomic hybridisation (aCGH) showed frequent microdeletions

involving 6p25.3 to be associated with outcome of *H. pylori* eradication in translocation negative gastric MALT lymphoma (Fukuhara *et al.*, 2007).

Recently, work in Professor Ming Du's group showed that *A20* deletion was associated with copy number gain at the TNFA/B/C locus and occurred preferentially in translocation negative MALT lymphoma of the ocular adnexa and salivary glands but not in the stomach, lung and skin (Chanudet *et al.*, 2009). In addition to the *A20* gene deletions and mutations, promoter methylation was shown to be an alternative mechanism for *A20* inactivation in MALT lymphoma and *A20* complete inactivation was significantly associated with a shorter lymphoma-free survival in ocular adnexal MALT lymphoma (Chanudet *et al.*, 2010). The *A20* protein (also known as Tumour Necrosis Factor Alpha-Induced Protein 3 or TNFAIP3) is a key player in the negative feedback regulation of NF-κB signalling in response to multiple stimuli. For example, A20 regulates tumour necrosis factor (TNF)-induced NF-κB activation. Recent genetic studies demonstrated a clear association between several single nucleotide polymorphism (SNPs) in the *A20* locus and autoimmune disorders such as Crohn's disease, Rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes (Vereecke *et al.*, 2009) further implicating its link with both autoimmune disease and lymphoma development.

More recently, inactivating mutations predicting truncated A20 products were identified in 6 (19%) of 32 marginal zone lymphomas, including 2 (18%) of 11 extranodal marginal zone lymphomas, 3 (33%) of 9 nodal marginal zone lymphomas, and 1 (8%) of 12 splenic marginal zone lymphomas (Novak *et al.*, 2009). Another study identified inactivating mutations in A20 in 21% MALT lymphoma patients (Kato *et al.*, 2009). Collectively, *A20* can be completely inactivated by homozygous deletion, hemizygous deletion plus mutation or promoter methylation and bi-allelic mutations. A20 inactivation abolishes the major negative

regulation of the NF-κB pathway, thus potentially contributing to the constitutive NF-κB activation in lymphoma subtypes such as DLBCL, particularly the (activated B-cell) ABC subtype, classic Hodgkin lymphoma and Burkitt's lymphoma.

1.4 NF-κB pathway

As mentioned in section 1.3, MALT lymphoma associated oncogenes target the NF-κB pathway, thus in this section the NF-κB pathway will be discussed.

Nuclear Factor-κB (NF-κB) (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB was first discovered in the laboratory of Nobel Prize laureate David Baltimore via its interaction with an 11-base pair sequence in the immunoglobulin light-chain enhancer in B cells (Sen *et al.*, 1986). NF-κB is a ubiquitous transcription factor found in almost all animal cell types and is involved in cellular responses to diverse stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low density lipoproteins, and bacterial or viral antigens (Gilmore, 2006).

1.4.1 NF-κB family members

NF-κB is a family of 5 inducible transcription factors, sharing a highly conserved reticulo-endotheliosis viral oncogene homology (REL) domain, REL homolog A (RelA, also known as p65), REL homolog B (RelB), NF-κB1 (also known as p50) and NF-κB2 (p52). All have a transactivation domain in their C-termini. 15 transcription factors can be formed from the homo- and heterodimerisation of the five NF-κB subunits (Gilmore, 2006). The NF-κB1 and NF-κB2 proteins are synthesised as large precursors, p105, and p100, which undergo processing to generate the mature NF-κB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective

degradation of their C-terminal region containing ankyrin repeats. Whereas the generation of p52 from p100 is a tightly-regulated process, p50 is produced from constitutive processing of p105. The particular dimers present are dependent on the cell type, its stage of differentiation, and environmental signals (Hayden *et al.*, 2004). As all dimers are able to bind to κB consensus sites in promoters and enhancers, they can all be termed as "NF-κB". The components of each dimer affect its specificity for DNA binding, its interaction with other proteins, and the set of genes that it controls (Hayden *et al.*, 2004). NF-κB dimers have the ability to activate or repress transcription of genes (Perkins, 2007). NF-κB dimers are sequestered in an inactive form in the cytoplasm by inhibitory proteins of the IκB family (also known as nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor, NFKBI). Diverse surface receptor signalling can activate the molecular pathways that allow NF-κB nuclear translocation and activate its target gene expression.

1.4.2 NF-κB activation pathways

There are two main pathways within a cell that result in NF-κB activation, namely canonical and non-canonical pathways (Figure 1.7). Recent data indicate the existence of a third NF-κB activation mechanism, referred to as linear ubiquitin chain assembly complex (LUBAC) pathway (Tokunaga *et al.*, 2009) though this needs further experimental validation.

1.4.2.1 Canonical pathway

Receptor signalling

Whilst many different stimuli are able to activate NF-κB via surface membrane receptor signalling, the major ones can be broadly categorised into the following groups (Hayden *et al.*, 2008):

1) Antigen receptors such as B-cell receptors (BCR) and T-cell receptors (TCR) which lead to NF-κB activation via antigenic stimulation
2) Toll-like receptors (TLRs) which respond to stimulations by bacterial or fungal products such as lipopolysaccharide (LPS), CagA and CpG, leading to NF-κB activation
3) Tumour necrosis factor receptor (TNFR) which lead to NF-κB activation by stimulation with TNF ligand and (TNF)-related apoptosis-inducing ligand (TRAIL)

Irrespective of the stimulus involved, the key event of canonical NF-κB activation is the phosphorylation of IκBα protein by an IκB kinase complex (IKK). This targets IκBα for ubiquitination and subsequent degradation, thus releasing NF-κB. NF-κB then translocates to the nucleus and binds the promoters of target genes to regulate their transcription (Figure 1.7a).

Signal transduction

As mentioned above, a number of surface receptor signals lead to activation of the canonical pathway in both B and T cells. In the context of this thesis, only the antigen receptor and TLR signalling will be outlined as this is most relevant to MALT lymphoma. Antigen receptor signalling triggers serial proximal signalling that causes the auto-phosphorylation of phosphoinositide-dependent kinase 1 (PDK1). In T cells, phosphorylated PDK1 binds Protein

Kinase Cθ (PKCθ), activating this kinase via phosphorylation of Thr538 (Lee *et al.*, 2005; Park *et al.*, 2009). Similarly, in B cells, antigen receptor signalling triggers phosphorylation and activation of PKCβ. Subsequently, PKCβ phosphorylates several residues in the "linker" region (also known as PKC receptor) of CARD11 (Matsumoto *et al.*, 2005; Sommer *et al.*, 2005). This phosphorylation event triggers a conformational change in CARD11 allowing its CARD domain to interact with the CARD of BCL10. Because BCL10 and MALT1 are constitutively associated in the cytoplasm (Su *et al.*, 2005), the CARD11-BCL10 interaction also brings MALT1 into the complex, thereby forming the CARD11-BCL10-MALT1 (CBM) complex (Figure 1.8) (Matsumoto *et al.*, 2005). CBM is further stabilised by a direct interaction between the coiled coil domain of CARD11 and the C-terminal portion of MALT1 (Che *et al.*, 2004) (Figure 1.8).

Coincident with or downstream of formation of the CBM complex, a proportion of cellular BCL10 and MALT1 becomes oligomerised, as demonstrated by biochemical methodology (Sun *et al.*, 2004) and FRET microscopy (Rossman *et al.*, 2006), and it is these oligomeric species that are active in NF-κB signal transduction (Sun *et al.*, 2004).

The active/oligomeric forms of BCL10 and MALT1 elicit the E3 ubiquitin ligase activity of the TRAF6 RING domain to synthesize a K63-linked polyubiquitin chain on TRAF6 itself (Stilo *et al.*, 2004) (Thome, 2004; Thome, 2008) (Rossman *et al.*, 2006; Sun *et al.*, 2004) (Figure 1.8). Auto-ubiquitination increases the catalytic activity of TRAF6, enabling TRAF6-mediated K63-polyubiquitination of specific protein targets (Lamothe *et al.*, 2007). Activated TRAF6 polyubiquitinates MALT1 on multiple C-terminal lysines and BCL10 on Lys31 and Lys63 (Oeckinghaus *et al.*, 2007; Wu *et al.*, 2008).

K63-polyubiquitination of BCL10 is enhanced by the presence of MALT1 (Wu *et al.*, 2008), suggesting that MALT1-associated TRAF6 may contribute substantially to K63-

polyubiquitin modification of BCL10 in the CBM complex. Wu and colleagues showed that K63-polyubiquitination of BCL10 is essential for CBM association with the IKK complex and for consequent activation of NF-κB (Wu *et al.*, 2008), while Duwel and colleagues showed an inability to detect K63-polyubiquitination of CBM-associated BCL10 (Duwel *et al.*, 2009). Overall, data are consistent with a model proposing that NEMO is able to bind to K63-polyubiquitin chains on both BCL10 and MALT1, and that blockade of either of these interactions impairs NF-κB activation, by preventing efficient physical association between the CBM and IKK complexes. However, it is also possible that ubiquitin modification of BCL10 and/or MALT1 affects the function of one or both proteins in other as yet unidentified ways that are crucial for maximal activation of the IKK complex and do not contribute directly to physical association with the CBM.

CBM complex recruits the IKK complex through a recently identified ubiquitin binding domain (UBD) in the NEMO protein. This specialised UBD, called the UBAN (UBD in ABIN proteins and NEMO), facilitates association between the IKK complex and the K63-polyubiquitinated CBM complex (Wagner *et al.*, 2008; Wu *et al.*, 2006; Wu *et al.*, 2008). The association of the CBM and IKK complexes then allows TRAF6 (in cooperation with Ubc13/Uev1A) to add K63-polyubiquitin chains to the C-terminal Zn-finger domain of NEMO at Lys399 and possibly additional lysines (Perkins, 2006).

Biochemical studies suggest that K63-polyubiquitination of NEMO enables physical association with the TAK1/TAB2/TAB3 kinase complex, via the UBD of TAB2. This association allows TAK1 to phosphorylate and activate the IKKβ catalytic subunit (Perkins, 2006). However, a more recent T-cell study has provided evidence that the phosphorylation of IKKβ is independent of CBM-mediated signalling and NEMO ubiquitination, suggesting that the mechanism of TAK1 association with the IKK complex remains to be defined

(Shambharkar *et al.*, 2007). Regardless of the details by which the IKK complex is modified, data from both groups strongly suggest that the combination of NEMO ubiquitination and IKKβ phosphorylation is required for activation of IKKβ kinase activity (Shambharkar *et al.*, 2007; Shinohara *et al.*, 2005). IKKβ then phosphorylates IκBα, triggering the terminal activation events of the canonical NF-κB activation cascade, as described above (Figure 1.7a).

Toll-like receptor signalling pathway

TLRs play a crucial role in the innate recognition of various molecular motifs in pathogens, termed pathogen associated molecular patterns (PAMPs) that are conserved in a large group of pathogens, including bacteria, viruses, fungi and parasites. At least 13 TLR family members in vertebrates and 10 TLRs in humans have been reported (Barton *et al.*, 2002). All of these TLRs have extracellular leucine-rich repeats and an intracellular Toll/IL-1 receptor homology (TIR) domain (Takeda *et al.*, 2003). These receptors transmit signals via several intracellular molecules, including myeloid differentiation factor-88 (MyD88), IL-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor (TRAF) 6 and mitogen activated protein kinases (MAPKs). When associated with TLR, MyD88 recruits members of the IRAK family through (death domain-death domain) homophilic interactions. IRAK1 and IRAK4 are serine-threonine kinases involved in the phosphorylation and activation of TRAF6. After phosphorylation by IRAKs, TRAF6 forms a complex with Ubc13 and Uev1A, and activates a MAPK kinase kinase (MAPKKK) called transforming growth factor ß–activated kinase (TAK-1) (Barton *et al.*, 2003). Activated TAK-1, in turn, can phosphorylate MKK3 and MKK6, the kinases upstream of p38 MAPKs and JNK which in turn lead to the activation of AP-1 protein (Takeda *et al.*, 2003). In addition, TAK-1 can activate the IKK

complex, which phosphorylates IκBα consequently leading to NF-κB activation as detailed above (Barton *et al.*, 2003).

Different TLRs show variable recognition for microbial lipo-polysaccharides (LPS). For example *Escherichia coli* (*E. coli*) LPS is a ligand for TLR4. *H. pylori* associated LPS is recognized by the receptor complex containing TLR2–TLR1 or TLR2–TLR6 but not that containing TLR4 (Yokota *et al.*, 2007).

Chemokine receptor signalling pathway

Chemokines are small peptides that are potent activators and chemoattractants for leukocyte subpopulations and some non-haemopoietic cells. Their actions are mediated by a family of 7-transmembrane G-protein–coupled receptors (Murphy *et al.*, 2000). Chemokine receptors are divided into different families, CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors that correspond to the 4 distinct subfamilies of chemokines they bind (Murphy *et al.*, 2000). Some chemokine and cytokines activate central cellular pathways. For example, when CXCL8 (IL-8) binds to its specific receptors, CXCR1 or CXCR2, a rise in intracellular calcium activates the enzyme phospholipase D (PLD) that goes on to initiate an intracellular signalling cascade by activating the NF-κB and MAP kinase pathway. The initiated NF-κB and MAP kinase pathways activate specific cellular mechanisms involved in chemotaxis, degranulation, release of superoxide anions and changes in the avidity of cell adhesion molecules called integrins (Murdoch *et al.*, 2000). In addition, the binding of chemokines to their respective leukocyte receptors initiates a series of cellular events including NF-κB and MAP kinase activation, all of which aim to eradicate the infiltrating inflammatory agents. These events include changes in cell shape leading to enhanced locomotion, secretion of lysosomal

enzymes, and production of superoxide anions. Once leukocytes reach the source of inflammation, a cytokine-rich milieu is generated that is sustained until the invading antigen is eliminated. In general, immune responses do not produce endothelial injury; however, on occasion acute or chronic inflammation may occur in which the endothelium and surrounding tissues become damaged (for example, by neutrophil generated products).

Several homeostatic chemokines have been shown to play an important role in mucosal immunology via germinal centre formation, homing mechanisms and local retention of activated mucosal B cells and dissemination of B cells to extra-intestinal secretary effector sites. Homing mechanisms play a role in B-cell recruitment to secondary lymphoid tissue. For example, recent mouse studies suggested that although the CCR7 ligand operating together with the CXCR4 ligand (CXCL12) are crucial for endothelial B-cell adhesion in lymph nodes, B-cell entry into Peyer's patches depends on CXCR5 (Okada *et al.*, 2002).

1.4.2.2 Non-canonical pathway

Receptor signalling

The non-canonical NF-κB pathway is activated largely through signalling via the following main receptors:

1) CD40 receptor (a TNFR family member) which leads to NF-κB activation via CD40 ligand stimulation
2) B-cell activating factor family (BAFF) receptor (BAFF-R)
3) lymphotoxin α and β (LTβ also known as TNFC) receptor (LTβ-R)

Signal transduction

Receptor signalling activates NF-κB inducing kinase (NIK), which phosphorylates IKKα. Activated IKKα induces the partial proteolysis of p100 into p52. Subsequently, p52 binds to

RelB, forming an NF-κB dimer, which migrates to the nucleus and transactivates its target genes (Figure 1.7b) (Jost *et al.*, 2007).

In contrast to the canonical signalling that relies upon NEMO-IKKβ mediated degradation of IκBα, -β, and –ε, the non-canonical signalling critically depends on NIK mediated processing of p100 into p52. Given their distinct modes of regulation, these two pathways were thought to be independent of each other. However, recent studies revealed that synthesis of the constituents of the non-canonical pathway, via RelB and p52, is controlled by the canonical IKKβ-IκB-RelA:p50 signalling (Basak *et al.*, 2008). Moreover, generation of the canonical and non-canonical dimers, via RelA:p50 and RelB:p52, within the cellular milieu are also mechanistically interlinked (Basak *et al.*, 2008). These data suggest that an integrated NF-κB system network underlies activation of both RelA and RelB containing dimer and that a malfunctioning canonical pathway will lead to an aberrant cellular response also through the non-canonical pathway. As with most cellular pathways, activation needs to be counter-balanced by inhibition or damping.

A variety of recent evidence, however, suggests that the control of the NF-κB pathway is more complex than simply IKK-mediated regulation of the IκB-NF-κB interaction. For example, RelA and p50 are regulated by ubiquitination, acetylation and prolyl isomerisation, and the transactivation activity of RelA and c-Rel can be affected by phosphorylation. In addition, as a consequence of the induction of NF-κB activity (at least by tumour necrosis factor), IKKα is also induced to enter the nucleus where it becomes associated with κB site promoters/enhancers to phosphorylate histone H3 which enhances the transcription of κB site-dependent genes (Chen *et al.*, 2007).

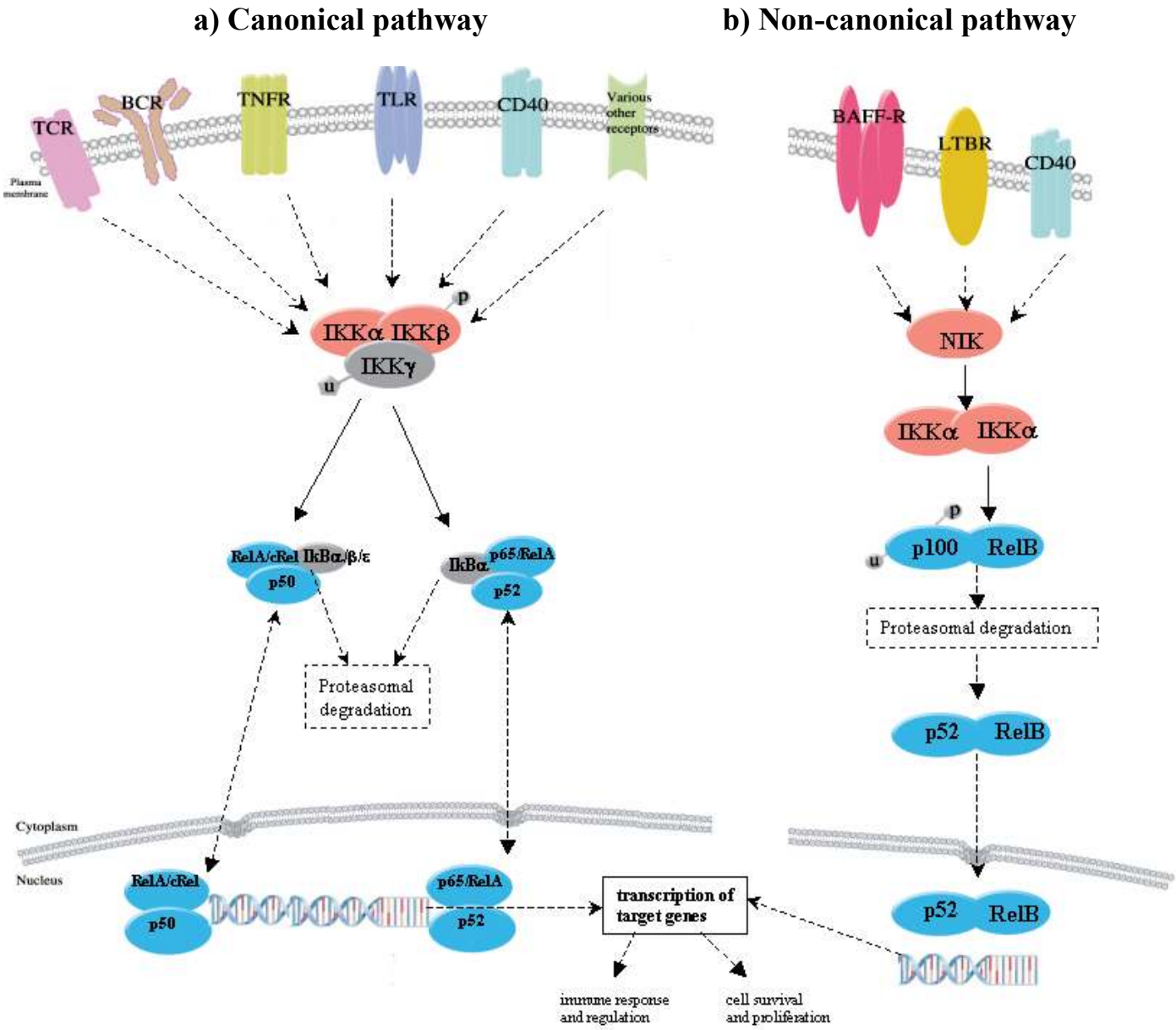


Figure 1.7 - Canonical (classical) and non-canonical (alternative) NF-κB pathway.
Inactive NF-κB dimers are sequestrated in the cytoplasm by inhibitory proteins of the IκB family, which includes IκBα, IκBβ, IκBγ, IκBδ, IκBε and BCL3, as well as p100 and p105 respective precursor of p52 and p50. In the canonical pathway, sequestrated NF-κB mainly consists of heterodimer p50-RELA. The IKK complex is composed of two kinase subunits (IKKα and IKKβ) and a regulatory subunit, NEMO (also known as NF-κB essential modifier NEMO). The activation of the IKK complex by phosphorylation (P) of IKKβ leads to the phosphorylation of IκB. This targets IκB for ubiquitination (U) and subsequent degradation. In the non-canoncial pathway, sequestrated NF-κB mainly consists of heterodimer p100-RELB. The IKK complex is a dimer of IKKα. Activated IKKα phosphorylates p100 and triggers its proteolysis, producing p52. This allows the formation of NF-κB active complex p52-RELB. Both the canonical and the non-canonical pathways ultimately lead to the translocation of NF-κB dimers into the nucleus, where they transactivate target genes critical for cellular mechanisms such as proliferation and survival. Some target gene products negatively regulate the NF-κB pathway.

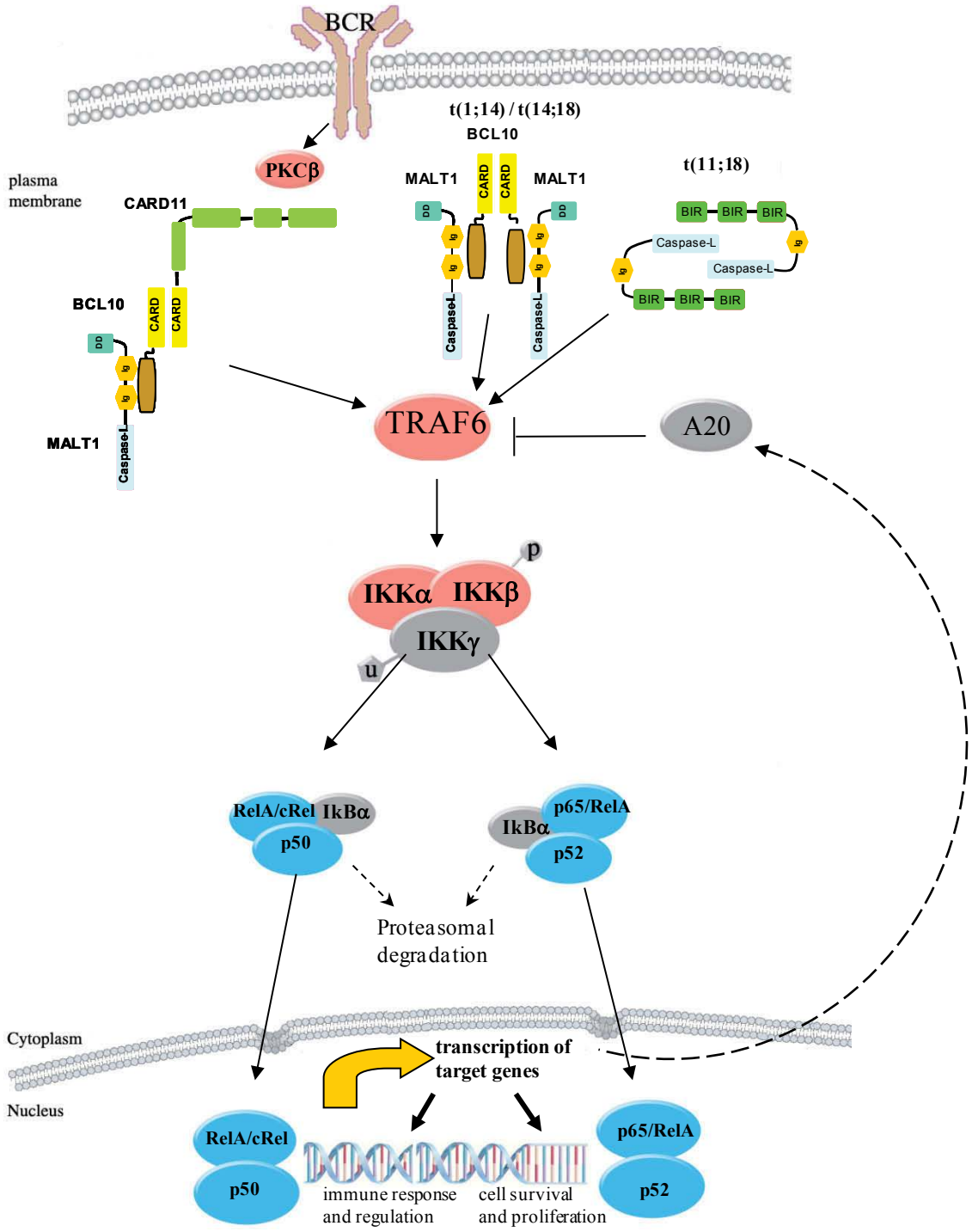


Figure 1.8 - B-cell receptor mediated NF-κB activation and constitutive NF-κB activation by MALT lymphoma associated translocations.
Following antigen recognition via PKCβ, CARD11 recruits BCL10 and MALT1 to the lipid raft at the activated receptor. Subsequently MALT1 binds TRAF6 activating its ubiquitin ligase. This results in TRAF6 and NEMO polyubiquitination and recruitment of kinase complex that phosphorylates IKKβ leading to IκBα phosphorylation and degradation. NF-κB is released and translocated into the nucleus, activating the transcription of target genes. The 3 MALT lymphoma associated translocations are believed to oligomerise and similarly recruit TRAF6 inducing constitutive NF-κB activation.

1.4.3 NF-κB negative regulators

As mentioned above, in unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB).

IκBs are related proteins in that they all have an N-terminal regulatory domain, followed by six or more ankyrin repeats and a PEST domain near their C terminus. Although the IκB family consists of IκBα, IκBβ, IκBγ and IκBε, and BCL3, the best-studied and major IκB protein is IκBα. Due to the presence of ankyrin repeats in their C-terminal halves, p105 and p100 also function as IκB proteins. The multiple copies of ankyrin repeats can mask the nuclear localisation signals (NLS) of NF-κB proteins sequestering them in an inactive state in the cytoplasm (Jacobs *et al.*, 1998).

Of all the IκB members, IκBγ is unique in that it is synthesised from the *NF-κB1* gene using an internal promoter, thereby resulting in a protein that is identical to the C-terminal half of p105 (Inoue *et al.*, 1992). The C-terminal half of p100, that is often referred to as IκBδ, also functions as an inhibitor (Basak *et al.*, 2007). IκBδ degradation in response to developmental stimuli, such as those transduced through LTβR, potentiate NF-κB dimer activation in of NIK dependent non-canonical pathway (Basak *et al.*, 2007). Unlike other members of the IκB family, BCL3 was found to function as a transcriptional activator and enhance NF-κB activity, primarily via forming a complex with the NF-κB p50 heterodimer to block the latter's mediated suppression of target gene expression (Li *et al.*, 2006).

Activation of NF-κB is initiated by the signal-induced degradation of IκB proteins via their ubiquitination by the proteosome. With the degradation of the IκB inhibitor, the NF-κB complex is then free to enter the nucleus where it can activate the expression of specific genes that have DNA-binding sites for NF-κB nearby. The activation of these genes by NF-κB then leads to the given physiological response, for example, an inflammatory or immune

response, a cell survival response, or cellular proliferation. Interestingly, these IκBs are part of NF-κB target genes where the newly synthesised IκB repressors masks the NLS of NF-κB sequestering it in the cytoplasm thus, forming a negative feedback loop, which results in tighter control and hence oscillating levels of NF-κB activity (Nelson *et al.*, 2004). This tight control is important as NF-κB regulates a multitude of target genes that play key roles in cell development and survival. In addition, NF-κB transactivates several other negative regulators such as A20 (Krikos *et al.*, 1992) and its adaptor molecules such as ABIN-1 to 3 (Verstrepen *et al.*, 2008).

1.4.4 NF-κB target genes and their biological implications

NF-κB activity is essential for development, activation, proliferation and survival of lymphocytes (Hoffmann *et al.*, 2006). NF-κB has the ability to regulate the transcription of an extensive variety of genes with a κB site in their promoter. Promoters that respond to NF-κB also contain consensus-binding sites for other transcription factors and these may be clustered into enhancers. This indicates that NF-κB may not act alone in transactivating gene expression (Hoffmann *et al.*, 2006).

Over 300 genes are regulated by NF-κB (http://people.bu.edu/gilmore/nf-kb/index.html) (Pahl, 1999), including NF-κB family members such as p50, p52 and RelB (Figure 1.9). A number of these NF-κB target genes encode NF-κB positive regulators, while several target genes encode negative regulators. According to their function, these NF-κB target genes can be categorised into the following groups:

Cytokines, chemokines and their modulators

NF-κB promotes the transcription of cytokines and chemokines (thus playing a critical role in inflammation) such as BAFF, TNF family members including TNFα, as well as numerous interleukins such as IL8 and IL2 and TRAIL (Apo2-Ligand) (Aggarwal, 2003).

Immunoreceptors

NF-κB target genes include those encoding immune system modulators such as Toll-like receptors (TLR) (TLR2 and TLR6), chemokine receptors (CCR5 and CCR2), CD40 (Hinz *et al.*, 2001), CD86 (Zou *et al.*, 2005), CD80 (Fong *et al.*, 1996), CD69 (Lopez-Cabrera *et al.*, 1995) and IRF4 (Grumont *et al.*, 2000). In all cell types studied thus far, the expression of most of the genes induced by inflammatory stimuli are upregulated by NF-κB (Li *et al.*, 2002) and interestingly the over-expression of surface immune receptor genes such as TLRs and CD40 can lead to positive feedback regulation of the NF-κB pathway.

Apoptosis regulators

NF-κB regulates genes involved in preventing apoptosis, such as *GADD45β* (Hoffmann *et al.*, 2006) and *BCL2* family members (Catz *et al.*, 2001) as well as IAP, FLIP, TRAF1 and TRAF2 (Karin *et al.*, 2002)

Cell cycle genes

NF-κB also regulates genes involved in cell cycle regulation cyclin D1 and MYC (Toualbi-Abed *et al.*, 2008) (Hoffmann *et al.*, 2006).

NF-κB negative regulators

NF-κB regulates the expression of its own inhibitors such as IκBα (Sun *et al.*, 1993), and IκBε (Tian *et al.*, 2005a) and NF-κB negative regulator genes such as *A20* (Krikos *et al.*, 1992) and ABIN (*A20* binding and inhibitor of NF-B) gene family including ABIN-1 to 3 (Verstrepen *et al.*, 2008) playing an important role in negative feedback regulation.

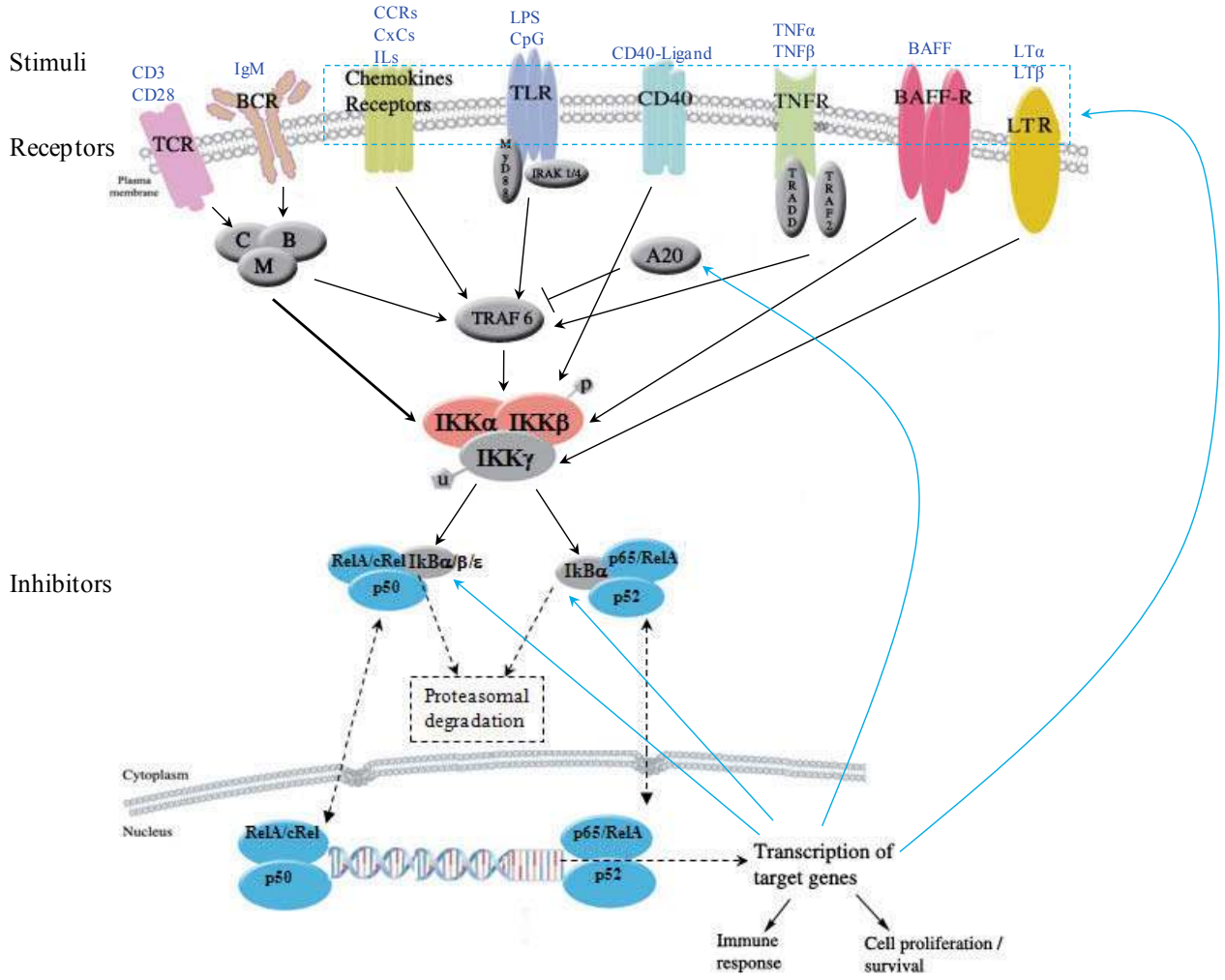


Figure 1.9 - Role of NF-κB target genes in immune response, cell proliferation and survival and maintaining cellular homeostasis.

NF-κB regulates the transcription (shown by the blue arrows) of many target genes involved in cell survival and proliferation as well as immune response and homeostasis. These can be broadly classed as positive and negative regulators of NF-κB. Positive regulators of NF-κB, shown in blue arrows, include immune surface receptors (shown in dotted blue box) that are essential for cell signalling such as chemokine receptors, TLRs, CD40, TNF receptor, BAFF receptor and lymphotoxin receptors. They respond to many pro-inflammatory and environmental stimuli such as LPS, chemokines and cytokines and ligands and lead to positive feedback loop causing constitutive NF-κB activation promoting tumour cell survival. Negative regulators of NF-κB include A20 and IκB family. Interactions between the pro-apoptotic and pro-survival pathways, as well as feedback loops within each pathway contribute to a tightly regulated balance of cell death and survival. B- and T- cell receptors are not target genes but lead to NF- κB activation via CARD11-MALT1-BCL10 (CBM) complex.

1.5 MALT lymphoma associated translocations target common molecular pathways that cause NF-κB activation

BCL10 and MALT1 are central to the transduction of signals from cell surface immune receptors to the classical NF-κB activation pathway in both B and T cells (Farinha *et al.*, 2005). The three common chromosomal translocations recurrently found in MALT lymphomas that involve the *BCL10* and *MALT1* genes are clearly implicated in the oncogenic process via deregulation of the NF-κB activation pathway. Mounting evidence indicates that the oncogenic activity of the three MALT-lymphoma associated chromosomal translocations is linked by roles of BCL10 and MALT1 in activating the NF-κB pathway in lymphocytes (Section 1.4) (Figure 1.8).

The API2-MALT1 fusion protein self oligomerises through a non-homotypic interaction mediated by the API2 region and is capable of activating NF-κB. However, neither MALT1 nor API2 alone is able to activate NF-κB *in vitro* (Du, 2007). Among the three BIR domains, only the first, BIR1, is essential for NF-κB activation by API2-MALT1 (Garrison *et al.*, 2009; Zhou *et al.*, 2005). BIR1 has been shown to contain an additional TRAF2/TRAF6 binding site, as well as a region that interacts with the C-terminal MALT1 responsible for a heterotypic oligomerisation that is critical for NF-κB activation (Garrison *et al.*, 2009). It is likely that the constituting oligomerisation of the API2-MALT1 fusion protein contributes to its ability to interact with and oligomerise downstream ubiquitin ligase such as TRAF2/TRAF6. TRAF2 and TRAF6 (together with the ubiquitin conjugating enzyme complex, Ubc13/Uev1A) trigger the K63-polyubiquitination of the API2-MALT1 fusion product and the K63-autoubiquitination of the TRAFs, themselves. The polyubiquitinated API2-MALT1 complex then binds to NEMO (Wagner *et al.*, 2008; Wu *et al.*, 2006). This

molecular association allows the TRAFs associated with API2-MALT1 to K63-ubiquitnate NEMO and causing the terminal events in the canonical NF-κB activation cascade.

In addition to binding to TRAF2/TRAF6, the API2-MALT1 protein has further activities that can augment signals to NF-κB (Zhou *et al.*, 2004). For example, some data suggest that API2-MALT1 itself possesses ubiquitin ligase activity that is capable of K63-polyubiquitinating NEMO and triggering NF-κB activation (Zhou *et al.*, 2005). Also, recent data indicate that the API2-MALT1 fusion protein binds to K63-polyubiquitinated NEMO via the UBA domain of API2 (Gyrd-Hansen *et al.*, 2008). It is possible that this interaction protects the NEMO protein from de-ubiquitination, thereby prolonging activation of the IKK complex. Another possibility is that the UBA-NEMO interaction stabilises the interaction between API2-MALT1 and the IKK complex, increasing the efficiency of NEMO K63-polyubiquitination by API2-MALT1-associated TRAF2 and TRAF6. There is also evidence that API2-MALT1, like MALT1, can mediate proteolytic cleavage of A20, a negative modulator of NF-κB signalling. This depends on the paracaspase activity of the caspase like domain (Coornaert *et al.*, 2008). A point mutation that disrupts this proteolytic activity decreases API2-MALT1 mediated NF-κB activation by approximately 3-fold (Hu *et al.*, 2006b). Thus, the proteolytic activity of API2-MALT1 appears to augment NF-κB activation by interfering with normal homeostatic mechanisms that serve to limit NF-κB activation. It remains to be investigated whether A20 is also cleaved when BCL10 or MALT1 is over-expressed.

BCL10 has been reported to interact with the API2-MALT1 fusion protein and synergistically enhance NF-κB activation in the absence of appropriate stimuli (Hu *et al.*, 2006b). In this study, it was shown that BCL10 interacts with a sub-fragment of API2 (1-

441), which contains the three BIR domains and the UBA domain (Gyrd-Hansen *et al.*, 2008) (Figure 1.4).

For the non-canonical (alternative) pathway, it has been shown that NF-κB signalling, once activated in a CD40-dependent immune response, is maintained and enhanced through deregulation of MALT1 or formation of an API2-MALT1 fusion (Ho *et al.*, 2005).

In contrast to MALT1 and API2-MALT1, BCL10 deregulation was demonstrated to promote survival of antigen-simulated B lymphocytes, by showing that over-expression of BCL10 in primary B cells activated *ex vivo* promoted the survival of these cells after removal of activating stimuli (Tian *et al.*, 2005b).

Although BCL10 and API2-MALT1 are potent activators of NF-κB, over-expression of these oncogenic products alone is not sufficient to induce malignant transformation. Transgenic mice expressing API2-MALT1 or BCL10 alone develop splenic marginal zone hyperplasia but not lymphoma (Baens *et al.*, 2006; Macintyre *et al.*, 2000; Li *et al.*, 2009). However, when transgenic mice expressing the API2-MALT1 fusion protein are immunised with the Freund's complete adjuvant, they develop splenic marginal zone lymphoma-like lymphoid hyperplasia (Sagaert *et al.*, 2006a). In line with this, expression of either API2-MALT1 or MALT1 in BJAB B cells enhanced the activation of IKK and NF-κB by CD40/CD40L stimulation (Ho *et al.*, 2005).

FOXP1-involved translocation occurs recurrently in MALT lymphoma and it is hypothesised that it may also confer oncogenesis through activation of NF-κB. Support for this hypothesis is provided by the observation that FOXP1 is highly expressed in the activated B-cell subtype of DLBCL in which NF-κB is constitutively active. However, these separate findings may be unrelated and offer no insight into the mechanism by which FOXP1 promotes oncogenesis. The full length and two short isoforms of FOXP1 were able to activate NF-κB alone and

synergistically with cell surface stimulation of B cells by LPS and T cells by CD3 and CD28. The mechanism of NF-κB activation is unknown but there is no evidence of activation of the canonical or non-canonical pathways in the cytoplasm, in keeping with the nuclear localisation of FOXP1 (unpublished data in Professor Ming Du's laboratory).

The extent of the oncogenic activities of these translocations is not yet fully understood as indicated by the aberrant pattern of BCL10 expression in some cases of MALT lymphoma. In normal B cells, including those of the marginal zone of B-cell follicles, BCL10 is expressed primarily in the cytoplasm (Ye *et al.*, 2000). However, in MALT lymphoma with t(1;14)(p22;q32), BCL10 is strongly expressed in the nuclei (Ye *et al.*, 2000). Moderate levels of nuclear BCL10 expression are also seen in up to 50% of t(1;14)(p22;q32)-negative MALT lymphomas, including almost all t(11;18)(q21;q21)-positive cases (Liu *et al.*, 2001b; Maes *et al.*, 2002; Ye *et al.*, 2003). Furthermore, BCL10 was found to be expressed at high levels in the nuclei of splenic marginal-zone B cells in transgenic mice in which BCL10 expression is driven by Ig enhancers (Li *et al.*, 2009). These observations indicate that aberrant nuclear BCL10 expression might have a role in MALT lymphoma development. However, the biological activity of nuclear BCL10 remains to be investigated.

1.6 Summary of current understanding of gastric MALT lymphoma

The sequential development of *H. pylori*-associated chronic gastritis, acquisition of MALT, low grade MALT lymphoma and transformation into DLBCL, clearly indicates a multistep process in the development of MALT lymphoma (Figure 1.10), primarily demonstrated by the histological presentation of these pathological conditions (Wotherspoon *et al.*, 1993). Recent studies provided further understanding at both the cellular and molecular levels. MALT lymphomagenesis recapitulates many aspects of the normal immune response and

lymphocyte development. The patterns of spread of lymphomas reflect the homing patterns of normal lymphocytes, both microscopically, within lymph nodes, and macroscopically, at a clinical level.

Correlation of the genetic aberrations with clinical outcome will permit improved diagnostic, prognostic and therapeutic sub-classification of MALT lymphomas. Whilst NF-κB deregulation as a consequence of the MALT lymphoma translocations is clearly indicated, further investigation is necessary to clarify the role of these events in oncogenesis. The equivalent events in translocation negative lymphoma also require elucidation.

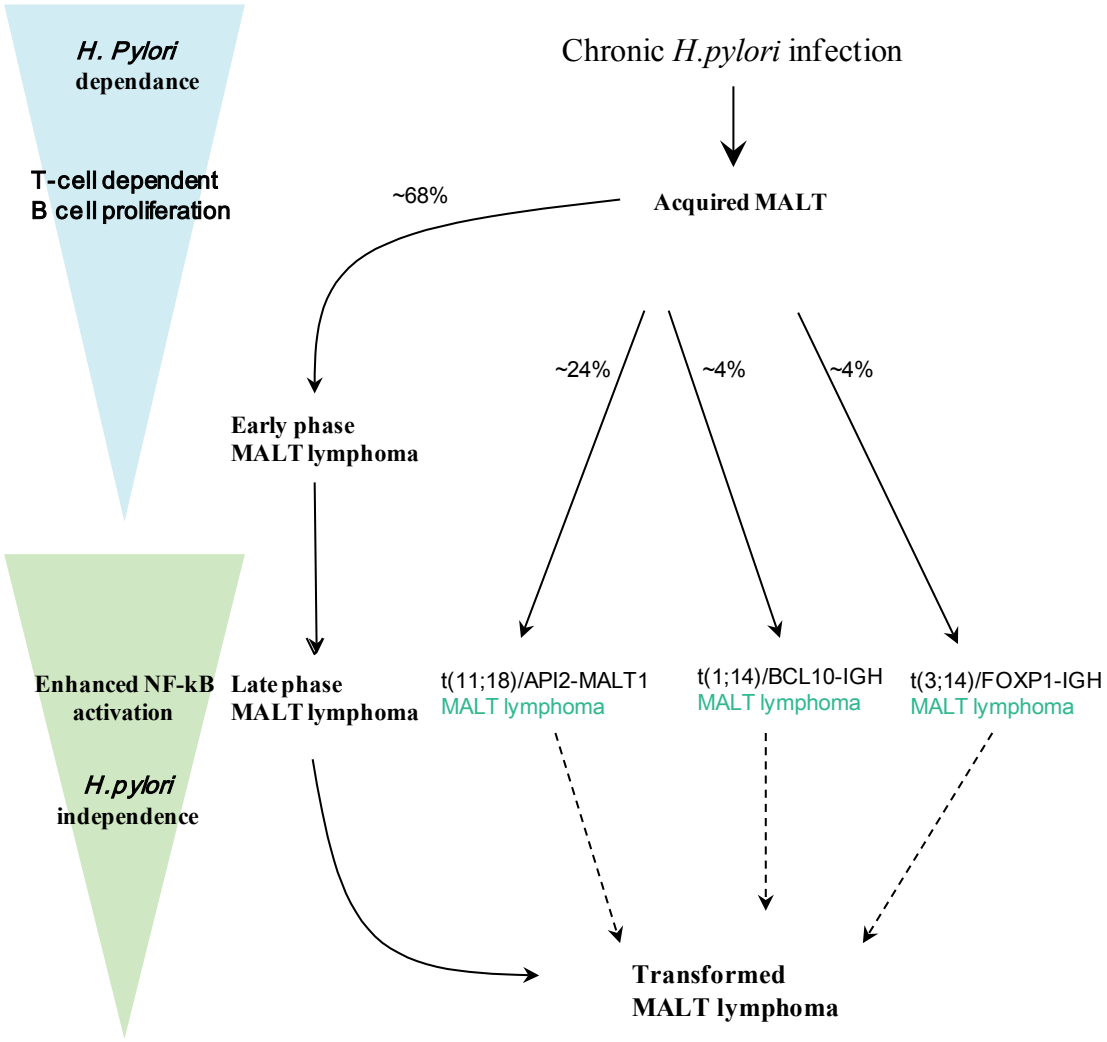


Figure 1.10 - Multistep development of gastric MALT lymphoma.

Figure adapted from Isaacson *et al.* (2004). Nature Review Cancer, 4, 644-653.

Infection by *H. pylori* first induces the formation of acquired mucosa-associated lymphoid tissue (MALT) in the gastric mucosa. The persistence of the bacterial infection results in chronic inflammation. Under sustained immunological stimulation, *H. pylori*-specific tumour-infiltrating T cells stimulate the proliferation of B cells. Genetic abnormalities such as chromosomal translocations (Figure 1.4) might be the result of oxidative stress associated with inflammation (Ye *et al.*, 2003). The acquisition of genetic abnormalities leads to malignant transformation. At this early stage, clonal expansion is *H. pylori*-dependent and the resulting MALT lymphoma can be effectively treated by *H. pylori* eradication with antibiotics. However, MALT lymphoma cells can gain autonomous growth ability, presumably through the acquisition of chromosomal translocations such as t(11;18) and t(1;14) or secondary genetic abnormalities such as A20 mutations, and thus, do not respond to *H. pylori* eradication.

The milieu of infection and chronic inflammation provide a common background for the development of MALT lymphomas. It is likely that NF-κB activation is involved in both translocation positive and negative MALT lymphoma. However despite the overlapping mechanisms there are important differences in the clinical and histological presentations between MALT lymphomas with and without chromosome translocation. Clinically, gastric MALT lymphomas with t(11;18) or t(1;14) are significantly associated with advanced clinical stages and resistance to *H. pylori* eradication (Liu *et al.*, 2002b; Ye *et al.*, 2006). Histologically, t(11;18) positive MALT lymphomas appear to be more monotonous, lacking apparent transformed blasts (Okabe *et al.*, 2003). These clinico-pathological characteristics may indicate the presence of significant differences in molecular mechanisms between MALT lymphomas with and without chromosome translocation. Thus, there are still many unanswered questions regarding MALT lymphomagenesis. Firstly, how chronic antigenic

stimulation in the presence of *H. pylori* or *C. psittaci* infection leads not only to chronic B-cell proliferation, but also to DNA damage which can result in the specific translocations that characterise most MALT lymphomas. Secondly, what are the molecular mechanisms that determine the response to *H. pylori* eradication. Thirdly, what is the molecular basis of NF-κB activation in MALT lymphoma. Fourthly, what are the molecular profiles and pathways targeted by MALT lymphoma with and without chromosomal translocations that can explain their differences in clinical and histological presentation. In order to answer some of these compelling questions, expression microarray investigations were applied in this thesis to MALT lymphoma cases with and without chromosomal translocation.

1.7 Gene expression microarray

Although all human somatic cells possess the same inherited genomic DNA, each cell transcribes different genes as mRNA according to the cell type. Further variation is provided by biological processes, both normal and abnormal conditions, amongst other parameters. The diversity in gene expression patterns has led to intensive research because of its biological and clinical relevance. Microarray technology allows the simultaneous profiling of the expression of tens of thousands of genes, thus painting a molecular portrait of the tissue or cell lines being studied. The technology, based on the micro-spotting of DNA, protein, tissue or small organic compounds, which can be probed with various labelled binding ligands (Howbrook *et al.*, 2003). Different microarray technologies are designed to address distinct biological questions. This thesis focuses on expression microarray technologies, which permit a broad overview of expression patterns.

1.7.1 Advantages of using gene expression microarray

It can be hypothesised within limits that changes in the phenotype of a cell or cell population should be reflected by concomitant transcriptional changes. Large-scale assessment of mRNA transcript abundance should thus provide a molecular signature of the state of gene activity of the biological system in question. Such assessment of gene expression has been considered a useful tool in the study of cellular biology in health and disease.

Currently, expression microarrays are much more reliable, optimised and permit higher throughput than any available proteome-assessing technology. Global and quantitative information on gene expression by measuring mRNA levels far outstrips the available proteomic technologies which are generally more technologically challenging and permit far lower throughput (Clarke *et al.*, 2001). Indeed, expression microarray technology has matured impressively in the last 8 years and now has a resolution of approximately one transcript per known gene. Currently available expression microarray platforms can assess gene expression of over 300,000 distinct transcripts in any single experiment. Nonetheless, the complementation of expression with data from high throughput proteomic technologies such as high throughput crystallography and protein-based microarrays is eagerly awaited (Howbrook *et al.*, 2003).

1.7.2 Limitations of gene expression microarray

The focus of expression microarray technology is the quantitative assessment of gene expression on a very large scale. Such an approach focusing on transcription is often questioned in relation to its ability to extrapolate findings at the protein level. This is one of the limitations of not only microarray-based, but all transcriptome-assessing technologies in

general. There is no doubt that results from expression technologies would be maximally effective if accompanied by and integrated with studies assessing global protein expression (Clarke *et al.*, 2001). However, even this combined approach would not suffice to describe a given cellular biological phenomenon in its entirety as an accurate description but would at least also require extensive study of protein post-translational modification and localisation.

Microarray technology is sensitive to cellular heterogeneity. In this context where RNA sample is obtained from complex tissues, expression results may relate to expression profiles from a number of distinct cell types. Thus it is important to use highly homogenous, carefully selected cell populations (for example lymphoma cells) as this can reduce the possibility of a given expression result being affected by cell types of secondary interest. The use of microdissected tissue may circumvent this problem by including mostly specific cell types in a given expression profiling experiment. Finally, the requirement of often large quantities of RNA (total or messenger) for these technologies may prove limiting especially for studies focusing on rare clinical material. The development of RNA amplification protocols, however, has addressed this issue successfully, significantly lowering the amount of RNA needed per microarray experiment. More recently, the discovery of microRNAs (miRNAs) (which are naturally occurring short non-coding RNA molecules that negative regulate eukaryotic expression through binding to the complementary sequences in the 3'-UTR of target mRNA) adds another limitation as some of the mRNA on the expression array may be negatively regulated by some of those miRNAs. Also studies have shown that some miRNAs such as miR-155, miR-210 and miR-21 are upregulated in the serum from DLBCL patients (Lawrie *et al.*, 2008a) whereas miR-150, miR-189, miR-223 and miR-768-3p are downregulated in DLBCL and BL patients suggesting that dysfunctional expression of miRNAs might be a feature in some haematological malignancies (Lawrie *et al.*, 2008b).

1.7.3 Microarray platforms

There are several expression microarray platforms, including complementary DNA (cDNA) microarrays (Schena *et al.*, 1995), oligonucleotide microarrays (Lockhart *et al.*, 1996) and serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995). The first two microarray technologies are the most commonly used platforms. The cDNA microarrays are based on standard microscopic glass slides on which cDNA fragments have been spotted. The oligonucleotide microarrays are constructed with oligonucleotides, 25- or 60-mer in length that are either synthesised *in situ* on a silicon wafer, or robotically spotted or injected on glass slides. The term commonly used to describe the DNA arrayed on a platform is "probe" and the cDNA or cRNA generated from a sample RNA, which represent the gene expression profile of the sample, are referred to as "target". The types of probes and targets used in each microarray platform differ, but these differences are becoming less significant. The main difference between the two platforms is the method by which the mRNA levels are determined. In cDNA microarrays, the quantitation is made by comparing a selected sample to a 'control' sample, while in oligonucleotide microarrays, a well-defined arbitrary unit is produced without the need to compare with a different sample. Generally, it has been shown that oligonucleotide arrays give better results than cDNA microarrays (Woo *et al.*, 2004). The most common oligonucleotide platform to study human genome expression is the Affymetrix HG-U133 GeneChip.

1.7.3.1 The Affymetrix HG-U133 GeneChip

The HG-U133 (Human Genome U133) GeneChip microarray set originally consisted of two GeneChips; HG-U133A and HG-U133B. Collectively, the HG-U133 set is capable of assessing a total of around 39,000 transcripts and variants, including more than 33,000 well-

substantiated human genes. The U133A chip alone assess 22,283 transcripts derived mostly from well-characterised genes and U133B can assess 22645 derived mostly from expressed sequence tags (ESTs). Sequences used in the design of the chip were selected from GenBank, dbEST and RefSeq. The sequence clusters were created from the UniGene database (Build 133, April 20, 2001) and then refined by analysis and comparison with a number of other publicly available databases. Subsequently around 2005, Affymetrix commercialised a new GeneChip that includes the whole human genome on one chip, named HG-U133 plus 2.0. This is capable of analysing the expression level of over 47,000 transcripts and variants, including 38,500 well-characterised human genes. It comprises more than 54,000 probe sets and 1,300,000 distinct oligonucleotide features. In addition, there are 9,921 new probe sets representing approximately 6,500 new genes. These gene sequences were also selected from GenBank, dbEST, and RefSeq. Sequence clusters were created from the UniGene database (Build 159, January 25, 2003) from manufacturer's datasheet at http://www.affymetrix.com/support/technical/datasheets/human_datasheet.pdf.

The unit of expression interrogation of the U133 chip is the probe set. Throughout this thesis, for the purpose of clarity, all genes mentioned will be associated to their probe set(s). The terms gene and probe set may be used interchangeably. It is stressed, however, that all observations refer to probe set signal intensity changes.

1.7.3.1.1 Probe versus Probe set

A major difference between spotted and GeneChip microarray technologies relates to probe design. Spotted microarray technologies employ probes that may either be synthetic oligonucleotides, long PCR products or cloned cDNAs. In all cases however, a single transcript is assessed by a single probe. For Affymetrix GeneChips, transcripts are

interrogated not by a single probe, but by a combination of probes collectively forming a probe set. Each probe in a probe set is designed to be complementary to a distinct region of the transcript queried. For example, the U133A Genechip used in this thesis, employs a total of 11 probes in the interrogation of each transcript assessed. These probes are referred to as perfect match (PM) as they are designed to be perfectly complementary to their respective transcript. In addition, for each probe set, a second set of mismatch probes (MM) with a mutation in the middle of the probe's sequence is employed to control for hybridisation specificity and background.

The generation of probe set values from respective probe values can be addressed in a number of ways. These differ mainly in relation to the use of MM probes and the statistical approaches adopted for obtaining single probe set expression values from individual probe intensities. In the most simplistic scenario, probe set level data can be obtained by initially correcting for background by subtracting each MM probe from its respective PM one and then averaging corrected PM probe intensities.

1.7.4 Data analysis

Regardless of the microarray platform, each experiment produces a data set containing tens to hundreds of thousands of values of gene expression. This overwhelming abundance of data requires the use of powerful statistical and analytical tools. After normalisation and non-specific filtering, there are two basic approaches to analysing gene expression data set. The supervised approach is based on determining genes that fit a predetermined pattern, usually used to correlate between gene expression and clinical data. The two most common supervised techniques are: nearest neighbour analysis (Golub *et al.*, 1999) and support vector machines (Brown *et al.*, 2000). The unsupervised approach is based on characterising the

components of the data set without the *a priori* input or knowledge of a training signal. This approach is usually used to identify a distinct subgroup of tumours that share similar gene expression profiles. The four most common unsupervised techniques are: principle-component analysis (Raychaudhuri *et al.*, 2000), hierarchical clustering (Eisen *et al.*, 1998), self organising maps (Tamayo *et al.*, 1999) and relevance networks (Butte *et al.*, 2000).

The first study utilising microarray technology demonstrated the power of this tool to classify and predict human acute leukaemias. These classifications and predictions were based solely on gene expression monitoring and were independent of previous biological knowledge (Golub *et al.*, 1999). Although histopathological evaluation, supplemented by cytogenetics and analysis of a few molecular markers, is still the gold standard in diagnosis and prognosis, gene expression profiling had proved capable of replacing these evaluations providing large numbers of patients are used in extracting the molecular signatures.

The rate-limiting step in functional genomics experiments is neither the handling of the biological samples nor the actual analysis, but instead the post-analytical work in determining what the results actually mean. This is largely restricted by the lack of biological knowledge of the gene studied, reliable bioinformatics methods in mining and interpretation of the massive data generated. Some advances have been achieved, such as the development of ONCOMINE, a cancer microarray database and web-based data-mining platform aimed at facilitating discovery from genome-wide expression analyses. To date, ONCOMINE contains 65 gene expression datasets comprising nearly 48 million gene expression measurements from over 4700 microarray experiments (Rhodes *et al.*, 2007) (http://www.oncomine.org). Other pathway and data mining software such as Ingenuity Pathway Analysis (http://www.ingenuity.com) and GeneGO (http://www.genego.com) help in the interpretation of microarray data. However, a precise method to map the genes that are differentially

expressed from the microarray studies to cellular pathways relevant to the cellular and molecular mechanisms of disease is required. Gene set enrichment analysis (GSEA) goes some way to achieve this. GSEA measures whether the individual genes in a signature are differentially expressed in a consistent fashion between two groups of samples (Mootha *et al.*, 2003). This method was originally used to demonstrate that genes involved in oxidative phosphorylation are decreased in expression in diabetic muscle (Mootha *et al.*, 2003). A recent enhancement of the GSEA method places added emphasis on those genes in a signature that are the most differentially expressed between two groups (Subramanian *et al.*, 2005).

1.7.5 Advances in lymphoma by gene expression microarray investigations

Gene expression profiling using microarrays has made huge improvement in disease classification, disease sub-classification, biomarkers identification, and deciphering the molecular pathogenesis in a number of lymphoma subtypes. Highlighted below are examples of such advances:

Disease sub-classification and characterisation

The most prominent examples of gene expression microarray in lymphoma sub-classification are studies of DLBCL. These studies revealed new unexpected subgroups of DLBCL which cannot be classified by conventional histology and immunophenotype. While 40% of patients respond well to chemotherapy, the remaining 60% succumbs to the disease. Using unsupervised analysis of gene expression data, Alizadeh *et al.* (Alizadeh *et al.*, 2000) identified two molecularly distinct forms of DLBCL, named germinal center B-cell-like (GCB) GCB-DLBCL and activated B-cell-like ABC-DLBCL. The different gene expression profiles apparently reflected the variation in tumour proliferation rate, host response and

differentiation state of the tumour. Importantly, this stratification proved to be clinically relevant in that activated B-cell (ABC) DLBCL is significantly associated with poor overall and event free survival. Subsequent studies showed that ABC-DLBCL is characterized by constitutive NF-κB activation and is more frequently associated with *CARD11* and *CD97B* inactivating mutations (Lenz *et al.*, 2008; Davis *et al.*, 2010).

Disease classification

Identification of molecular profiles that differentiate the lymphoma in question from other B-cell lymphomas can be seen in Burkitt's lymphoma microarray studies. Burkitt's lymphoma is a rare, aggressive B-cell lymphoma that accounts for 30% to 50% of lymphomas in children but only 1% to 2% of lymphomas in adults (NHL Lymphoma Classification Project, 1997; Swerdlow *et al.*, 2008). The main diagnostic challenge in Burkitt's lymphoma is to distinguish it from diffuse large B-cell lymphoma. The tumour cells of Burkitt's lymphoma are characteristically medium-sized (smaller than the cells of most diffuse large-B-cell lymphomas) and very high fractions are proliferating. Dave *et al.* (Dave *et al.*, 2006) and Hummel *et al.* (Hummel *et al.*, 2006), used gene expression microarray technology to improve the accuracy of the diagnosis of Burkitt's lymphoma. The two studies differ in many important ways, but both reach the same conclusion: the gene-expression profiling of cases classified as Burkitt's lymphoma by expert pathologists identifies a characteristic genetic signature that clearly distinguishes this tumour from cases of diffuse large-B-cell lymphoma. Furthermore, the microarray method seems to outperform the expert pathologists: 17% (Dave *et al.*, 2006) and 34% (Hummel *et al.*, 2006) of cases with the gene-expression signature of Burkitt's lymphoma had been called diffuse large-B-cell lymphoma or unclassifiable high-grade B-cell lymphoma; 0.4% (Dave *et al.*, 2006) and 4% (Hummel *et al.*, 2006) of cases

without the Burkitt's signature had been called classic or atypical Burkitt's lymphoma; and 3% (Dave *et al.*, 2006) and 8% (Hummel *et al.*, 2006) of cases diagnosed as diffuse large B-cell lymphoma or unclassifiable high-grade B-cell lymphoma had a Burkitt's signature.

Deciphering the molecular basis of lymphomagenesis

Examples of the use of expression microarrays in understanding the molecular pathogenesis of lymphomas can be seen in mantle cell lymphoma (MCL) and follicular lymphoma (FL). The common cytogenetic alteration in MCL patients, t(11;14), leads to cyclin D1 over-expression. Nevertheless, overexpressing cyclin D1 in transgenic mice was not sufficient to induce lymphomas, and other oncogenic factors, were required (Bodrug *et al.*, 1994). Thus, different mechanisms must be required for the development and progression of MCL. The first use of microarrays in studying MCL demonstrated altered apoptosis pathways, in addition to the known over-expression of cyclin D1 (Hofmann *et al.*, 2001). A comparison of MCL with normal human B cells revealed a distinct gene expression signature affecting lymphocyte trafficking, differentiation and growth regulation (Ek *et al.*, 2002). In follicular lymphoma, gene expression profiling showed that an interaction of tumour cells and the microenvironment determine the clinical behaviour (Glas *et al.*, 2007) and that transformation of follicular lymphoma to diffuse large B-cell lymphoma is preceded by distinct oncogenic mechanisms (Davies *et al.*, 2007).

Biomarker identification

Blenk *et al.* (Blenk *et al.*, 2007) analysed a large data set on DLBCL gene-expression (248 patients, 12196 spots) and identified specific, activated B-cell-like (ABC) and germinal center B-cell-like (GCB) distinguishing genes. These include early (e.g. CDKN3) and late (e.g. CDKN2C) cell cycle genes. Independently from previous classification by marker genes

they confirmed a clear binary class distinction between the ABC and GCB subgroups. The biomarkers set distinguishing marked over-expression in ABC from that in GCB, is built by: ASB13, BCL2, BCL6, BCL7A, CCND2, COL3A1, CTGF, FN1, FOXP1, IGHM, IRF4, LMO2, LRMP, MAPK10, MME, MYBL1, NEIL1 and SH3BP5. It predicts and supports the aggressive behaviour of the ABC subgroup and help to understand target interactions, improve subgroup diagnosis, risk prognosis as well as therapy in the ABC and GCB DLBCL subgroups.

1.7.6 Gene expression microarray studies of MALT lymphomas

There are so far three gene expression microarray studies on MALT lymphomas. O'Rourke *et al.* (O'Rourke, 2008) investigated gastric mucosa of BALB/c mice infected with *H. pylori* and correlated transcriptional profile with histological changes during the progression from chronic inflammatory infiltrate to MALT lymphoma. Huynh *et al.* (Huynh *et al.*, 2008) compared gene expression profiles of 21 gastric MALT lymphomas with corresponding *H. pylori* associated MALT B-cell follicles and aggregates. The study showed that gastric MALT lymphoma has a distinct gene expression profile, characterized by up-regulation of several surface receptor markers of haematopoietic cells such as CD1c, CD40, CD44, CD53, CD83 and CD86 and members of the HLA-D family, indicating antigen-dependent survival of lymphoma cells. Chng *et al.* (Chng *et al.*, 2009) attempted to classify 35 cases of pulmonary MALT lymphoma (10 with t(11;18), 3 with t(14;18) translocations and 22 negative for all known MALT lymphoma translocations) with other B- and T- cell lymphomas. They showed that MALT lymphoma is a distinct entity with a prominent T-cell signature and a marginal zone/memory B-cell profile. Fifty genes were differentially expressed between MALT lymphoma and all the other samples, 13 of which showed over-

expression in MALT lymphoma. Only 4, MMP7, SIGLEC6, WSB1, and PRO1853, were specifically overexpressed in MALT lymphoma, and 2 of these, MMP7 and SIGLEC6 were validated using immunohistochemistry on MALT lymphoma tissue microarrays. Hierarchical clustering of pulmonary MALT lymphoma with and without translocations showed overlapping transcriptional profiles with over-expression of NF-κB and chemokine signalling pathways in MALT lymphomas with t(11;18). Additionally, spiked expression analysis showed high expression of MALT1 and RARA. Samples with plasmacytic differentiation had high FKBP11 expression, and samples with high RGS13 expression tended to have trisomy 3 and reactive follicles. However, the main criticism of this study is that only 7 out of the 33 cases used had 70% or higher tumour content and including cases that had tumour content as low as 15% (Chng *et al.*, 2009). This can lead to false positives and under-powered study. In addition the study only focused on t(11;18) and t(14;18) in pulmonary MALT lymphomas only which can exclude other important genes implicated in MALT lymphoma such as BCL10.

In summary, none of the MALT lymphoma microarray studies thus far, convincingly showed the key molecular events explaining its lymphomagenesis.

1.8 Objectives of the thesis

1) To characterise the expression profile of MALT lymphoma with and without translocations with the aim to understand the common and unique molecular mechanisms underlying the disease;
2) To investigate the cooperation between the expression of MALT lymphoma oncogenes and immunological stimulation on NF-κB activation;
3) To identify novel phenotypic markers for MALT lymphoma by comparing expression microarray data of MALT lymphoma with other lymphomas such as FL, MCL, CLL and SMZL.

CHAPTER 2 – Materials and methods

2.1 Materials

2.1.1 Tissue materials and clinical data

2.1.1.1 Ethical considerations

The patient materials included in this study were archival fresh frozen and formalin-fixed paraffin-embedded tissues. Tissue material was retrieved from Professor Ming Du's laboratory and his international collaborators. Local ethical guidelines were followed for the use of these archival tissues for research with the approval by the local ethics committees of the relevant institutions.

2.1.1.2 Tissue materials for expression microarray and phenotypic marker studies

A total of 26 well characterised MALT lymphomas, 14 SMZL, 7 nodal FL and 8 nodal MCL were used for the expression microarray study. The MALT lymphoma cases included 9 positive for t(11;18)(q21;q21)/API2-MALT1 (8 gastric and 1 pulmonary), 4 positive for t(1;14)(p22;q32)/BCL10-IGH or t(1;2)(p22;p11)/BCL10-IGk (3 gastric and 1 pulmonary), 2 positive for t(14;18)(q32;q21)/IGH-MALT1 (1 hepatic and 1 ocular adnexal), 1 positive for t(3;14)(p13;q32)/IGH-FOXP1 and 10 gastric cases negative for all known MALT lymphoma associated chromosome translocations (Table 2.1). In all cases, fresh frozen tissue from surgical resection specimens was available. Tumour content was checked based on histological examination of Haematoxylin and Eosin (H&E) slides and where necessary crude microdissection was performed to ensure at least 70% tumour cells were used for molecular investigations. The chromosomal translocation status in these cases was investigated in previous studies using conventional cytogenetics, interphase FISH and RT-PCR where appropriate (Table 2.1). Extensive immunophenotyping including BCL10,

MALT1 and FOXP1 immunohistochemical data was available from previous studies (Goatly *et al.*, 2008; Liu *et al.*, 2001b; Liu *et al.*, 2001a; Liu *et al.*, 2004a; Ye *et al.*, 2000; Ye *et al.*, 2006).

Table 2.1 - Summary of clinico-pathological, molecular and immunohistochemical data of MALT lymphoma cases used in gene expression microarray studies.

Case No.	Sex	Age	Site	Diagnosis	Translocation status	Stage	Treatment	Follow-up	BCL10 immunohistochemistry
1	M	41	Stomach	MALT lymphoma	t(11;18)(q21;q21)	IIIE			Moderate nuclear
2	F	72	Stomach	MALT lymphoma	t(11;18)(q21;q21)	IVE			Moderate nuclear
3	M	NA	Stomach	MALT lymphoma	t(11;18)(q21;q21)				Moderate nuclear
4	F	52	Stomach	MALT lymphoma	t(11;18)(q21;q21)	IIE			Moderate nuclear
5	F	75	Lung	MALT lymphoma	t(11;18)(q21;q21)	I	Surgical resection,		Moderate nuclear
6	M	51	Stomach	MALT lymphoma	t(11;18)(q21;q21)	I	total gastrectomy	CR during 6 year follow up	Cytomplasmic
7	M	54	Stomach	MALT lymphoma	t(11;18)(q21;q21)	I	total gastrectomy	CR during 4 year follow up	Cytomplasmic
8	M	48	Stomach	MALT lymphoma	t(11;18)(q21;q21)	II1	total gastrectomy	CR during 10 year follow up	Cytomplasmic
9	F	62	Stomach	MALT lymphoma	t(11;18)(q21;q21)	I	Gastrectomy, no evidence of lymphoma during 3 years follow-up		Cytomplasmic
10	M	71	Stomach	MALT lymphoma	t(1;14)(p22;q32)	IVE	Gastrectomy		Strong nuclear
11	NA	NA	Lung	MALT lymphoma	t(1;14)(p22;q32)				Strong nuclear
12	F	55	Stomach	MALT lymphoma	t(1;14)(p22;q32)				Strong nuclear
13	M	67	Stomach	MALT lymphoma	t(1;2)(p22;q12)	IIIE	Total gastrectomy		Strong nuclear
14	F	62	Liver	MALT lymphoma	t(14;18)(q32;q21)	I	Surgical resection, no evidence of lymphoma during 6 years follow-up		Strong cytoplasmic
15	F	56	Ocular adnexa	MALT lymphoma	t(14;18)(q32;q21)	I	Treated by radiotherapy, no evidence of lymphoma during 10 years follow-up		Strong cytoplasmic
16	M	NA	Stomach	MALT lymphoma	Negative				Moderate nuclear
17	M	62	Stomach	MALT lymphoma	Negative				Moderate nuclear
18	NA	NA	Stomach	MALT lymphoma	Negative				Moderate nuclear
19	M	64	Stomach	MALT lymphoma	Negative				Cytoplasmic
20	M	NA	Stomach	MALT lymphoma	Negative				Weak cytoplasmic
21	M	18	Stomach	MALT lymphoma	Negative				Cytoplasmic
22	M	57	Stomach	MALT lymphoma	Negative	II1	Total gastrectomy	CR during 13 year follow up	Cytoplasmic
23	F	64	Stomach	MALT lymphoma	Negative	I	Total gastrectomy	CR during 6 year follow up	Cytoplasmic
24	M	50	Stomach	MALT lymphoma	Negative	I	Total gastrectomy	CR during 2 year follow up	Cytoplasmic
25	M	55	Stomach	MALT lymphoma	Negative				Cytoplasmic
26	NA	NA	Stomach	MALT lymphoma	t(3;14)(p14;q32)				Cytoplasmic

CR: complete remission

2.1.1.3 Tissue materials for qRT-PCR and immunohistochemistry

For qRT-PCR and immunohistochemical validation of the microarray study, an additional 73 cases of well-characterised MALT lymphoma were recruited. They included 18 cases positive for t(11;18), 8 cases positive for t(1;14) or variant, 9 cases positive for t(14;18), and 38 cases negative for these translocations.

2.1.2 Reagents

2.1.2.1 Reagents used in gene expression microarray and qRT-PCR

5x First Strand buffer for double stranded cDNA synthesis (Invitrogen)
250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$

5x Second Strand buffer for double stranded cDNA synthesis (Invitrogen)
100mM Tris-HCl (pH 6.9), 450mM KCl, 23mM $MgCl_2$, 0.75mM β-NAD+, 50mM $(NH_4)_2SO_4$

5x Fragmentation buffer
200mM Tris acetate pH 8.2, 500mM potassium acetate, 150mM magnesium acetate

1x Hybridisation buffer
100mM MES, 1M [Na+], 20mM EDTA, 0.01% Tween20

Hybridisation cocktail per chip
3nM Control Oligonucleotide B2, Eukaryotic Hybridisation Controls (*bioB*, *bioC*, *bioD* and *cre*, prepared in concentrations of 1.5, 5, 25 and 100pM respectively), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1x Hybridisation buffer to a final volume of 275µl with ultrapure deionised water

MES (2-(N-Morpholino) ethanesulfonic acid sodium salt)
To make 12X MES Stock (1.22M MES, 0.89M [Na+]) in 1000 ml, add :

70.4g MES free acid monohydrate (Sigma)
193.3g MES Sodium Salt (Sigma)
800ml of Molecular Biology Grade water
Mix and adjust volume to 1000ml with DEPC-treated water
The pH should be between 6.5 and 6.7. Filter through a 0.2 µm filter.

Wash buffer
100mM MES, 0.1M [Na+], 0.01% Tween20

Streptavidin-Phycoerythrin (SAPE) solution per chip
10µg/ml SAPE stain, 1x MES stain buffer, 2mg/ml acetylated BSA to a final volume of 600µl with double distilled water

Biotinylated anti-streptavidin per chip
2mg/ml acetylated BSA, 0.1mg/ml normal goat IgG (Sigma), 0.5mg/ml biotinylated antibody (Vector Lab, Burlingane, California, USA), 1x MES stain buffer to a final volume of 600µl with double distilled water

2.1.2.2 Reagents used in immunohistochemistry

PBS-Tween
100µl of Tween 20 (Sigma-Aldrich) was added to 200ml of PBS to give a final concentration of 0.05%

Diaminobezidine tetrahydrochloride (DAB) substrate solution (DAKO)
20µl of Dako REAL DAB Chromogen (Dako Cytomation) was added to 1ml of Dako REAL Substrate Buffer (Dako Cytomation) to make the substrate working solution

Diaminobezidine tetrahydrochloride (DAB) substrate solution (Kem-En-Tec)
This solution was always prepared fresh just before use. One tablet of DAB (Kem-En-Tec, Denmark) was dissolved in 10ml of distilled water. 10µl of 30% hydrogen peroxide solution was added to the solution just before application

Haematoxylin
Mayers haematoxylin was filtered and two drops of Tween 20 (Sigma-Aldrich) were added per 500ml

Citrate Buffer pH 6.0
8.82g of sodium citrate tribasic dehydrate (Sigma-Aldrich) were dissolved in 3 litres of distilled water and the pH was adjusted to 6.0 with 1M HCl

Peroxidase block solution
This solution was prepared fresh before use and was composed of 200µl of 30% hydrogen peroxide (Sigma-Aldrich) in 12ml of methanol

Tris-buffered saline pH 7.6 (TBS)
6.05g of Tris (hydroxymethyl) aminomethane (Sigma-Aldrich) and 80g of NaCl were dissolved in 8 litres of distilled water, the pH was adjusted to 7.6 with 1M HCl and the volume brought up to 10 litres with distilled water

TBS-Tween
Tween 20 (Sigma-Aldrich) was added to TBS to give a final concentration of 0.05%

2.1.2.3 Reagents used in tissue culture

RPMI 1640 10% fetal calf serum (FCS) medium & 10% P/S
50ml of FCS (Invitrogen) were added to 450ml of RPMI 1640 medium (Invitrogen). When culturing BJAB Tet-On and TRex cells, tetracycline (Tet) free FCS was used to prevent inadvertent induction of expression. Add penicillin/streptomycin (P/S) to a final concentration of 100U/ml penicillin and 100µg/ml streptomycin

10x Phosphate buffered saline (PBS)
146.6g sodium chloride, 47.2g hydrogen phosphate and 26.4g sodium dihydrogen phosphate for 2 litres distilled water – pH 7.2

Freezing medium
2ml of DMSO were added to 18ml of FCS

2.1.2.4 Reagents used in Western blotting and co-immunoprecipitation

Triton Lysis Buffer
5ml of 50mM Tris pH 7.4, 30ml of 300mM NaCl, 10ml of 1% Triton X-100 (Sigma-Aldrich), and 400µl of 2mM EDTA were added to 100ml with sterile distilled water and mixed to make a stock solution. One tablet of Protease Inhibitor Cocktail (Roche, Penzberg, Germany) for inhibition of proteases was added to 10 ml of stock solution before use

Protein lysis buffer (2X)
Lysis buffer was composed of 100mM Tris-HCl (pH8.0), 300mM NaCl, 0.04% sodium azide, 0.2% sodium dodecyl sulphate (Sigma-Aldrich), 2% nonidet P-40 (BDH), 1% sodium deoxycholate (BDH), 2mM EDTA, 100mg/ml phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 1mg/ml leupeptin (Sigma-Aldrich)

Sample loading buffer
4µl of β-mercaptoethanol (BDH) was added to 96µl of 4X NuPAGE LDS Sample Loading Buffer (Invitrogen)

Running Buffer
50ml of 20X NuPAGE Running Buffer (Invitrogen) were added to 950ml of deionised water

Transfer Buffer
50ml of 20X NuPAGE Transfer Buffer (Invitrogen) and 100ml of methanol were added to 850ml of deionised water

TBST
40ml of 1M Tris pH 7.4, 18g of NaCl and 2ml of Tween 20 (Sigma-Aldrich) were made up to 2 litres with deionised water and mixed

4% milk/TBST
4g of milk powder (Marvel, Premier Foods, UK) dissolved in 100ml of TBST

Stripping buffer
100ml of 10% SDS (10g in 100ml deionised water) were combined with 31.25ml of 1M Tris-HCl pH 6.8 and made up to 500ml with deionised water

2.1.2.5 Reagents used in cloning and DNA sequencing

LB medium
Autoclaved Luria-Bertani (LB) medium was provided in-house: 1% sodium chloride, 0.5% yeast extract and 1% tryptone (Sigma)

LB agar plates
15g/L bacteriological agar (Sigma) added to LB medium, autoclaved and poured into 100*15mm Petri dishes (Sigma)

1x Blue dextran mix
100mg/ml blue dextran (Sigma), 25mM EDTA

1x Tris-borate-EDTA electrophoresis buffer (TBE)
89mM Tris base (Sigma), 89mM boric acid (BDH), 2mM EDTA

Gel solution
To 900ml of Automatrix 4.5% 29:1 acrylamide:bisacrylamide ready made 6M urea gel solution (National Diagnostics, Hull) add 10ml of 1x TBE

2.1.3 Cell lines

BJAB cells: BJAB is a human Burkitt's lymphoma cell line and is Epstein-Barr virus (EBV) negative (Steinitz *et al.*, 1975). BJAB cells were a kind gift from Dr. Rolf Renne (University of Florida Shands Cancer Center, Florida, USA).

BJAB-TetON cells: BJAB-TetON cells were a kind gift from Dr. Rolf Renne (University of Florida Shands Cancer Center, Florida, USA) (An *et al.*, 2005). Tetracycline free FCS was used when carrying out experiments involving inducible expression of the oncogene of interest.

T-REx Jurkat cells: Jurkat cells are a human T-cell line derived from a patient with acute lymphoblastic leukemia (Schneider *et al.*, 1977). T-REx Jurkat cells (Invitrogen, Paisley, UK) stably express the tetracycline repressor protein. Tetracycline free FCS was used when carrying out experiments involving inducible expression of the oncogene of interest.

BaF-3 cells: An IL-3-independent clone of this murine pro-B cell line was established from the peripheral blood of a BALB/c mouse and was the kind gift of Dr Heike Laman, (University of Cambridge, UK).

WEHI cells: Immature murine pro-B cell line established from the peripheral blood of BALB/c mouse and was the kind gift of Dr Heike Laman, (University of Cambridge, UK).

2.2 Methods

2.2.1 Overview of the study plan

Figure 2.1 outlines the study plan in this thesis integrating the investigations using the primary lymphoma materials and *in vitro* cell lines.

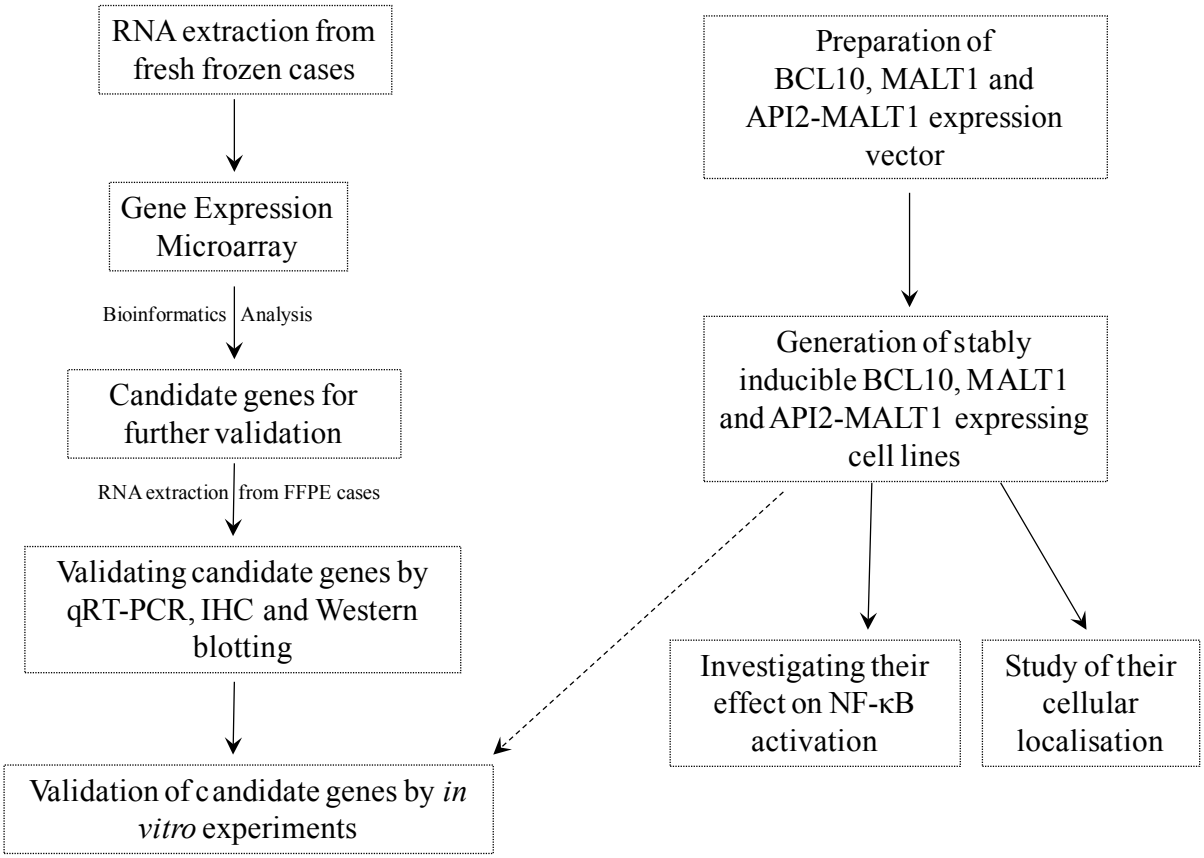
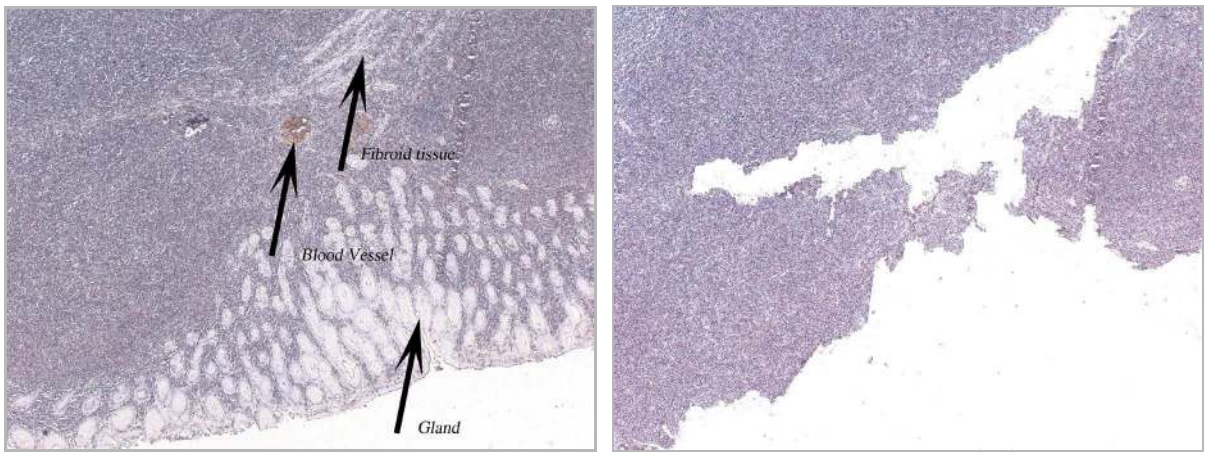


Figure 2.1 - Summary of the study plan.

2.2.2 Crude microdissection

4μm sections of FFPE tissue were cut routinely and mounted on charged glass microscope slides. Sections were dried overnight at 56°C, deparaffinised in xylene and rehydrated using decreasing concentrations of ethanol (100%, 10 minutes; 95%, 5 minutes; 75%, 5 minutes) and immersed in distilled water for 5 minutes, and stained in Haematoxylin for 5 seconds.

The sections were covered in 50% ethanol and the unwanted tissue was scraped away using a needle (Figure 2.2). The selected cell populations were then scraped off the slide into sterile 1.5ml Eppendorf tubes. The microdissected cells were dried and used for RNA extraction.



(a) (b)

Figure 2.2 - Crude microdissection of gastric MALT lymphoma.
(a) Gastric MALT lymphoma with constitutive normal tissues
(b) Gastric MALT lymphoma after crude microdissection

2.2.3 RNA preparation

2.2.3.1 RNA extraction from fresh frozen tissue

RNA extraction was carried out using RNeasy™ Mini kit (Qiagen, East Sussex, UK) according to the manufacturer's instructions. Briefly, crudely microdissected tumour cells from fresh frozen tissue sections (<5 μm) were lysed in 600 μl of RNeasy lysis buffer (Buffer RLT) containing 1% β-mercaptoethanol. The crude lysate was homogenised by spinning through a QIAshredder column (Qiagen, East Sussex, UK). After addition of absolute ethanol, the samples were applied onto an RNeasy spin column. The column was washed with buffers RW1 and RPE before elution with RNase-free water. To remove all traces of DNA from the samples, they were treated with Turbo DNAse (Applied Biosystems,

Warrington, UK) for 30 minutes at 37°C. The DNAse was inactivated using the supplied Inactivation Reagent and the RNA yield was quantified.

2.2.3.2 RNA extraction from formalin fixed paraffin-embedded tissue

This was carried out on microdissected tissue using the Ambion RecoverAll Total Nucleic Acid Isolation kit (Applied Biosystems, Warrington, UK) essentially according to the manufacturer's protocol with the exception that the tissue was digested in protease K at 50°C for 3.5 hours.

2.2.3.3 RNA extraction from cell lines

Around 7 million cells were centrifuged at 1200rpm for 5 minutes and the resulting pellet was resuspended in RLT buffer containing 1% β-mercaptoethanol before application to a QIAshredder column. RNA was extracted from cells and the contaminating DNA removed using the protocol described in section 2.2.3.1. The RNA quantity and quality were then measured (Section 2.2.3.5).

2.2.3.4 RNA linear amplification

For gene expression microarray using the HG-U133B GeneChips, there was insufficient RNA to carry out the hybridisation. Therefore a linear RNA amplification method was developed in house and tested with the RNA extracted from fresh frozen tissue. The in house method was based on data showing that reverse transcription using random pentadecamer primers increases yield and quality of resulting cDNA (Stangegaard *et al.*, 2006) and the use of T7 and T3 RNA polymerase can be incorporated into the protocol to generate sense and anti-sense RNA (Marko *et al.*, 2005; Xiang *et al.*, 2003). The goal was to develop a strategy based upon the Eberwine method (Van Gelder *et al.*, 1990) but with the ability to produce

sense RNA from small quantities of total or poly-(A)+ RNA extracted from both ideal samples (e.g. cell line RNA) and "real world" samples (e.g. tumours or tissues). This protocol avoids the need for PCR steps and requires two primers only. Additionally, the protocol is cost effective, efficient, and technically simple to perform. Finally, the method gives results consistent with similar amplification techniques when used with subsequent microarray analysis. The in house method used a T7 tagged oligo dT primer to generate a double stranded cDNA from the 3 prime end, followed by T3 tagged pentadecamer to generate a second cycle of double stranded cDNA. This avoided the problems of amplifying degraded RNA and allowed small amounts of RNA to be amplified faithfully for expression microarray studies. 1ng of total RNA was used to generate around 20µg of amplified anti-sense RNA ready to be used for the labelling step before hybridisation to microarray (Section 2.2.4).

2.2.3.5 RNA quantification and quality control

RNA concentration and quality were assessed spectrophotometrically using the GeneQuant Pro (Amersham Pharmacia Biotech, Uppsala, Sweden), and considered to be acceptable for further analysis at a concentration of greater than 500ng/µl RNA with an A_{260}/A_{280} ratio between 1.7 and 2.0. For gene expression microarray studies, the RNA quality was assessed further by running a 100-300ng aliquot on an Agilent 2100 Bioanalyser (Agilent, Berkshire, UK) using Agilent RNA Nano Labchips. RNA extracted from all 26 cases of MALT lymphoma for microarray studies was of good quality varying from 1µg to 5µg total RNA with an A260/A280 ratio between 1.7 and 2.0.

2.2.4 Expression microarray

2.2.4.1 Affymetrix HG-U133 GeneChips

Microarray experiments, during the early phase of the study, were carried out on MALT lymphoma cases using the HG-U133A and B, while in the latter phase of the study, FL and MCL cases were analysed using the updated HG-U133plus2 GeneChips.

2.2.4.2 Preparation of biotinylated cRNA target for Affymetrix GeneChips

Total RNA extracted was used to generate double stranded cDNA which acts as a template to generate biotin labelled cRNA that was fragmented using heat and high salt buffer and hybridised to the Affymetrix HG-U133 GeneChip as described below (Figure 2.3).

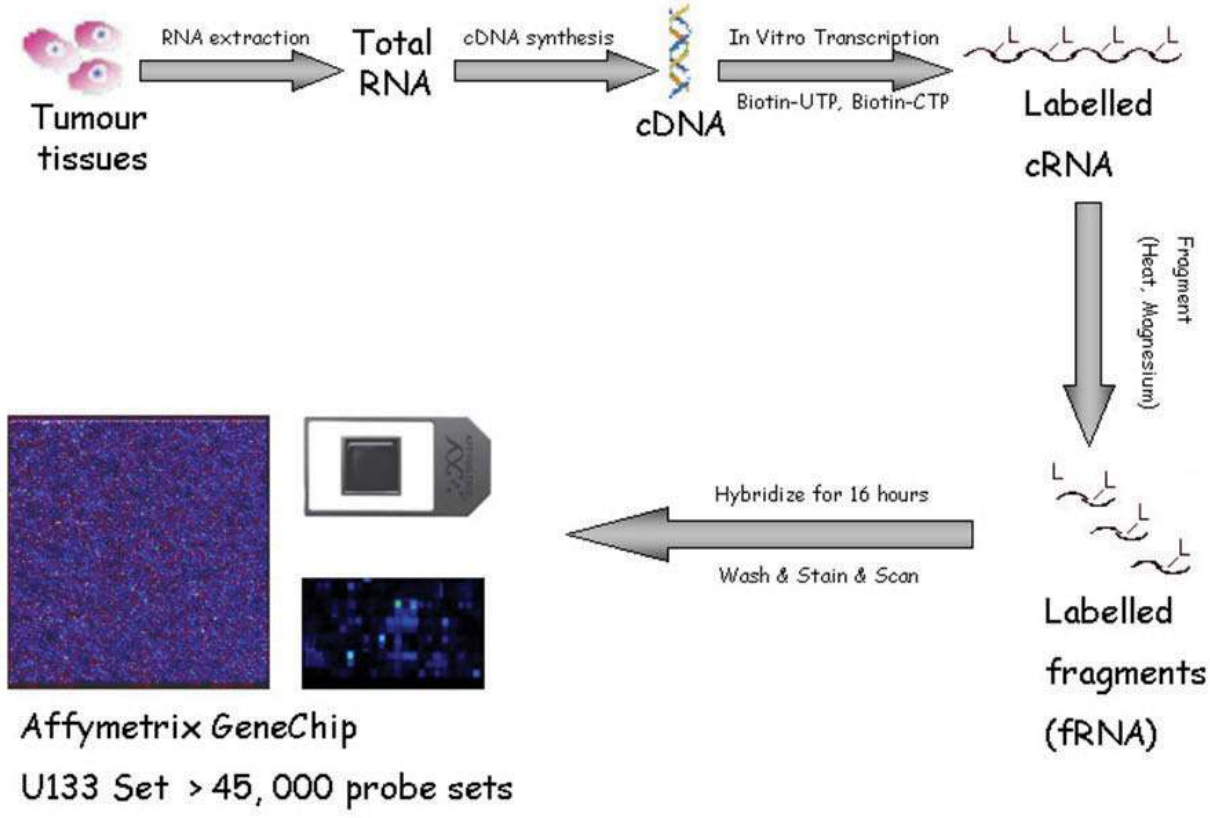


Figure 2.3 - Strategy for generating hybridisation target from total RNA.

2.2.4.2.1 Double stranded cDNA synthesis

First strand cDNA was synthesised from 5µg RNA using 100 pmol HPLC purified oligo(dT) primer conjugated to a T7 promoter (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG $(dT)_{24}$-3') (Thermo Scientific, Surrey, UK), hybridised to the poly-A tail of the mRNA primer. Samples were incubated at 70°C for 10 minutes to denature the secondary RNA structures and placed on ice for the primer to anneal. First strand synthesis was performed in a reaction mix containing; 1× First Strand Buffer, 10mM DTT, 500µM each dNTP (Invitrogen, Paisley, UK) and 100U Superscript II reverse transcriptase (Invitrogen, Paisley, UK) at 42°C for 1 hour. The samples were incubated on ice for 2 minutes followed by brief centrifugation.

The second strand synthesis was carried out in a reaction mixture containing; 1× Second Strand Reaction Buffer, 200µM each dNTP, 10U *E. coli* DNA Ligase, 40U *E. coli* DNA Polymerase I, and 2U *E. coli* RNase H (Invitrogen, Paisley, UK) at 16^{o}C for 2 hrs. T4 DNA Polymerase (Invitrogen, UK) was added at a final concentration of 10U followed by incubation at 16^{o}C for a further 5 minutes. The reaction was terminated by addition of 10µl 0.5M EDTA, pH 8.0 and the double stranded cDNA immediately purified using the cDNA purification columns from the GeneChip sample cleanup module kit (Affymetrix, High Wycombe, UK). The purified cDNA was eluted with 12µl of ultrapure water.

2.2.4.2.2 cRNA labelling using IVT (In-Vitro Transcription)

The generation of cRNA target was carried out using the Enzo Bioarray HighYield RNA Transcript Labelling Kit (Affymetrix, High Wycombe, UK). The reaction was carried out in a 40µl mixture containing: 12µl template cDNA from second strand synthesis reaction, 4µl 10× HY Buffer, 4µl Biotin Labelled Ribonucleotides, 4µl DTT, 4µl RNase Inhibitor Mix,

2µl T7 RNA Polymerase, 10µl water at 37°C for 4.5 hours. The cRNA was purified using the cRNA purification columns from the GeneChip sample cleanup module kit (Affymetrix, High Wycombe, UK). 1µl of each cRNA sample was used to check for sample concentration and purity (Section 2.2.3.5) and the remainder stored at –20°C awaiting array hybridisation.

2.2.4.2.3 Fragmentation of cRNA

20µg biotinylated cRNA was fragmented by incubation in fragmentation buffer and RNase free water at 94°C for 40 minutes followed by 10 minutes incubation on ice. The fragmented cRNA was stored at –20°C until use for hybridisation.

2.2.4.3 Hybridisation to HG-U133 Affymetrix GeneChips and Data Acquisition

2.2.4.3.1 Hybridisation mix preparation

A hybridisation cocktail was prepared in a microfuge tube by adding reagents at the following concentrations; 3nM Control Oligonucleotide B2, Eukaryotic Hybridisation Controls (*bioB, bioC, bioD and cre*, prepared in concentrations of 1.5, 5, 25 and 100pM respectively), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1x hybridisation buffer to a final volume of 275µl.

2.2.4.3.2 Hybridisation to HG-U133 GeneChips

The cRNA target samples were hybridised to HG-U133 chips. The chips were pre-equilibrated with 200µl 1x hybridisation buffer at 45°C for 10 minutes in a GeneChip 450 Hybridisation Oven (Affymetrix, High Wycombe, UK). The hybridisation cocktail containing the biotinylated cRNA was denatured at 99°C for 5 minutes, and transferred to a 45°C heat block for 5 minutes to pre-equilibrate before being added to the hybridisation chamber and incubated in a 45°C oven for 16 hours with rotation at 60rpm.

2.2.4.3.3 **Hybridisation to Test3 GeneChip**

A proportion of the biotinylated cRNA target samples was hybridised to Test chips (GeneChip Test3 Array) (Affymetrix, High Wycombe, UK) to ensure successful cRNA biotin labelling using IVT. This was similarly performed as above, with the exception that 80µl 1× hybridisation buffer was used for gene chip pre-equilibration, followed by 80µl hybridisation cocktail.

2.2.4.3.4 Staining of GeneChips

Hybridisation cocktail was removed and replaced with a non-stringent wash buffer (6× SSPE, 0.01% Tween 20). HG-U133A chips were placed in the corresponding slots of the Gene Chip Fluidics Station 450 (Affymetrix, High Wycombe, UK) and subjected to the washing and staining protocol EukGE WASH-WS2V4_450. The GeneChips were washed with a series of non-stringent and stringent wash buffers. Staining was carried out using the following procedure; 40 minutes incubation in Streptavidin-Phycoerythrin (SAPE) solution, then by 20 minutes incubation with biotinylated anti-streptavidin followed by a final 20 minutes incubation in SAPE solution.

2.2.4.4 HG-U133 GeneChip data acquisition and quality control

Chips were scanned at pixel value 2.5µm, wavelength 570 nm using argon laser Affymetrix GeneArray scanner 3000. Data were analysed using Affymetrix MAS and GCOS software. The array was inspected manually for image artefacts such as scratches, overall background, image intensity fluctuations and intensity of hybridisation controls. A grid was automatically placed over the image and correct alignment of grid to image was checked. The Microarray suite (MAS) software (version 5.0) (Affymetrix, High Wycombe, UK) was used to analyse

the scanned image and generate transcript expression data. To allow comparisons between samples, a global scaling technique was used to set the average intensity of each probe set within the array to a target intensity of 100.

The quality control of gene expression microarray varies between different studies and different platforms. Based on Brune *et al.* (Brune *et al.*, 2008) and similar papers involving human tissue, the following quality control parameters for the HG-U133A and HG-U133plus2 GeneChips were used; overall background should be less than 130, scaling factor should be less than 10, the 5'/3' ratio of the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) should be less than 4, hybridisation control *bioB* is called 'present' by the software at least 50% of the time and *bioC*, *bioD* and *cre* called 'present' with increasing signal values representing their relative concentrations and percentage 'present' call of genes should be reproducible between tissues and higher than 20%.

In this study, the HG-U133B GeneChip samples were processed using RNA linear amplification protocol (Section 2.2.3.4) thus the quality control parameters according to (The Tumor Analysis Best Practices Working Group, 2004) and (Brune *et al.*, 2008) were as follows; overall background should be less than 130, scaling factor should be less than 20, the 5'/3' ratio of the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) should be less than 13, hybridisation control *bioB* is called 'present' by the software at least 50% of the time and *bioC*, *bioD* and *cre* called 'present' with increasing signal values representing their relative concentrations and percentage 'present' call of genes should be reproducible between tissues and higher than 10%.

2.2.5 Bioinformatics and statistical analysis of gene expression microarray data

In general, bioinformatics analysis of gene expression microarray starts with normalisation followed by elimination of non-variant probes. The remaining variant probes are the ones that are changing across the samples, and are used for downstream analyses such as unsupervised clustering and differential expression analysis. Full bioinformatics comparison of methods and strategies are presented in Appendix I.

2.2.5.1 Normalisation

After testing many different strategies as outlined in Appendix I.I, the best strategy for generating variant probes that are differentially expressed between the groups was to normalise the array sets using gcRMA and MAS5 and cross reference the probes that passed through non-specific filtering based on coefficient of variation and absolute value thresholding. Raw gene expression data from Affymetrix CEL files were uploaded to bioconductor where MAS5 and gcRMA normalisation were performed separately for each Affymetrix platform. All MAS5 data were scaled to a target intensity of 100. The MAS5 normalised data was imported into Genespring 7.3.1 where they were log-transformed and median centred for further analysis. For comparison between microarray data obtained from HG-U133A&B and HG-U133 plus2 platforms, an additional median polishing normalisation step was applied. MAS5 normalised data were used for unsupervised clustering and fold change calculations; while gcRMA normalised data were used for gene set enrichment analysis (GSEA). Both MAS5 and gcRMA normalised data were subjected to non-specific filtering.

2.2.5.2 Non-specific filtering

To filter out non-variant genes, a combination of noise and variance filtering was applied. To filter out non-expressed genes, only probes with a value of 50 or higher in the MAS5 dataset in 2 or more samples were selected, since the minimum number of cases with a particular translocation, i.e. t(14;18)/IGH-MALT1, was two. To eliminate non-variant genes, only those with a coefficient of variation (CV) value of 10% or higher in the gcRMA dataset across all cases were considered to be variant and thus selected. CV was calculated as the mean / standard deviation of each gene across all cases. Finally, the genes that passed the above two filtering methods were intersected to obtain a common set of variant genes.

For comparison of microarray data between HG-U133A/B and HG-U133 plus2 platform where indicated, the non-specific filtering was similarly performed separately for each of these platforms as outlined above, then intersected to generate a final common set of variant genes. All the above analyses were carried out using scripts written in R programming language (Appendix II.I). The above procedure for analysis of expression microarray data from HG-U133A/B and HG-U133 plus2 platform was validated by a serial empirical testing using the published pulmonary MALT lymphoma expression microarray data from the HG-U133 plus2 platform as a reference (Gene Expression Omnibus: GSE13314) (Chng *et al.*, 2009).

2.2.5.3 Clustering analysis

The microarray dataset after the above normalisation and filtering was used for unsupervised clustering and this was carried out using Pearson correlation coefficient and average linkage as the similarity measure and clustering algorithm respectively with Genespring GX 7.3.1.

Separate clustering was performed among all MALT lymphoma, FL and MCL cases and also within the MALT lymphoma cases.

2.2.5.4 Gene Set Enrichment Analysis (GSEA)

GSEA was used to identify gene sets differentially regulated between MALT lymphoma with and without chromosome translocation and this was performed essentially as previously described with minor modification (Subramanian *et al.*, 2005). As the original GSEA only identifies the gene set showing either uniformly up or down regulation, for the gene sets displaying both up and down regulated genes, absolute GSEA was additionally performed as previously described (Saxena *et al.*, 2006). A total of 4395 gene sets were analysed and they included:

1) NF-κB target genes, which were collated from online data base (http://www.nf-kb.org), published works (http://bioinfo.lifl.fr/NF-KB and http://people.bu.edu/gilmore/nf-kb/target/index.html) and systematic bioinformatics search

2) Biological pathways involved in inflammatory and immune response from human immunome database (http://bioinf.uta.fi/Immunome) (Ortutay *et al.*, 2006; Ortutay *et al.*, 2009), Gene Ontology (http://wiki.geneontology.org/index.php/Immunology, http://www.geneontology.org/GO.immunology.shtml), and Ingenuity (http://www.ingenuity.com/)

3) Gene sets from Molecular Signature database (http://www.broad.mit.edu/gsea/msigdb/index.jsp). The GSEA results were ranked according to the nominal P value and False Discovery Rate (FDR)

For the gene sets differentially regulated between MALT lymphoma with and without translocation, leading edge analysis was carried out to identify the biologically important gene subset (Subramanian *et al.*, 2005). For absolute enrichment, a modification on the original GSEA was made to extract the leading edge set from either end of the list. When generating gene sets, for each sample, only the maximum expression value of the multi-probes for a given gene was used for GSEA as described previously (Subramanian *et al.*, 2005), thus avoiding any potential biased representation due to multiple probes for the same gene.

2.2.5.5 Analysis of differential gene expression in MALT lymphomas with and without translocation

Differential gene expression between MALT lymphomas with and without chromosome translocation was investigated using one-way ANOVA with Cross-Gene Error Model in GeneSpring with P value of ≤ 0.05 considered to be significant. The MAS5 normalised and filtered dataset was used for this analysis and the genes differentially expressed between translocation positive and negative MALT lymphomas were obtained. For each of these significantly differentially expressed genes, fold change calculation was carried out and those showing more than 2 fold differences were selected for functional annotation using gene ontology.

2.2.5.6 Functional annotation using gene ontology (GO)

To further assess the biological implications of differential gene expression in MALT lymphomas with and without chromosome translocation, we measured the representation of gene ontology (GO) terms (association of gene products with regard to their associated biological processes, cellular components, and molecular functions) in the above

differentially expressed genes using Genespring and hypergeometric tests provided in the R package GOstats, version 2.8.0. This allowed us to examine whether any GO term was over or under-represented as compared to chance variations. Independent analyses of GO categories were performed for both over and under-expressed genes in translocation positive MALT lymphoma.

2.2.5.7 Phenotypic marker analysis

The 26 MALT lymphoma cases were compared to 14 SMZL, 7 FL, 8 MCL and 22 CLL. All expression microarrays were performed in house except for CLL which was from a previous study by Calin *et al.* (Calin *et al.*, 2008) and the raw CEL files downloaded from ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) using the query "E-MEXP-1482". Normalisation and non-specific filtering was carried out as in sections 2.2.4.1 and 2.2.4.2.

The 26 MALT lymphoma HG-U133A and HG-U133B and FL, MCL and SMZL HG-U133plus2 data were normalised separately using RMA algorithm (Irizarry *et al.*, 2003) and combined using median polish step. Multivariate one-way ANOVA (Welch test) (using GeneSpring 7.3) and Bayesian statistical analysis (using in house R scripts) were carried out independently on all 77 lymphoma cases. The data from both algorithms were intersected to generate a list of common probes to both analyses. The common probes were subjected to SOM and Volcano plot analysis to further filter the gene set. Biological insight, literature search and pathway analysis were used to select the most meaningful phenotypic marker for the study.

2.2.5.8 Statistical analyses

Fisher's exact test ("stats Package" in R version 2.8.0) is a non-parametric test used to assess the statistical significance of the association between two variables in a 2 by 2 contingency

table. The test is specifically adapted for small sample size, including unequally distributed data among the cells of the table, unlike a Chi-square test. Thus, it was more robust to compare categorical variables from the immunohistochemical staining of each antibody among the various MALT lymphomas with different translocation status.

Student's t-test is a statistical hypothesis test in which the test statistic follows a Student's t distribution if the null hypothesis is supported. It is most commonly applied when the test statistic would follow a normal distribution if the value of a scaling term in the test statistic were known. It was used to calculate the statistics for comparing reporter assay experiments.

Mann–Whitney U is a non-parametric test for assessing whether two independent samples of observations have equally large values. It is one of the best known non-parametric significance tests. It is identical to performing an ordinary parametric two-sample t-test on the data after ranking over the combined samples. Thus it was used to compare the qRT-PCR data of each transcript between the MALT lymphomas with various translocation statuses.

In all the above statistical methods, the null hypothesis corresponded to the independence of the chosen variables. The null hypothesis was rejected if the probability value of the test of association was less than 0.05, meaning the variables were significantly associated.

2.2.6 Quantitative Real-time RT-PCR

2.2.6.1 Primer design

Primers were designed for RT-PCR to validate the expression of candidate genes obtained from expression microarray analysis. All primers were designed using primer3 software (http://frodo.wi.mit.edu/primer3) initially and for difficult regions such as GC rich, Oligos software (Institute of Biotechnology, Finland) was used. Where possible, primers were designed to contain a GC clamp at the 3' end. All primers were checked for any possible

primer dimer formation or self-complementarity and primer sequences were checked using BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST) from National Centre for Biotechnology Information (NCBI). Details of the primers used for each candidate gene are shown in Table 2.2. Where possible, all gene-specific primer pairs were designed to span exons in order to make them suitable for degraded paraffin-embedded tissue nucleic acid where amplicons of less than 150 base pairs are desired (Liu *et al.*, 2002a).

Table 2.2 - Primers used to investigate candidate genes by qRT-PCR.

Gene name	Exon targets	Primer	Primer sequence	Amplicon size	Accession Number
18SrRNA	N/A	Sense	TGACTCAACACGGGAAACC	114bp	NR_003286
	N/A	Anti-sense	TCGCTCCACCAACTAAGAAC		
N-MALT1	Exon1	Sense	CTCCGCCTCAGTTGCCTAGA	104bp	NM_006785
	Exon2	Anti-sense	CAACCTTTTTCACCCATTAACTTCA		
BCL10	Exon1	Sense	GAAGTGAAGAAGGACGCCTTAG	80bp	NM_003921
	Exon2	Anti-sense	AGATGATCAAAATGTCTCTCAGC		
NR4A3	Exon6	Sense	TTCCATCAGGTCAAACACTGC	84bp	NM_173198
	Exon7	Anti-sense	AATCCACGAAGGCACTGAAG		
CD86	Exon1	Sense	GGAATGCTGCTGTGCTTATGC	121bp	NM_006889
	Exon2/3	Anti-sense	AGCACCAGAGAGCAGGAAGG		
CD69	Exon2	Sense	CCACCAGTCCCCATTTCTCAA	125bp	NM_001781
	Exon3	Anti-sense	TTGGCCCACTGATAAGGCAAT		
TLR6	Only has one exon	Sense	AACAAGTACCACAAGCTGAAG	100bp	NM_006068
		Anti-sense	CTCTAATGTTAGCCCAAAAGAG		
CCR5	Exon2	Sense	ATCCGTTCCCCTACAAGAAACTC	100bp	NM_000579
	Exon3	Anti-sense	GCAGGGCTCCGATGTATAATAA		
CCR2A	Exon3	Sense	GCGTTTAATCACATTCGAGTGTTT	77bp	NM_001123041
	Exon3	Anti-sense	CCACTGGCAAATTAGGGAACAA		
BCL2	Exon3	Sense	TTGCTTTACGTGGCCTGTTTC	94bp	NM_000633
	Exon3	Anti-sense	GAAGACCCTGAAGGACAGCCAT		
IRF4	Exon7	Sense	GCCCAACAAACTGGAGAGAG	123bp	NM_002460
	Exon8	Anti-sense	AAGCATAGAGTCACCTGGAAT		
Lactoferrin	Exon1	Sense	GCCACAAAATGCTTCCAATGG	116bp	NM_002343
	Exon2	Anti-sense	GCCCTGTTTTCCGCAATGG		

2.2.6.2 Complementary DNA (cDNA) synthesis

cDNA was generated using the Superscript III Reverse Transcriptase kit (Invitrogen, Paisley, UK) with gene specific primers. Typically, the reaction mixture contained: 1µM of each gene-specific anti-sense primer, 1µl of 10mM dNTP, and 200ng of RNA per reverse primer. After incubation at 65°C for 5 minutes the following were added: 2µl of 10 RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT, 1µl of RNAse inhibitor and 2U of Superscript III and incubated at 50°C for 50 minutes followed by incubation at 85°C for 15 minutes. RNAse H was added and the mixture incubated at 37°C for 20 minutes to remove the original RNA.

2.2.6.3 Quantitative PCR (qRT-PCR)

The expression of each target transcript was normalised against the level of expression of 18S rRNA in each case. Real-time PCR was performed using the iCycler IQ system (BioRad, Hertfordshire, UK) using SYBR Green I Supermix (BioRad, Hertfordshire, UK).

The conditions for real-time PCR were optimised prior to result collection. The specificity of the primers in producing PCR products was confirmed by melt-curve analysis. The efficiency of each primer was designed to be between 95% and 110% by generating standard curves for each candidate gene from serial dilutions of cDNA produced from tonsillar RNA. All qRT-PCR were carried out in triplicate and standard deviation of less than 0.5 was deemed to be acceptable. The average correlation coefficient value (R^2) for each standard curve was above 0.99. The slope of the standard curves was used to determine the exponential amplification and efficiency of the qPCR reaction by the following equation (Tichopad *et al.*, 2003):

Efficiency = $10^{(-1/slope)}$

cDNA was diluted 1/1000 for detection of 18S rRNA as it is highly expressed in most tissues (Ye *et al.*, 2005). All samples were amplified by qRT-PCR on 96-well plates in triplicate using the following parameters: initial denaturation at 95°C for 3 minutes, followed by 45

cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. To ensure minimal intra-plate variation, a calibrator sample consisting of high and low C_t values was used in triplicate on each plate and was used to adjust the C_t. Only those samples that showed specific amplification by melt curve analysis were used for data analysis. The ΔC_t value for each sample was calculated by subtracting the C_t value for 18S from that of the target transcripts. The higher the ΔC_t value, the lower the expression of a transcript and *vice versa*. For all qRT-PCR, two negative controls were included; a negative control of water instead of RNA that goes through the cDNA synthesis and qPCR step and a negative control of RNA in the qPCR step to ensure that there is no genomic DNA contamination.

2.2.7 Immunohistochemistry

Immunohistochemistry was carried out with the help of Dr. Hongtao Ye, using the protocol described below. Paraffin sections (4μm) were deparaffinised in xylene (BDH, Leicestershire, UK), rehydrated using decreasing concentrations of ethanol (BDH Leicestershire, UK), and incubated in peroxidase blocking solution for 10 minutes to block the endogenous peroxidase activity. Antigen retrieval was carried out prior to immunostaining. Antigen retrieval conditions and primary antibody dilutions are detailed in Table 2.3. Sections were incubated with primary antibody at an optimal dilution for 1 hour followed by biotinylated secondary antibody (1:200 – 1:300) and peroxidase conjugated ExtroAvidin (1: 200) for 30 minutes, respectively. Finally, the sites of antibody binding were visualised with DAB in H_2O_2 (Kem-En-Tec, North Carolina, USA) and counter-stained with Mayer's haematoxylin. The slides were washed in TBS-Tween, three times for 5 minutes each between all incubation steps and mounted with cover slips for viewing.

Table 2.3 - Immunohistochemistry antibodies and conditions.

Protein	Primary antibody	Source	Antigen retrieval method	Conditions for immunohistochemistry or Western blot
BCL10	Mouse monoclonal antibody to human BCL10 (clone 151)	In house	Microwave in DAKO target retrieval solution pH 6.0 for 25-35 minutes	For immunohistochemistry:primary antibody (1/50), 1 hour at RT; biotinylated rabbit antimouse antibody, 30 mins at RT;peroxidase-conjugated extroAvidin, 30 mins at RT.
MALT1	Mouse monoclonal antibody to human C-MALT1	In house	Microwave in DAKO target retrieval solution pH 9.9 for 25 minutes	For immunohistochemistry:primary antibody (1/50), 1 hour at RT; biotinylated rabbit antimouse antibody, 30 mins at RT;peroxidase-conjugated extroAvidin, 30 mins at RT.
BCL2	Mouse monoclonal antibody	Novocastra	Pressure cooking with citrate buffer pH 6.0 for 3 minutes	For immunohistochemistry: primary antibody (1/120), 1 hr at RT; biotinylated rabbit antimouse antibody, 30 mins at RT;peroxidase-conjugated extroAvidin, 30 mins at RT.
CD69	Mouse monoclonal antibody	NeoMarkers	Pressure cooking with 1mM EDTA for 3 minutes	For immunohistochemistry: primary antibody (1/20), 1hr at RT;followed by polymer amplification system.
CD86	Sheep CD86 polyclonal antibody	R & D	Pressure cooking with citrate buffer pH 6.0 for 3 minutes	For immunohistochemistry: primary antibody (1/60), 1 hr at RT; biotinylated donkey antisheep antibody, 30 mins at RT;peroxidase-conjugated extroAvidin, 30 mins at RT.
IRF4	Mouse monoclonal antibody	Dako Cytomation	Microwave in DAKO target retrieval solution pH 9.9 for 25 minutes	For immunohistochemistry: primary antibody (1/50), 1 hr at RT; biotinylated rabbit antimouse antibody, 30 mins at RT;peroxidase-conjugated extroAvidin, 30 mins at RT.
Lactoferrin	Rabbit polyclonal antibody	Abcam	Pressure cooking with citrate buffer pH 6.0 for 3 minutes	For immunohistochemistry: primary antibody (1/50), 1 hr at RT; biotinylated rabbit antimouse antibody, 30 mins at RT;peroxidase-conjugated extroAvidin, 30 mins at RT.

2.2.8 Expression constructs preparation

2.2.8.1 Modification of pIRES vectors containing HA and FLAG tag sequences

pIRES contains internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one mRNA thus ensuring that the gene of interest is expressed together with a puromycin antibiotic resistance gene in the vector. This reduces the rate of false positives by ensuring that after selection with puromycin, nearly all surviving colonies will stably express the gene of interest (Jang *et al.*, 1988). A forward oligo was designed containing the second half of the *Nhe*I restriction site, an ATG translation start site and the sequence of the FLAG tag

(5'CTAGCATGGATTACAAGGATGACGACGATAAGG) or HA tag (5'CTAGCATGTACCCATACGATGTTCCAGATTACGCTG). A reverse oligo was designed containing the first half of the *Eco*RI restriction site, ATG translation start site and the sequence of the FLAG tag (5'AATTCCTTATCGTCGTCATCCTTGTAATCCTAG) or HA tag (5'AATTCAGCGTAATCTGGAACATCGTATGGGTACATG). The two oligos for each tag were annealed by mixing 1μg of each oligo with 100mM sodium chloride, 10mM Tris pH7.5 and 1mM EDTA and boiling for 90 seconds, before being allowed to cool at room temperature for 30 minutes. This generated a piece of double stranded sequence containing the *Nhe*I and *Eco*RI "sticky ends". The pIRESpuro2 vector (Clontech Laboratories, UK) (Figure 2.4) was digested with *Nhe*I and *EcoR*I. The FLAG or HA tag sequence was ligated into the cut vector with T4 DNA ligase (NEB, UK) to generate FLAG and HA tagged pIRESpuro which were used to generate constructs to make the stable cell lines as described in section 2.2.9.

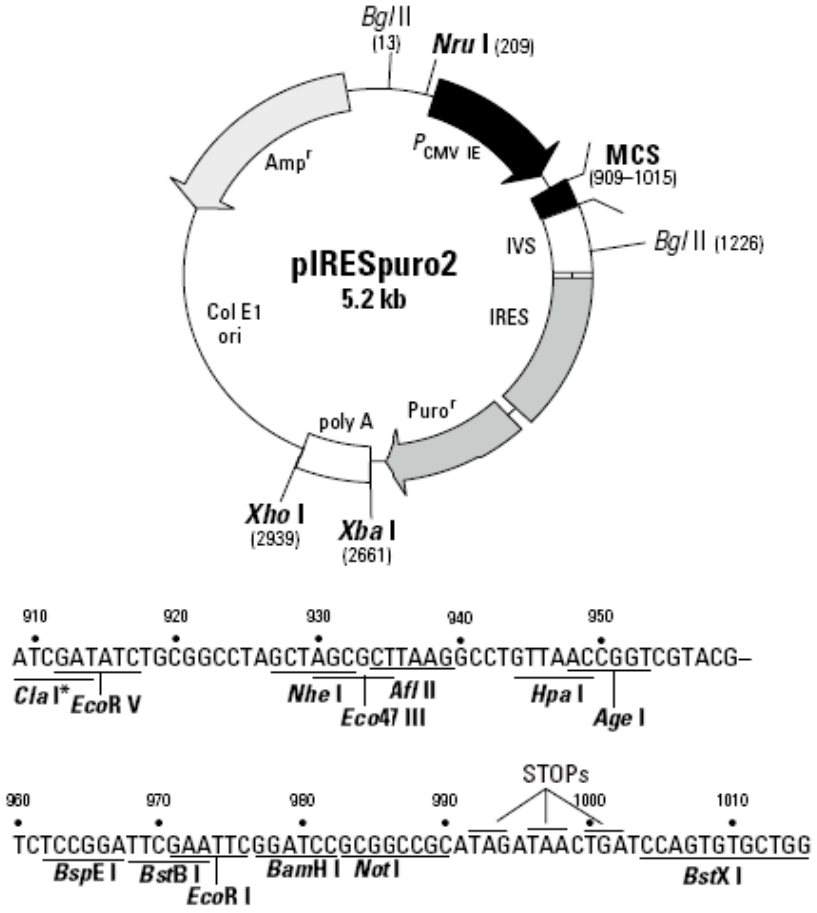


Figure 2.4 - pIRESpuro vector.

In order to generate cell lines that mimic the 3 main chromosome translocations; t(11;18), t(14;18) and t(1;14) three constructs were prepared, namely HA-tagged *BCL10*, FLAG-tagged *MALT1* and FLAG-tagged *API2-MALT1* (Table 2.4).

Table 2.4 - Primers used in construct preparation.

Construct	Primer Sequence (sense and antisense in 5' to 3')
BCL10	Lead *Eco*RI TGAT GAATTC BCL10 Exon1 ATGGAGCCCACCGCACCGTCC
	Lead *Not*I TGAT GCGGCCGC BCL10 Exon3 TCATTGTCGTGAAACAGTACGTG
MALT1	Lead *Bam*HI TGAT GGATCC MALT1 Exon1 ATGTCGCTGTTGGGGGACCCGCTACAG
	Lead *Not*I TGAT GCGGCCGC MALT1 Exon17 TCATTTTTCAGAAATTCTGAGCCTGTC
API2-MALT1	Lead *Eco*RI TGAT GAATTC API2 Exon1 ATGAACATAGTAGAAAACAGCATATTC
	Lead *Not*I TGAT GCGGCCGC MALT1 Exon17 TCATTTTTCAGAAATTCTGAGCCTGTC
pIRES	ATCGATATCTGCGGCCTAGC
	CCAGCACACTGGATCAGTTATC
pTRE2	GGATCCTCTAGTCAGCTGACG
	TCTAGAGATATCGTCGACAAGC

2.2.8.2 Generation of tagged BCL10, MALT1 and API2-MALT1 expression constructs

A standard 25µl PCR reaction was carried out using 2mM $MgCl_2$, 0.2mM of each dNTP, 0.2µM of each primer and 1 unit of *Pfu* polymerase (Stratagene, Leicestershire, UK) on a Hybaid Px2 thermal cycler (Thermo Scientific, Surrey, UK). The PCR reaction was heated to 94°C for 2 minutes, followed by 30 cycles of denaturation for 45 seconds at 94°C, primer annealing for 45 seconds starting at 55°C and extension for 90 seconds at 72°C with a final extension time of 10 minutes at 72°C. PCR products were visualised by electrophoresis using 1.5% agarose gels.

2.2.8.2.1 Preparation of HA-tagged BCL10

The full length cDNA sequence of BCL10 was generated by RT-PCR of a t(1;14) MALT lymphoma and sub-cloned into TOPO-TA cloning vector (Invitrogen, Paisley, UK). The full length BCL10 cDNA sequence from the TOPO-TA cloning vector was then amplified by PCR using *Pfu* polymerase with sense primer containing an *Eco*RI site and anti-sense primer containing a *Not*I site. The PCR products were cut with *Eco*RI and *Not*I to produce "sticky ends" and cleaned by gel extraction using the QIAQuick Gel Extraction Kit (Qiagen, East Sussex, UK). The HA-tag containing pIRESpuro2 vector was linearised by *Pvu*I restriction followed by *Eco*RI and *Not*I restriction and then ligated with the above BCL10 products. The vector sequences were checked for absence of mutations and to ensure that the gene of interest was in frame by sequencing in both directions.

2.2.8.2.2 Preparation of FLAG-tagged API2-MALT1 and FLAG- tagged MALT1

pcDNA3.1 vectors containing full length MALT1 and full length API2-MALT1 (joining nucleotides 1 to 2048 of API2 to nucleotides 814 to 2475 of MALT1) (Ho *et al.*, 2005) were kindly provided by Dr. Liza Ho, Department of Clinical Pathology, Geneva, Switzerland. This API2-MALT1 product represents the most common breakpoint found in 93% of t(11;18) MALT lymphomas. Sense primer was designed with the addition of an *Eco*RI restriction site targeting exon1 of the API2 gene while an anti-sense primer was designed with an addition of a *Not*I site targeting exon 17 of the MALT1 gene. For the MALT1 construct, the sense primer has a *Bam*HI restriction site targeting exon1 of MALT1 and the anti-sense primer has a NotI restriction site targeting exon 17. Both MALT1 and API2-MALT1 sequences were amplified by a standard PCR reaction using *Pfu* polymerase. The PCR products were cut with *Eco*RI (for API2-MALT1) or *Bam*HI (for MALT1) and *Not*I to produce "sticky ends" and cleaned

by gel extraction using the QIAQuick Gel Extraction Kit (Qiagen). The FLAG-tag containing pIRESpuro2 vector was cut with *Eco*RI (API2-MALT1) or *Bam*HI (MALT1) and *Not*I and the MALT1 or API2-MALT1 ligated with the above product using T4 DNA ligase. The vector sequences were checked for absence of mutations and to ensure that the gene of interest was in frame by sequencing in both directions.

2.2.8.2.3 Sub-cloning of oncogenes into pTRE2

pTRE2 vector (Clontech, Saint-Germain-en-Laye, France) is a response plasmid that can be used to express a gene of interest in Clontech's Tet-On and Tet-Off cell lines. pTRE2 contains a multiple cloning site (MCS) immediately downstream of the Tet-responsive promoter (Figure 2.5).

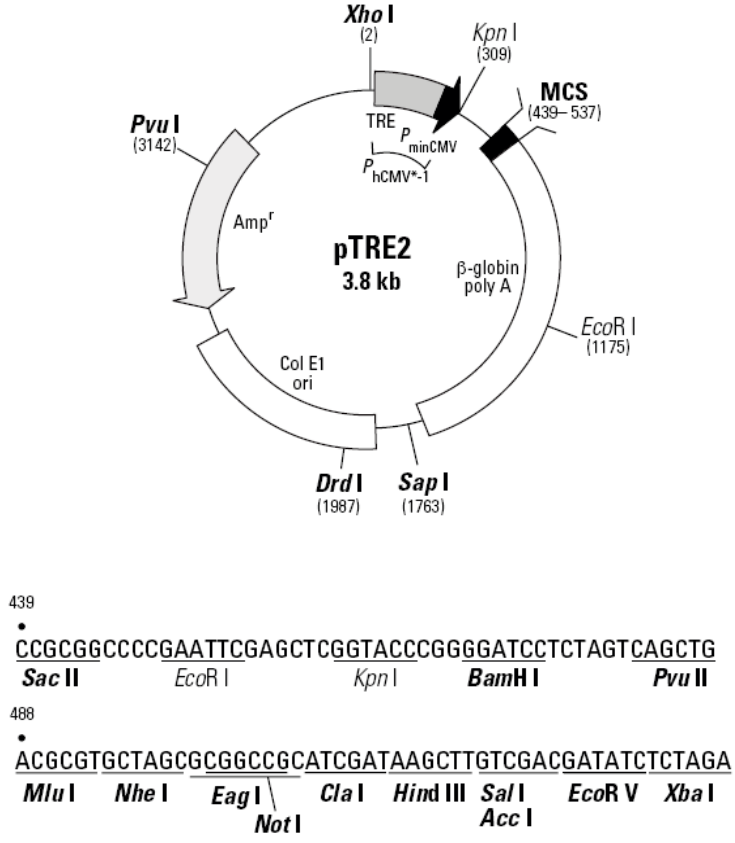


Figure 2.5 - pTRE vector.

cDNA or genes inserted into the MCS will be responsive to the rTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems respectively. $P_{hcmv\ -1}$ contains the Tet response element (TRE), which consists of seven copies of the 42-bp tet operator sequence (tetO). The TRE is just upstream of the minimal CMV promoter ($P_{min\ cmv}$), which lacks the enhancer that is part of the complete CMV promoter. Consequently, $P_{hcmv\ -1}$ is silent in the absence of binding of TetR or rTetR to the TetOs' sequences. pTRE2 puro and pTRE hygro were used to generate inducible BCL10, MALT1 and API2-MALT1 expression cell lines.

In order to generate the cell lines, each oncogene construct and the pTRE vector was cut using *Nhe*I and *Not*I and the insert containing the oncogene in the correct reading frame was ligated into the pTRE vector with T4 DNA Ligase. Before transfection to the cell lines the vector containing the oncogene of interest was linearised by cutting with *Pvu*I restriction enzyme to facilitate its integration into the host genome.

2.2.8.3 Toll-like receptors expression constructs

Toll-like receptor (TLR) 1, 2 and 6 in pFLAG-CMV-1 vector were a kind gift from Dr. Koichi Kuwano (Department of Bacteriology, Kurume University, Japan) (Shimizu *et al.*, 2007). pFLAG-CMV-1 expression vector is used for expression and secretion of N-terminal FLAG fusion proteins in mammalian cells, its MCS has preprotrypsin which binds the expressed protein to the plasma membrane. All 3 vectors were verified by sequencing from both ends using pFLAG-CMV-1 vector primers.

2.2.8.4 DNA Sequencing

The sequencing reaction was carried out according to Hamoudi *et al.* (Hamoudi *et al.*, 2002) using the dRhodamine Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The sequencing reaction was carried out in a 10µl reaction mixture

containing 500ng of plasmid DNA, 4µl dRhodamine, 2µl of 5µM primer and 4µl ultrapure water in a thermal cycler (Hybaid Px2 thermal cycler, Thermo Scientific) using the following cycling protocol; 96°C for 30 seconds, 50°C for 15 seconds and 60°C for one minute, for 25 cycles. The products were precipitated with 3 volumes of ethanol and 0.5 volume of 3M sodium acetate at pH 5.2. DNA pellets were resuspended in 1µl of denaturing blue dye and denatured for 2 minutes at 90°C before loading on polyacrylamide gels (29:1 acrylamide:bisacrylamide ready made 6M urea) (National Diagnostics, Hull, UK), and electrophoresis was carried out using an ABI 377 DNA Sequencer (Applied Biosystems). Data was collected via the associated DNA Sequencer Collection software version 2.0 (Applied Biosystems) and the results were analysed using the BioEdit sequence alignment editor version 7 (Ibis Biosciences, California, USA), Sequence Navigator version 1.1 (Applied Biosystems) and online BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.9 Cell culture

Cells were cultured in RPMI 1640 with 10% Fetal Calf Serum (FCS) and incubated at 37°C, 5% CO_2 in a humidified, automatically controlled incubator. All cells were checked for the presence of mycoplasma by PCR using VenorGeM kit (Minverva Biolabs, Germany).

2.2.9.1 Protocol for freezing cells

The cells to be frozen were centrifuged at 1200rpm for 10 minutes and the pellet was resuspended in Freezing Medium then transferred to cryotubes (Corning, Dorset, UK). The cell suspension was placed at –80°C overnight and then in liquid nitrogen for storage.

2.2.9.2 Protocol for thawing cells

The frozen cells were thawed at 37°C for 5 minutes with gentle shaking, briefly washed with 8ml of warm culture medium and transferred to a sterile tissue culture flask with appropriate culture medium and antibiotics.

2.2.9.3 Cell clot preparation

Around 5×10^5 cells were centrifuged at 1200rpm for 5 minutes and immediately placed on ice. Five drops of plasma were added to the cell pellet followed by gentle vortexing for 10 seconds. Three drops of thrombin (Diagnostic Reagents Ltd, Oxford, UK) were added and mixed using a plastic pipette in order to clot the cells. The clotted cells were transferred to Speci-wrap paper, which was folded and placed inside a formalin cassette. The cassette was placed inside a formalin jar and left for 4 hours. The samples were processed using a Shandon Excelsior machine to generate formalin fixed paraffin embedded blocks.

2.2.9.4 Transient transfection of cells

Baf-3 and WEHI cell lines were transiently transfected. The required number of cells were centrifuged to form a pellet and washed in PBS (Invitrogen, Paisley, UK). The clean cell pellet was resuspended in 100µl of appropriate Amaxa Cell Line Nucleofector Solution (Lonza, Berkshire, UK). The vector DNA was added at a ratio of around 1µg of DNA to 1 million cells and the cells were transfected using the appropriate programme on a Nucleofector I machine (Lonza, Berkshire, UK). The transfected cells were then routinely cultured. Nucleofector Solution T with programme T16 was used to transfect BJAB, Jurkat T-Rex and Baf-3 cells whereas solution V with programme T30 was used to transfect WEHI cells. These conditions were optimized by transfection of pmaxGFP and subsequent analysis

of the transfected cells by Flow cytometry using FACSCalibur flow cytometer (Beckton Dickson, Oxford, UK).

2.2.9.5 Generation of stably inducible MALT lymphoma associated oncogenes cells

pTRE-BCL10, pTRE-MALT1, pTRE-API2-MALT1 and pTRE wild type were used to generate stable inducible cell lines, whilst pIRESpuro-BCL10 and pIRESpuro wild type were used to generate stable cell lines. 20μg of each different construct were linearised by digestion with *Pvu*I, purified by phenol/chloroform extraction and resuspended in 20μl of sterile water to a final concentration of 1μg/μl. For each transfection, 2×10^7 of BJAB, BJAB-TetON or Jurkat TetON (T-REx) cells were washed with PBS, resuspended in 700μl of PBS, mixed with 20μl of the linearised construct and transferred to a 4mm Gene Pulser cuvette. After 5-10 minutes on ice, electroporation was carried out using the Gene Pulser Apparatus (BioRad) at 250 volts and 950μFD (a time between 15 and 20 milliseconds indicates successful electroporation). The samples were incubated on ice for 10 minutes and transferred to a Falcon tube containing 20ml of RPMI 1640 medium with 10% FCS, 30% conditioned medium containing 1mM sodium pyruvate, 50μM α-thioglycerol and 20nM bathocuproindisulfonic acid disodium salt (reagents needed for cell growth and survival) (Brielmeier *et al.*, 1998). Various concentrations of transfected cells (1.5×10^5, 3.75×10^3 and 1.25×10^3 cells) were seeded in 96-well plates for 24 hours in medium containing 4μg/ml puromycin. Clones were checked after 10 to 14 days, and transferred to a 24-well culture plate, then to a small 10ml flask. The cloned cells were subjected to Western blot analysis to check their oncogene expression. Induction of the oncogene expression was carried out by adding 1μg/ml of doxycycline.

A total of 14 cell lines expressing MALT lymphoma associated oncogenes were generated and they included :

Inducible expression cell lines

1) BJAB Tet-ON pTREpuro wild type (control)
2) BJAB Tet-ON pTREpuro FLAG-MALT1
3) BJAB Tet-ON pTREpuro FLAG-API2-MALT1
4) BJAB Tet-ON pTREhygro HA-BCL10
5) BJAB Tet-ON pTREhygro HA-BCL10 and pTREpuro FLAG-MALT1
6) BJAB Tet-ON pTREhygro HA-BCL10 and pTREpuro FLAG-API2-MALT1
7) Jurkat Tet-ON (T-REx) pTREpuro wild type (control)
8) Jurkat Tet-ON (T-REx) pTREpuro FLAG-MALT1
9) Jurkat Tet-ON (T-REx) pTREpuro FLAG-API2-MALT1
10) Jurkat Tet-ON (T-REx) pTREhygro HA-BCL10
11) Jurkat Tet-ON (T-REx) pTREhygro HA-BCL10 and pTREpuro FLAG-MALT1
12) Jurkat Tet-ON (T-REx) pTREhygro HA-BCL10 and pTREpuro FLAG-API2-MALT1

Stable expression cell lines

13) pIRESpuro wild type (control)
14) pIRESpuro HA-BCL10

2.2.10 Protein analysis

2.2.10.1 Protein extraction from whole cell lysate

Cells were harvested by centrifugation and cell pellets were washed twice in 1×PBS then lysed in Triton Lysis Buffer, on ice for 30 minutes. The cell lysate was centrifuged at 7300rpm for 20 minutes at 4°C, and the supernatant was transferred to a new tube and quantified (2.2.10.4). Samples were stored at -80°C until required.

2.2.10.2 Preparation of protein homogenate from frozen tissue

Frozen tissue sections were homogenised in Triton lysis buffer by gentle pipetting at 4°C for 30 minutes. The lysate was centrifuged at 7300rpm for 20 minutes at 4°C and the supernatant

was transferred to a new tube and quantified as described in 2.2.10.4. Samples were stored at -80°C until required.

2.2.10.3 Protein extraction from nuclear and cytoplasmic fractions

Preparation of nuclear and cytoplasmic protein extracts from cells was carried using CelLytic NuCLEAR Extraction kit (Sigma, Dorset, UK) according to the manufacturer's instructions. The procedure for nuclear protein extraction is to allow cells to swell with hypotonic buffer. The cells are then disrupted and cytoplasmic fraction is removed, and the nuclear proteins are released from the nuclei by a high salt buffer.

2.2.10.4 Protein quantitation

The concentrations of extracted proteins were measured using the Quant-iT Protein Assay kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions with the Victor 3 illuminometer (Perkin Elmer, Warrington, UK).

2.2.10.5 Antibodies used for functional and cellular work

Monoclonal and polyclonal antibodies used in the functional studies described in this thesis are shown in Table 2.5.

Table 2.5 - Primary and secondary antibodies used in co-immunoprecipitation and Western blotting.

Antibody	Source (Part No.)	Dilution	Application	Type	Host species and (primary/secondary)
C-MALT	In-house	1:500	Co-IP and Western blotting	Monoclonal	Mouse - primary
BCL10	In-house	1:10,000	Co-IP and Western blotting	Monoclonal	Mouse - primary
HA	Sigma-Aldrich (H9658)	1:10,000	Western blotting	Monoclonal	Mouse - primary
FLAG	Sigma-Aldrich (F3165)	1:10,000	Western blotting	Monoclonal	Mouse - primary
β-actin	Sigma-Aldrich (A5441)	1:1,000,000	Western blotting	Monoclonal	Mouse - primary
TLR6	Abcam (ab62569)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
IκBβ	Cell Signaling Technology (9248)	1:20,000	Western blotting	Polyclonal	Rabbit - primary
IκBα	Santa Cruz Biotechnology (sc-203)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
IκBε	Cell Signaling Technology (9249)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
p65	Santa Cruz Biotechnology (sc-7151)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
p50/p105	Santa Cruz Biotechnology (sc-7178)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
p100/p52	Cell Signaling Technology (3017)	1:10,000	Western blotting	Monoclonal	Rabbit - primary
c-Rel	Cell Signaling Technology (4727)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
RelB	Cell Signaling Technology (4954)	1:10,000	Western blotting	Polyclonal	Rabbit - primary
CD3	eBioscience (16-0037)	1:10,000	Western blotting	Monoclonal	Mouse - primary
CD28	Sigma-Aldrich (C7831)	1:10,000	Western blotting	Monoclonal	Mouse - primary
Donkey anti-rabbit HRP-conjugated	GE Healthcare (NA934)	1:20,000	Western blotting	Not applicable	Donkey - secondary
Sheep anti-mouse HRP-conjugated	GE Healthcare (NA931)	1:20,000	Western blotting	Not applicable	Sheep - secondary

2.2.10.6 Western blot

Protein lysates were mixed with 4X NuPAGE (Invitrogen, Paisley, UK) loading buffer containing 4% β-mercaptoethanol (BDH, Leicestershire, UK), heated to 99^{o}C for 5 minutes and left on ice for 2 minutes then separated on NuPAGE gels (Invitrogen, Paisley, UK) by electrophoresis at 120V for 90 minutes using SeeBlue size standard (Invitrogen, Paisley, UK).

The proteins were transferred to an Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, USA) using XCell II Blot Module (Invitrogen, Paisley, UK) running at 30V for 90 minutes.

The membrane was first placed in TBST for 10 minutes followed by 4% milk/TBST for 1 hour whilst shaking to block non-specific binding then probed with primary antibody in 4% milk/TBST with 10% FCS overnight at 4°C. After washing in TBST, the membrane was incubated with an anti-species specific (according to Table 2.5) Horseradish Peroxidase (HRP)-conjugated secondary antibody in 4% milk/TBST for a minimum of 1 hour at room temperature. Finally, the protein was detected by the addition of an Immobilon HRP Substrate Peroxide Solution (Millipore Corporation, Watford, UK) and Immobilon HRP Substrate Luminol Reagent (Millipore Corporation, Watford, UK) for 5 minutes. The HRP chemiluminescent reaction resulted in the catalysed oxidation of luminol by peroxide. Oxidised luminol emits light as it decays. The chemiluminescence was detected by exposing an X-ray film to the membrane.

2.2.10.7 Stripping of Western blot

Bound antibody was removed from membranes by immersion in Stripping Buffer at 62^{o}C for 2 hours, washed in TBST and placed in 4% milk/TBST for 1 hour, then probed and detected with the appropriate antibodies (Table 2.5) as described in section 2.2.10.6. The loading control antibody, β-actin was used on stripped blots. Also in section 5.4.5, IκBα were stripped and re-probed with IκBβ which was stripped and re-probed with β-actin.

2.2.10.8 Co-immunoprecipitation

Co-immunoprecipitation was carried out using the Anti-HA immunoprecipitation kit (Sigma, Dorset, UK) according to the manufacturer's instructions. Some of the immunoprecipitated protein was then subjected to silver staining using the ProteoSilver Stain kit (Sigma, Dorset, UK) and dried for long term storage using the DryEase kit (Invitrogent, Paisley, UK) according to the manufacturer's instructions.

2.2.11 Dual luciferase reporter assays

2.2.11.1 NF-κB luciferase reporter assay

NF-κB activity was measured in tissue culture cells using the Dual Luciferase Reporter Assay System (Promega, Southampton, UK). Cells were resuspended in Nucleofector Solution (Lonza, Berkshire, UK) and transfected with the pRL-TK (Promega, Southampton, UK) and pNF-κB-luc (Stratagene, Leicestershire, UK) reporter vectors, in combination with a pIRES vector containing the oncogene of interest or the pIRES control vector by electroporation or using an inducible stable cell using the Amaxa Nucleofector I machine (Lonza, Berkshire, UK). Luciferase is expressed from pNF-κB-luc after binding of NF-κB subunits to the vector. pRL-TK contains a cDNA encoding Renilla luciferase which was used as a control for

normalisation of the data. These cells were incubated at 37°C and 5% CO_2 overnight and then stimulated as follows :

1) BaF3 cells with 10μg/ml LPS (Sigma, Dorset, UK) for 6 hours
2) Jurkat cells with 1μg/ml anti-CD3 (Sigma, Dorset, UK) and 1μg/ml anti-CD28 (Sigma, Dorset, UK) for 6 hours. For TLRs experiments, cells were stimulated with 10μg/ml LPS (Sigma, Dorset, UK) for 6 hours
3) WEHI cells with 10μg/ml LPS (Sigma, Dorset, UK) for 6 hours or 0.1μg/ml CD40 ligand (with 1μg/ml CD40 enhancer) or 10μg/ml anti-IgM

The cells were centrifuged to form a pellet and washed in 1X PBS before resuspension in 1X Passive Lysis Buffer (Promega, Southampton, UK). The cell suspension was rotated for 30 minutes to allow the cells to lyse completely. 15μl of whole cell extract was added to 45μl of Luciferase Assay Reagent 2 (LAR) (Promega, Southampton, UK) and the level of fluorescence produced by the firefly luciferase was detected with the Victor 3 luminometer (Perkin Elmer, Warrington, UK). The reaction was stopped with the addition of 45μl of Stop and Glo (Promega, Southampton, UK) and the fluorescence level produced by the Renilla luciferase was measured by the luminometer. The reading for firefly luciferase divided by the reading for Renilla luciferase gave the NF-κB activity.

2.2.11.2 AP-1 luciferase reporter assay

AP-1 activity was measured in transiently transfected BJAB cells using the Dual Luciferase Reporter Assay System (Promega) similar to that described for NF-κB luciferase reporter assay in section 2.2.11.1. The AP-1 vector used was the AP-1 cis-Reporting system (Stratagene, Leicestershire, UK). pRL-TK was used as the normalisation vector.

CHAPTER 3 – Characterisation of the gene expression profiles of MALT lymphoma with and without chromosome translocation

3.1 Introduction

As detailed in chapter 1, t(11;18)(q21;q21)/*API2-MALT1*, t(1;14)(p22;q32)/*IGH-BCL10* and t(14;18)(q32;q21)/*IGH-MALT1* are specifically associated with MALT lymphoma, occurring at variable frequencies in different anatomic sites. Although these translocations involve different oncogenes, their resultant oncogenic products commonly target the canonical NF-κB activation pathway. Expression of API2-MALT1, BCL10 and MALT1 (in the presence of BCL10) induces the activation of the NF-κB transcriptional factor, which transactivates a number of genes important for cellular proliferation and survival. The capacity of these oncogenic products to activate NF-κB is believed to be the crucial molecular mechanism underlying their oncogenic activity. Nonetheless, over-expression of these oncogenic products alone is insufficient for malignant transformation as both *Eμ-API2-MALT1* and *Eμ-BCL10* transgenic mice developed splenic marginal zone hyperplasia, but not lymphoma (Morris, 2001; Sagaert *et al.*, 2006b; Li *et al.*, 2009). *In vitro* assay showed that expression of both API2-MALT1 and MALT1 enhanced by the CD40 induced NF-κB activation in B cells (Ho *et al.*, 2005). It is possible that such immunological stimulation is operational in MALT lymphoma. The extent and the nature of potential cooperation between MALT lymphoma associated oncogenic products and immune surface receptor signalling remain to be investigated.

There are important differences in the clinical and histological presentations between MALT lymphomas with and without chromosome translocation. Clinically, gastric MALT

lymphomas with t(11;18) or t(1;14) are significantly associated with advanced clinical stages (Liu *et al.*, 2001b; Ye *et al.*, 2006) and resistance to *H. pylori* eradication (Isaacson *et al.*, 2004; Liu *et al.*, 2001a; Liu *et al.*, 2002b; Ye *et al.*, 2006). Histologically, t(11;18) positive MALT lymphomas appear to be more monotonous, lacking apparent transformed blasts (Okabe *et al.*, 2003). These distinct clinico-pathological characteristics indicate the presence of significant differences in molecular mechanisms between MALT lymphomas with and without chromosome translocation. In order to investigate this and understand further the molecular mechanism of MALT lymphomagenesis, the transcriptional profiles of a well characterised series of MALT lymphomas with different chromosome translocation status were determined.

3.2 Aims of the study

1) To characterise gene expression profiling of MALT lymphoma with and without chromosome translocation

2) To identify the molecular mechanisms involved in translocation positive MALT lymphoma

3) To identify the molecular mechanisms involved in translocation negative MALT lymphoma

3.3 Experimental design

3.3.1 Case selection

Fresh frozen tissues from 24 well-characterised MALT lymphomas (case numbers 1 to 24 in Table 2.1), 7 nodal follicular lymphomas (FL) and 8 nodal mantle cell lymphomas (MCL) were used for this part of the gene expression microarray analysis. The MALT lymphoma cases included 9 positive for t(11;18)(q21;q21)/*API2-MALT1* (8 from stomach and 1 from lung), 4 positive for t(1;14)(p22;q32)/*BCL10-IGH* or t(1;2)(p22;p11)/*BCL10-IGκ* (3 from stomach and 1 from lung), 2 positive for t(14;18)(q32;q21)/*IGH-MALT1* (1 from liver and 1 from ocular adnexa) and 9 cases negative for all known MALT lymphoma associated chromosome translocations (all from stomach with two having nuclear BCL10 staining). MALT lymphomas case number 25 was excluded from the analysis due to the fact that it was a mixture of MALT and diffuse large B-cell lymphoma and case number 26 was excluded due to the fact that it is the only fresh frozen case of t(3;14(p14;q32)/*IGH-FOXP1* and a minimum of two cases are needed to obtain useful mechanistic information. However both cases were included in the phenotypic marker analysis carried out as described in chapter 6. The percentage of tumour cells are estimated on haematoxylin & eosin stained sections and where necessary, crude microdissection was carried out to enrich tumour cells and ensure that a sample containing at least 70% tumour cells was used for expression microarray analysis.

3.3.2 RNA quality check using Agilent nanochip

Total RNA, complementary RNA and fragmented RNA were subjected to quality control assessment using the Agilent nanochip. The criteria for passing the quality control were as follows: for total RNA both the 18S and 28S bands should be around 2000 and 4000bp

respectively, for cRNA the mean of the size distribution should be around 1000bp and for fragmented RNA, the mean of the size distribution should be around 60 to 100bp.

3.3.3 Validation of the in house linear RNA amplification protocol

To validate the fidelity of the linear amplification protocol developed in house, RNA from a fresh frozen tissue specimen of a t(11;18) positive MALT lymphoma was amplified, then labelled and hybridised to HG-U133A chip according to the method described in section 2.2.5. Pearson correlation comparing the values of all 22283 probes from the amplified RNA and total RNA of HG-U133A GeneChip from the same case was carried out.

3.3.4 Unsupervised clustering analysis

After normalisation and non-specific filtering, the 24 MALT lymphoma cases derived from HG-U133A and HG-U133B, 7 FL and 8 MCL on the HG-U133plus2 platform were subjected to unsupervised clustering using Pearson correlation coefficient and average linkage as the similarity measure and clustering algorithm respectively within Genespring GX 7.3.1. Unsupervised clustering was also carried out within the MALT lymphoma group to investigate whether there are distinct subsets.

3.3.5 Characterisation of gene expression features of MALT lymphoma with and without chromosome translocation

Normalisation followed by non-specific filtering was used to derive set of variant probes which were collapsed and used in interrogating 4395 pathways using GSEA (Section 2.2.5.4). The same set of probes was subjected to one-way ANOVA multivariate analysis to derive a set of differentially expressed genes between MALT lymphoma with and without chromosomal translocation (Section 2.2.5.5). The differentially expressed genes were subjected to Gene Ontology (GO) analysis using hypergeometric testing (Section 2.2.5.6).

Results from both GSEA and GO analysis were compared to characterise the features of MALT lymphoma with and without translocation.

3.4 Results

Details of the bioinformatics analysis including choice of algorithms and software are given in Appendix I. In this section, results of the analysis of gene expression microarray of MALT lymphoma are presented.

3.4.1 RNA quality control

In each case the total RNA, complimentary RNA and fragmented RNA were of good quality as shown by electrophoresis on RNA nanochip (Figure 3.1).

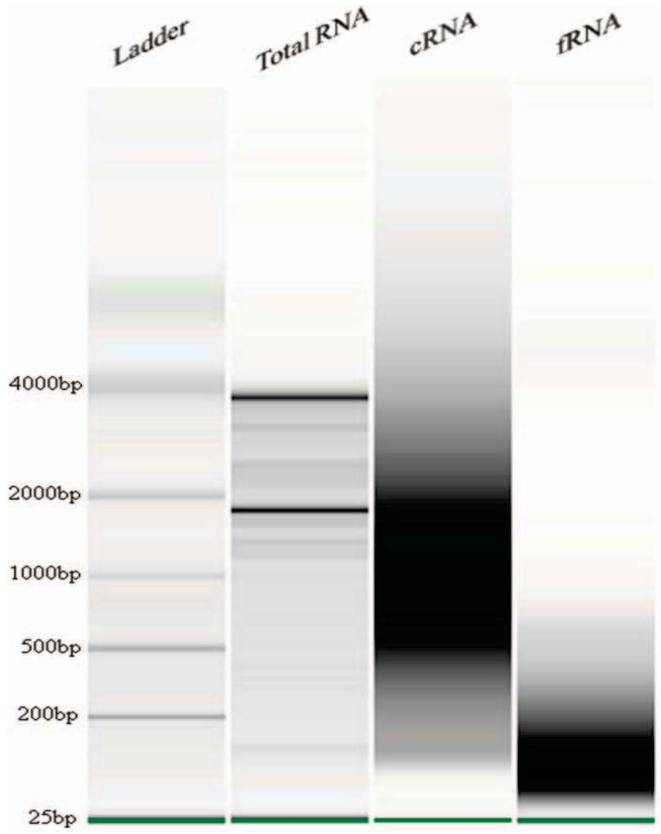


Figure 3.1 - Typical good quality data of total, complementary and fragment RNA on RNA nanochip.

3.4.2 Comparison of total RNA with amplified RNA

Total RNA and linearly amplified RNA from the same t(11;18) positive MALT lymphoma fresh frozen specimen were hybridised on a HG-U133A GeneChip. The Pearson correlation coefficient (R^2) between the two RNA preparations was 97.2% with $p < 0.001$ (Figure 3.2), indicating that linearly amplified RNA faithfully amplifies the same distribution of the starting total RNA.

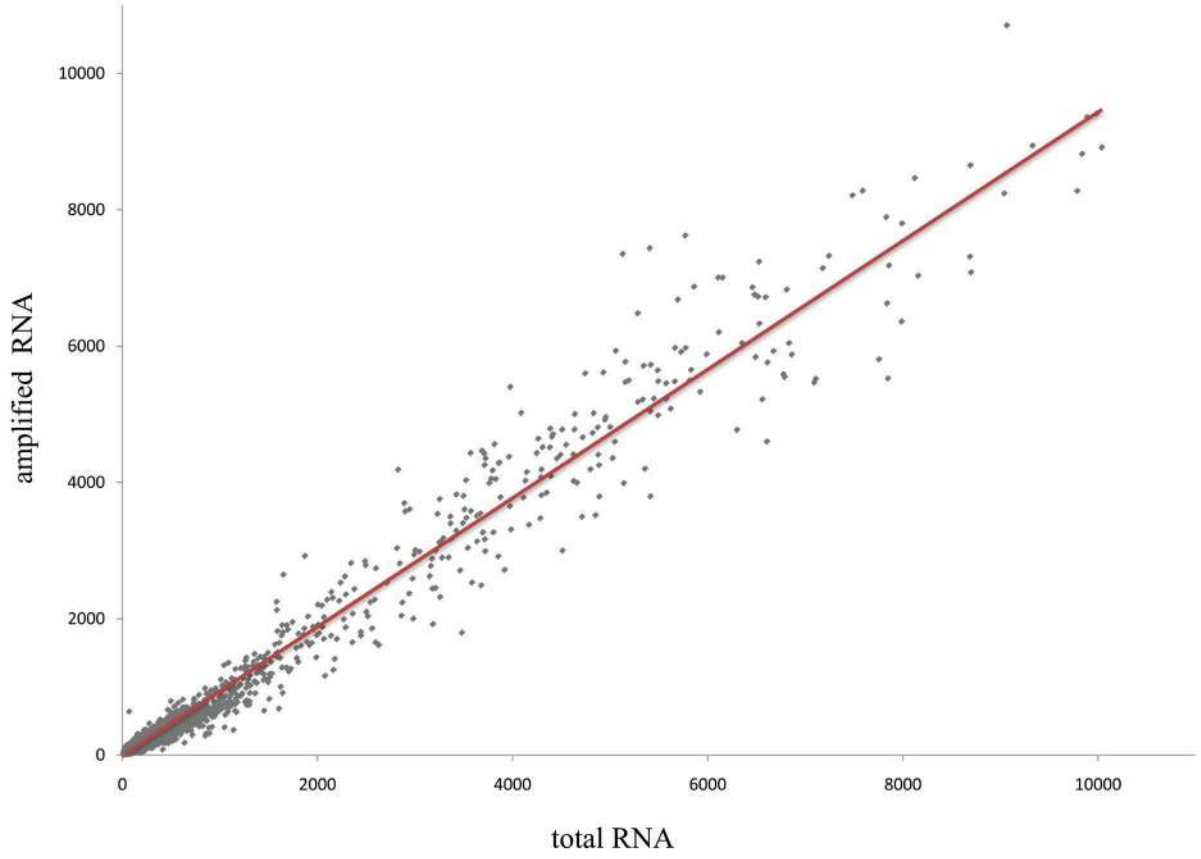


Figure 3.2 - Correlation between total and amplified RNA from the same fresh frozen specimen of t(11;18) positive MALT lymphoma.
Red line is the line of best fit through the points with a correlation coefficient of 97.2%.

3.4.3 Validation of microarray data using biological controls

As expected, the hybridisation signal of the N-terminal MALT1 probe was the highest in the two cases of t(14;18) positive MALT lymphoma, whereas the signals of two C-terminal MALT1 probes were much higher in the cases with t(11;18) than those with t(1;14) or without translocation (Figure 3.3). Cyclin D1 was highly expressed in MCL, while CD10 and BCL6 showed the highest expression in FL. The CD10 expression in MALT lymphoma was low, suggesting the presence of minimal reactive B cells components in the fresh frozen materials used for expression microarray. Despite the fact that *BCL2* t(14;18)(q32;q21)/*IgH-BCL2* translocation is the genetic hallmark of follicular lymphoma, it was not in the hallmark genes probably because *BCL2* was shown to be highly expressed in translocation positive MALT lymphomas (Section 3.4.6.1). Overall, the expected results from the biological controls indicated that the labelling, hybridisation and basic bioinformatics analysis were successfully carried out.

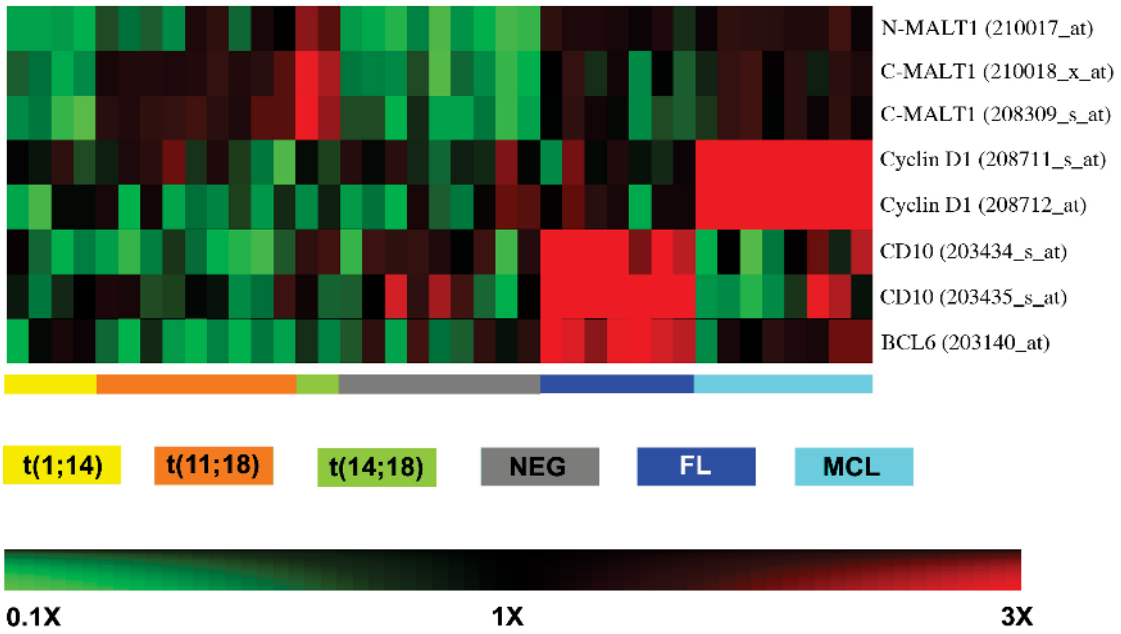


Figure 3.3 - Expression of hallmark genes in MALT lymphoma, FL and MCL.
Correlation of known expression levels of genes with different lymphoma types. N-MALT1 probe (210017_at) detects only wild type MALT1 transcripts, while C-MALT (210018_x_at & 208309_s_at) probes detect both MALT1 and API2-MALT1 transcripts. The chromosome translocation status of MALT lymphoma is indicated by colour. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale showed at the bottom of the figure.

3.4.4 Unsupervised clustering between MALT, MCL and FL defines MALT lymphoma as a distinct entity

The methodology used to combine MALT lymphoma HG-U133A with FL and MCL HG-U133plus2 is summarised (Figure 3.4).

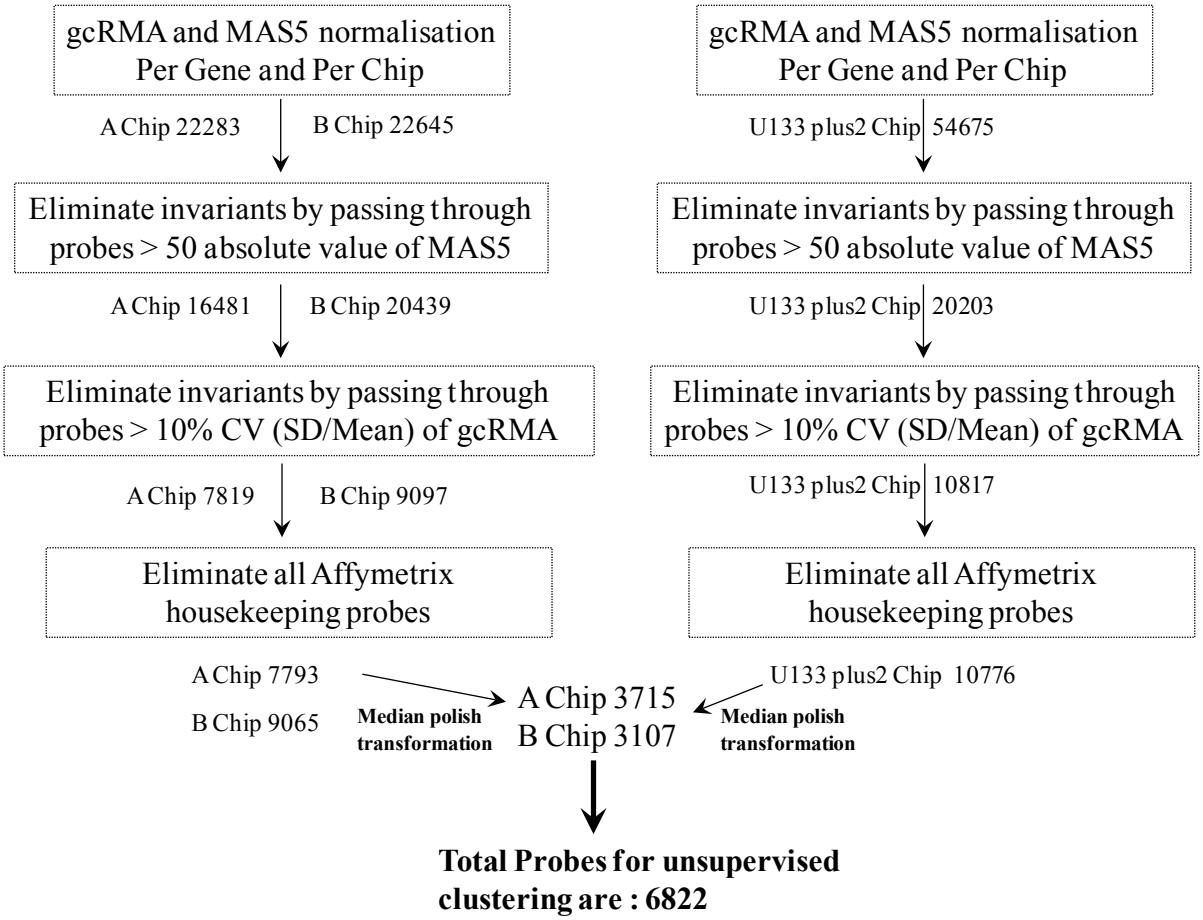


Figure 3.4 - Summary of bioinformatics strategy to combine FL and MCL with MALT microarray GeneChips.
Normalisation is followed by non-specific filtering and exclusion of housekeeping probes for both U133AB and U133plus2. The probes from the two platforms are combined using median polish transformation and the probes used for unsupervised clustering.

Analysis of HG-U133A and B and plus2 were done separately then a median polish step was used to normalise the data from the two different platforms giving rise to 6822 probes. These were used for unsupervised clustering of the 24 MALT lymphomas, 7 follicular lymphoma (FL) and 8 mantle cell lymphoma (MCL).

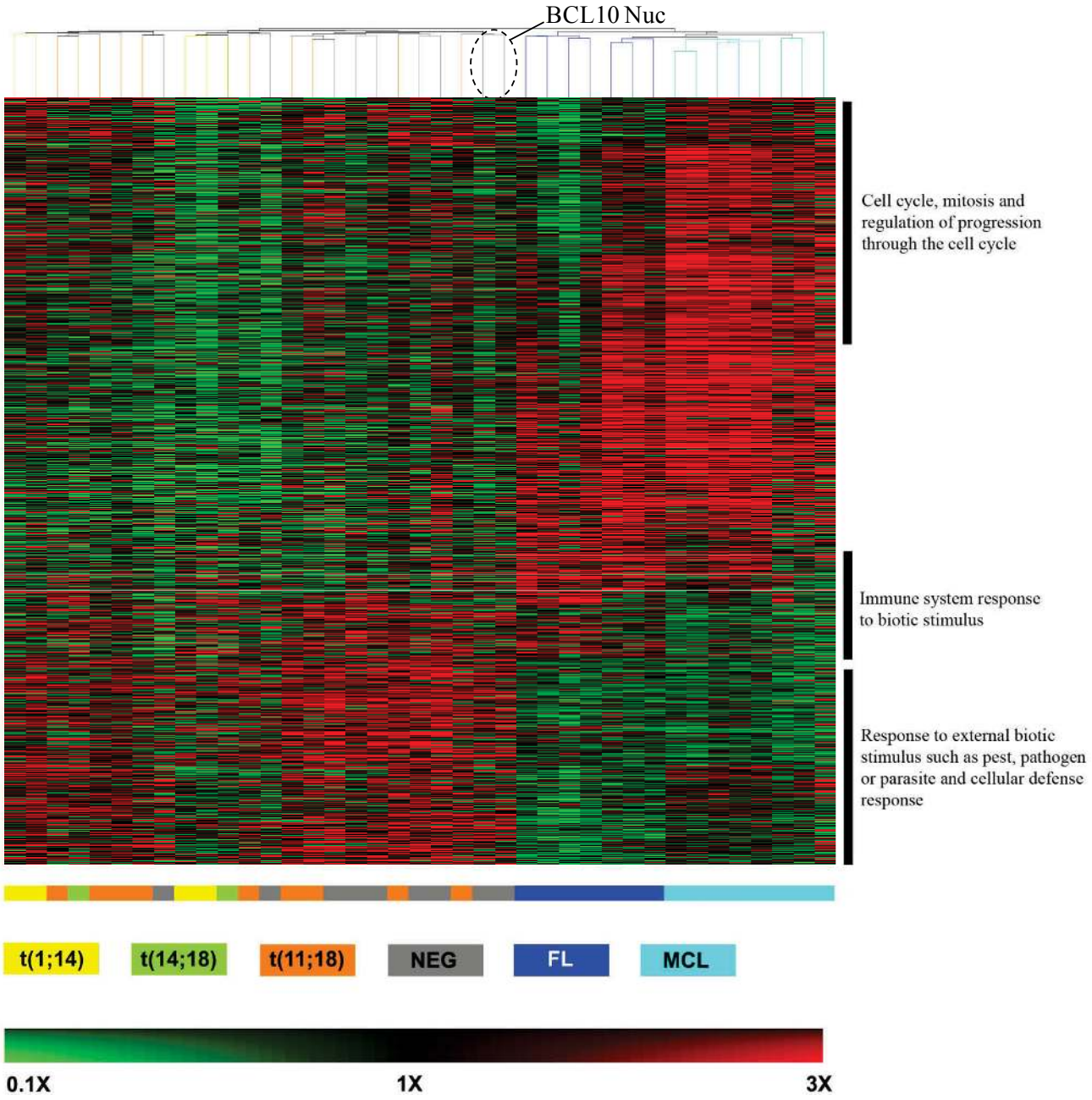


Figure 3.5 - MALT lymphoma shows distinct gene expression profiles from follicular lymphoma and mantle cell lymphoma using unsupervised hierarchical clustering.
After normalisation and filtering across both U133A&B and U133plus2 sets, a set of 6822 probes were obtained and used for unsupervised hierarchical clustering analysis. FL: follicular lymphoma; MCL: mantle cell lymphoma; NEG: translocation negative MALT lymphoma. The chromosome translocation status of MALT lymphoma, FL and MCL are indicated by colour. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale shown at the bottom of the figure.

MALT lymphomas were clustered as a single branch, irrespective of their origin from different anatomic sites or chromosome translocation. Within the MALT lymphoma group, the chromosome translocation status appeared to have little impact on the hierarchical clustering as translocation positive MALT lymphomas were intermingled with translocation negative cases (Figure 3.5). The two translocation negative cases with BCL10 nuclear staining clustered together (Figure 3.5).

In order to derive the functional groups in Figure 3.5, the gene tree was ordered according to biological processes defined by gene ontology, and the gene clusters enriched for a particular biological process in a lymphoma subtype as shown by hypergeometric testing as indicated. The gene sets for cell cycle (GO:7049) and regulation of progression through cell cycle (GO:74) were highly enriched in MCL, while those for immune response (GO:6955) and immune response to biotic stimulus (GO:9607) were enriched in FL. The gene sets for immune response to external biotic stimulus such as pest, pathogen or parasite (GO:9613) and cellular defence response (GO:6968) were highly enriched in MALT lymphoma.

3.4.5 Unsupervised clustering confirms overlapping features in gene expression profiling between MALT lymphoma with and without chromosome translocation

The strategy used to compare MALT lymphoma with and without translocation is summarised in Figure 3.6.

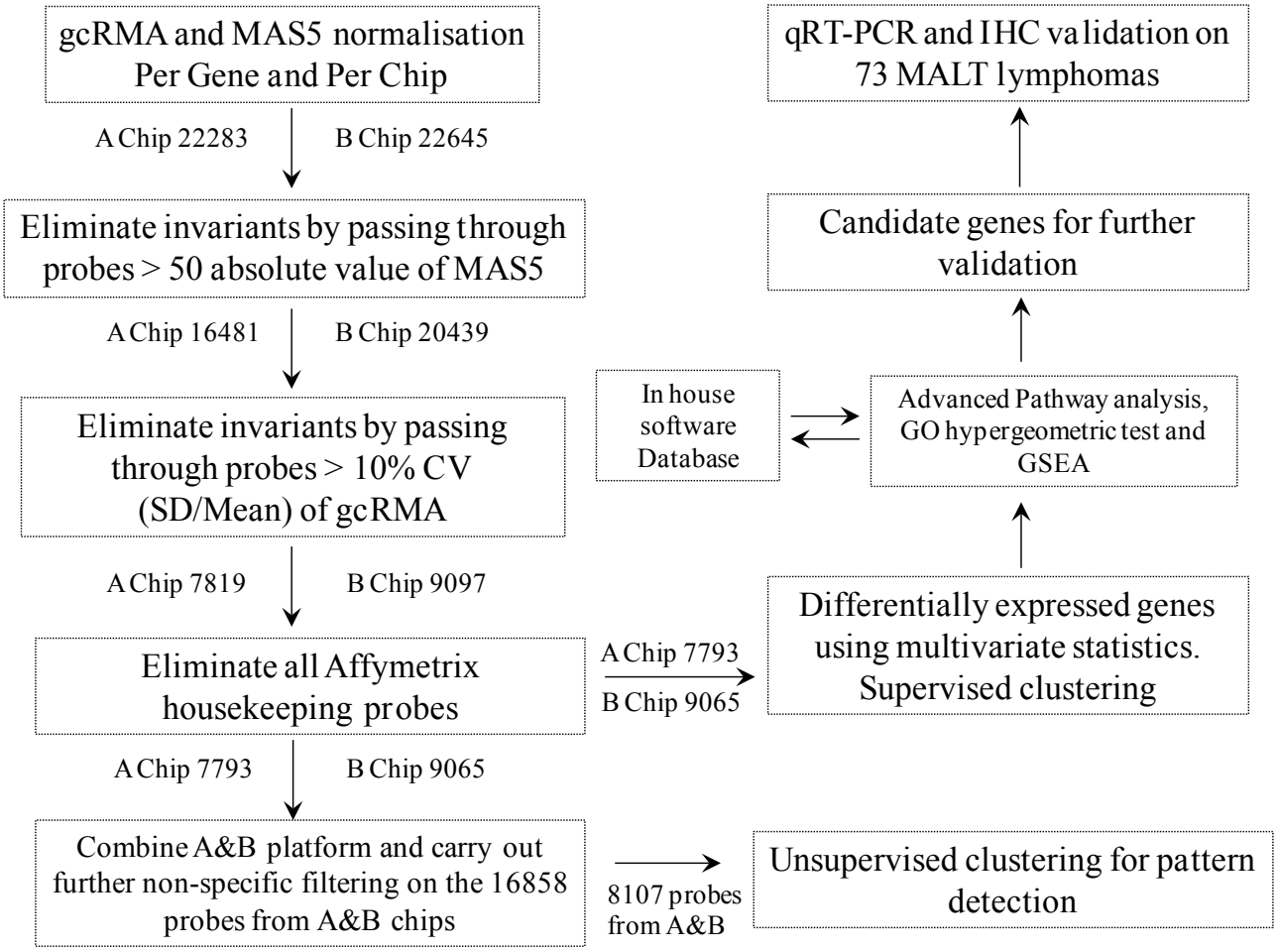


Figure 3.6 - Summary of bioinformatics strategy used to compare MALT lymphoma with and without chromosome translocations.
Normalisation is followed by non-specific filtering and exclusion of housekeeping probes. The probe sets were used to determine differentially expressed genes using multivariate analysis, this is followed by pathway analysis using GSEa and GO and in house datamining. Candidates form this analysis were validated further by qRT-PCR and immunohistochemistry (IHC).

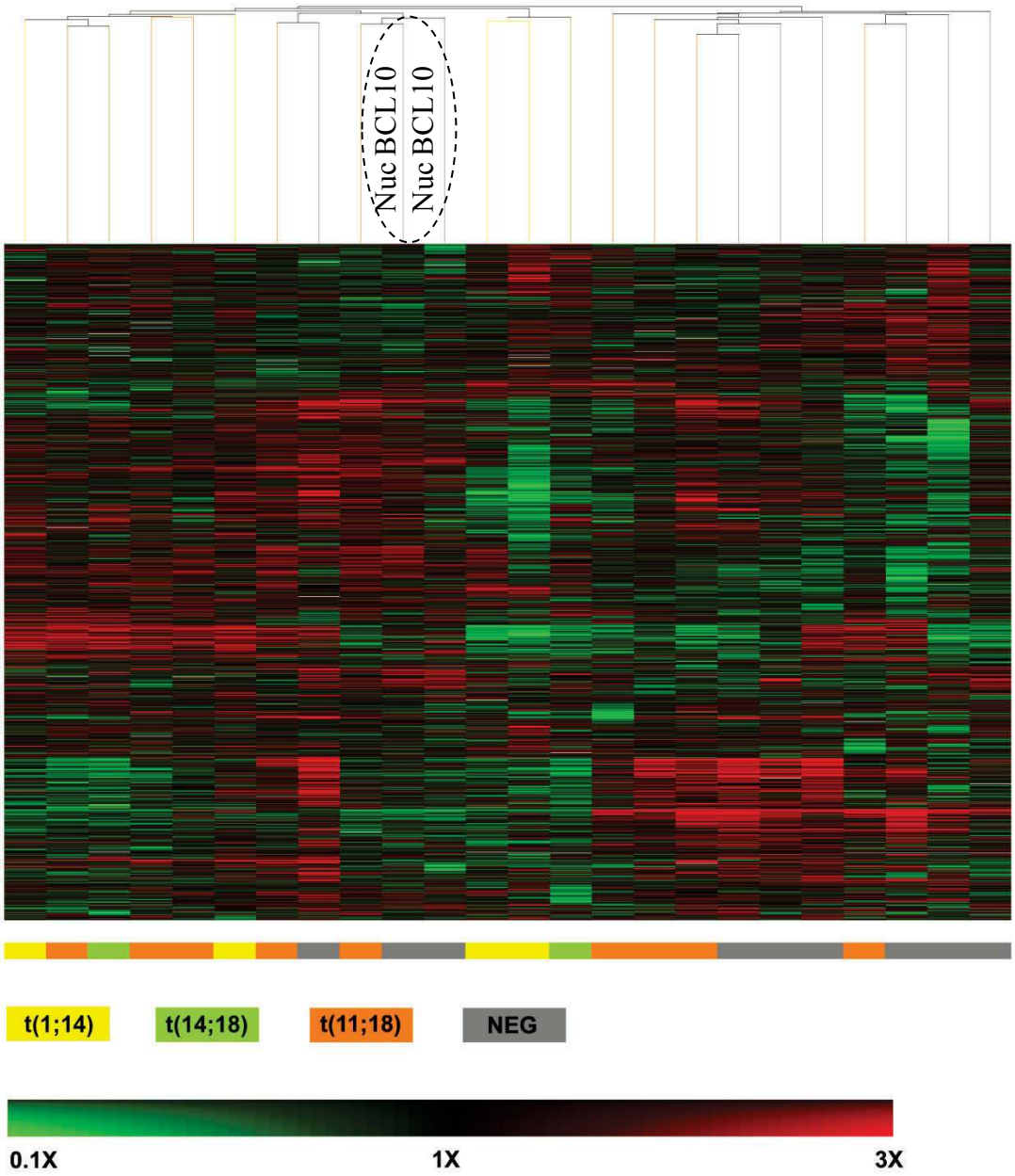


Figure 3.7 - Unsupervised hierarchical clustering of MALT lymphoma with different translocation status.
After normalisation and filtering, a set of 8107 probes were obtained and used for unsupervised hierarchical clustering analysis. MALT lymphomas with and without chromosome translocation are intermingled together. The chromosome translocation status of MALT lymphoma is indicated by different colour scheme. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale shown at the bottom of the figure.

The result of unsupervised clustering of MALT lymphoma cases again showed that the chromosome translocation status had little impact on the hierarchical clustering as translocation positive MALT lymphoma cases were intermingled with translocation negative cases (Figure 3.7). The results suggest an overlap in gene expression profile and hence molecular mechanisms between MALT lymphoma with and without chromosome translocation. The clustering showed that the two translocation negative cases with BCL10 nuclear staining clustered together with a group consisting of predominantly translocation positive MALT lymphomas (Figure 3.7). In order to investigate this further, supervised clustering with 733 probes was carried out on the four t(1;14) and nine translocation negative cases including two with nuclear BCL10 staining. The 733 probes were derived by applying one-way ANOVA multivariate analysis across MALT lymphoma with and without translocation groups to the 8107 probes obtained after normalisation and non-specific filtering. The two translocation negative MALT lymphomas with nuclear BCL10 expression still clustered with the four cases with t(1;14) (Figure 3.8).

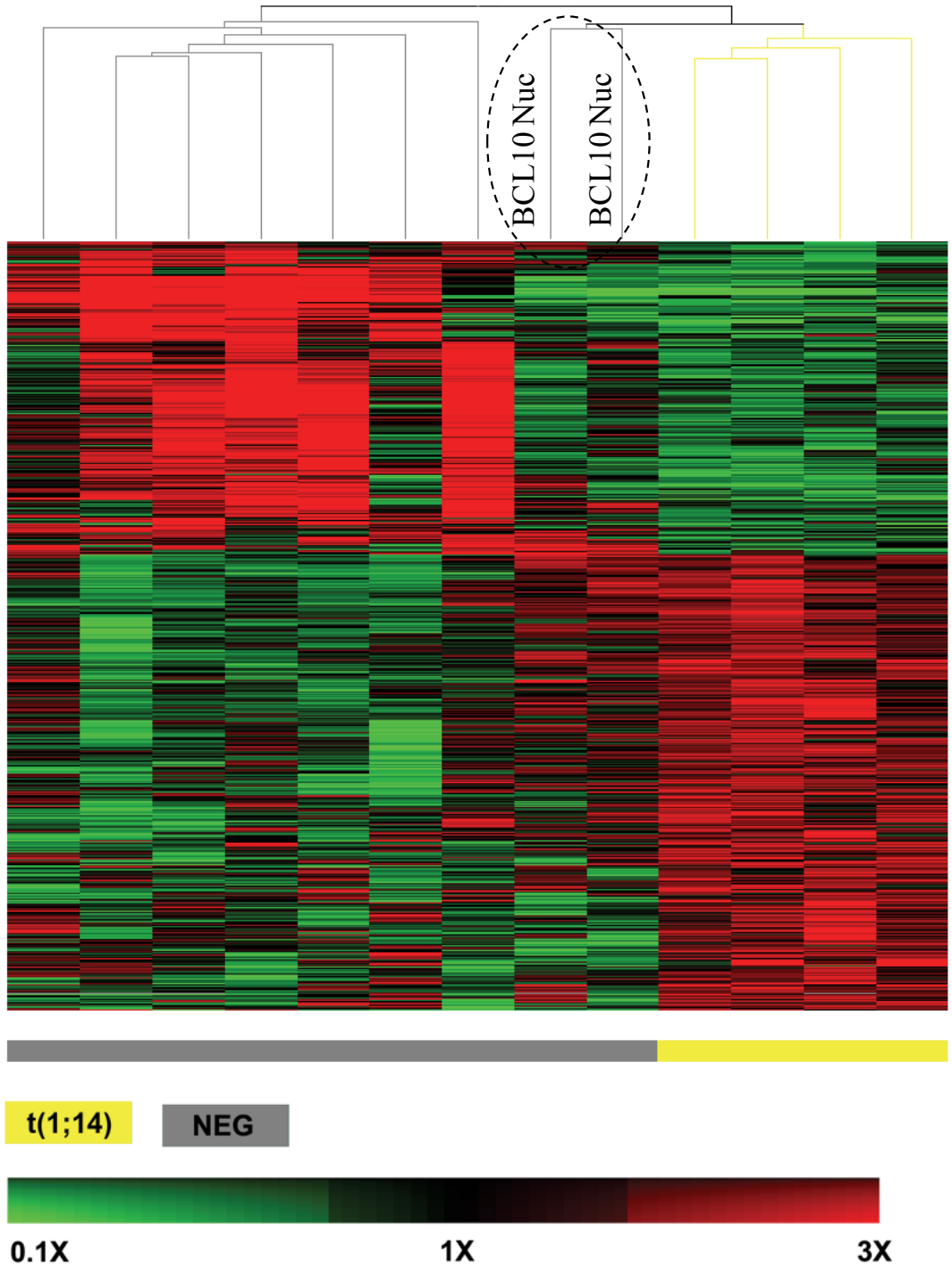


Figure 3.8. Supervised hierarchical clustering of MALT lymphoma with different translocation status.

After normalisation and filtering, a set of 733 probes were obtained and used for supervised hierarchical clustering analysis. MALT lymphoma cases without chromosome translocation but with nuclear BCL10 staining are clustering with t(1;14) positive cases. The chromosome translocation status of MALT lymphoma is indicated by different colour scheme. On the heatmap, red represents up-regulated genes and green down-regulated genes, with the scale shown at the bottom of the figure.

3.4.6 Characterisation of the gene expression profiles of MALT lymphoma with and without chromosome translocation

To gain further insights into the potential difference in molecular mechanisms between MALT lymphomas with and without chromosome translocation, GSEA and absolute GSEA were performed on 4395 gene sets covering various cellular pathways, biological processes or molecular functions derived from in house analysis and molecular signature database (Subramanian *et al.*, 2005) as described in Appendix I.III. Results of GSEA was cross validated using GO hypergeometric testing as described in section 2.2.5.6. Summary of the methodology used for GSEA (Figure 3.9).

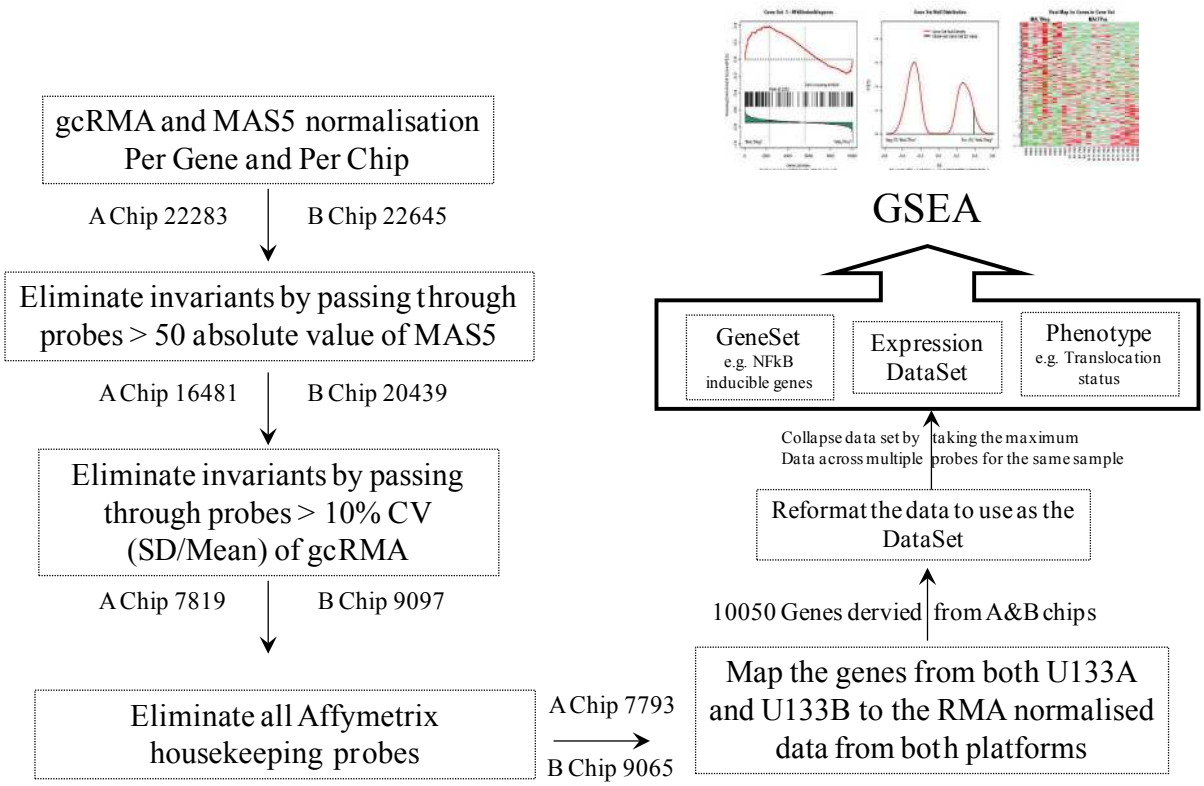


Figure 3.9 - Summary of bioinformatics strategy used for GSEA on MALT lymphoma with and without chromosome translocation.
Normalisation is followed by non-specific filtering and exclusion of housekeeping probes. The probes were then combined and mapped to the RMA normalised data. The probes are collapsed and used as an expression dataset in GSEA together with the relevant geneset and phenotype.

GSEA identified a total of 51 gene sets (not including those with very general terms) with a statistical significance ($P<0.05$ and $FDR<0.05$) and were thus differentially over-represented between MALT lymphomas with and without translocation. Remarkably, the NF-κB target gene signature was the most differentially enriched gene set ($p < 0.0001$ and $FDR < 0.0001$) (Table 3.1).

Table 3.1 - Gene sets differentially over-represented between MALT lymphoma with and without chromosome translocation.

Gene Set	Size	Source	ES	Norm ES	Nominal *P* value	FDR *q* value	FWER *P* value	Tag %	Gene %
NF-κB related									
NF-κB target genes	167	Appendix III.I for details	0.531	1.826	0.0001	0.0001	0.0000	0.455	0.249
positive regulation of IKK and NF-κB cascade	62	GO_0043123	0.466	1.541	0.0088	0.0446	0.1235	0.274	0.157
Inflammation and immune responses									
Inflammation	73	Immunome database	0.590	1.865	0.0000	0.0065	0.0076	0.548	0.257
Cellular cation homeostasis	56	GO:0030003	0.519	1.842	0.0000	0.0116	0.0710	0.429	0.264
Inflammatory response	166	GO_0006954	0.530	1.775	0.0022	0.0116	0.0128	0.482	0.267
Response to other organism	47	GO:0051707	0.575	1.829	0.0000	0.0124	0.0840	0.404	0.183
Locomotory behaviour	54	GO:0007626	0.601	1.800	0.0000	0.0153	0.1210	0.556	0.257
Chemokine	128	Immunome database	0.510	1.725	0.0022	0.0163	0.0293	0.367	0.175
Immune responses	119	GO_0006955	0.517	1.648	0.0067	0.0185	0.0266	0.429	0.259
Defence response	135	GO:0006952	0.511	1.757	0.0000	0.0210	0.1870	0.467	0.274
B-cell activation	26	GO_0042113	0.623	1.779	0.0000	0.0222	0.0117	0.423	0.152
Innate immune response	52	GO_0045087	0.506	1.595	0.0065	0.0311	0.0724	0.519	0.301
Cell adhesion molecules	86	HSA04514	0.456	1.812	0.0000	0.0321	0.0480	0.326	0.209
Lymphocyte activation	77	GO_0046649	0.525	1.647	0.0292	0.0322	0.0501	0.338	0.152
Response to biotic stimulus	71	GO:0009607	0.507	1.687	0.0000	0.0378	0.3500	0.423	0.262
Cellular homeostasis	74	GO:0019725	0.448	1.692	0.0000	0.0380	0.3310	0.351	0.264
HumoralImmunity	65	Immunome database	0.545	1.526	0.0550	0.0395	0.1312	0.477	0.257
Inflammatory response	66	GO:0006954	0.534	1.662	0.0065	0.0420	0.4020	0.455	0.274
TLR sigalling pathway from GeneGo	33	GeneGO database	0.520	1.616	0.0088	0.0421	0.0674	0.606	0.366
Cellular immunity	47	Immunome database	0.542	1.537	0.0214	0.0456	0.1269	0.489	0.262
Chemokine	29	GO_0042379	0.638	1.519	0.0338	0.0463	0.1438	0.655	0.257
Cell adhesion	50	GO:0016337	0.483	1.634	0.0058	0.0482	0.4860	0.42	0.259
MAPK pathway realted									
Rceptor signalling protein activity	54	GO:0005057	0.529	2.061	0.0000	0.0015	0.0010	0.574	0.355
Regulation of MAP kinase activity	41	GO:0043405	0.493	1.979	0.0000	0.0026	0.0140	0.537	0.292
Regulation of protein kinase activity	95	GO:0045859	0.455	1.894	0.0000	0.0066	0.0340	0.453	0.316
Regulation of kinase activity	96	GO:0043549	0.450	1.875	0.0000	0.0079	0.0420	0.448	0.316
Activation of MAPK activity	22	GO:0000187	0.549	1.845	0.0020	0.0118	0.0690	0.591	0.287
MAPKKK cascade	61	GO:0000165	0.443	1.847	0.0000	0.0121	0.0660	0.623	0.44
Transmembrane receptor protein tyrosine kinase signalling pathway	48	GO:0007169	0.465	1.832	0.0000	0.0125	0.0820	0.354	0.213
JNK cascade	28	GO:0007254	0.444	1.727	0.0000	0.0282	0.2550	0.5	0.343
MAPK signalling pathway	152	HSA04010	0.442	1.826	0.0000	0.0383	0.0380	0.467	0.343
G protein coupled receptor protein signalling pathway	125	GO:0007186	0.424	1.670	0.0061	0.0412	0.3870	0.32	0.256
Protein kinase cascade	184	GO:0007243	0.421	1.672	0.0021	0.0414	0.3830	0.413	0.317
JAK STAT cascade	20	GO:0007259	0.559	1.661	0.0151	0.0419	0.4070	0.55	0.3
Peptide GPCRS	25	C2 genmapp	0.609	1.829	0.0020	0.0457	0.0200	0.32	0.107
G protein signalling coupled to cyclic nucleotide second messenger	36	GO:0007187	0.502	1.644	0.0083	0.0475	0.4560	0.278	0.154
Cell cycle related									
Regulation of cell growth	29	GO:0001558	0.632	2.014	0.0000	0.0013	0.0060	0.448	0.205
Negative regulation of growth	24	GO:0045926	0.681	2.019	0.0000	0.0016	0.0060	0.458	0.183
Positive regulation of cell proliferation	87	GO:0008284	0.500	1.958	0.0000	0.0027	0.0140	0.483	0.308
Regulation of cell proliferation	179	GO:0042127	0.465	1.813	0.0000	0.0136	0.1030	0.43	0.308
Negative regulation of cell cycle	49	GO:0045786	0.548	1.690	0.0000	0.0370	0.3380	0.51	0.282
Cell cycle arrest	36	GO:0007050	0.564	1.640	0.0022	0.0480	0.4680	0.472	0.223
Others									
Cation homeostasis	57	GO:0055080	0.509	1.820	0.0000	0.0131	0.0970	0.421	0.264
Reproductive process	59	GO:0022414	0.516	1.803	0.0000	0.0150	0.1150	0.424	0.257
Ion homeostasis	63	GO:0050801.	0.481	1.748	0.0021	0.0218	0.2010	0.54	0.377
mitochondrion organization and biogenesis	30	GO:0007005	0.551	1.690	0.0000	0.0376	0.3360	0.4	0.23
Positive regulation of transcription	80	GO:0045941	0.414	1.681	0.0000	0.0379	0.3620	0.325	0.282
Angiogenesis	22	GO:0001525	0.563	1.648	0.0206	0.0474	0.4400	0.364	0.137
Cyclic nucleotide mediated signalling	36	GO:0019935	0.502	1.644	0.0083	0.0475	0.4560	0.278	0.154
Protein processing	26	GO:0016485	0.511	1.642	0.0019	0.0475	0.4600	0.385	0.26
Carbohydrate biosynthetic process	20	GO:0016051	0.542	1.636	0.0200	0.0484	0.4810	0.45	0.294

ES: Enrichment score; Norm ES: Normalised ES; FDR: False discovery rate; FWER: Family wise-error rate; Tag%: the percentage of gene tags before (for positive ES) or after (for negative ES) the peak in the running enrichment score; Gene %: the percentage of genes in the gene list before (for positive ES) or after (for negative ES) the peak in the running enrichment score.

3.4.6.1 NF-κB target genes are significantly differentially expressed between MALT lymphoma with and without chromosome translocation

GSEA showed that the expression of the NF-κB target genes was enhanced in both translocation positive and negative cases but with a different signature for each group. A subset of the NF-κB target genes was over-represented in translocation positive MALT lymphomas, while another subset was enriched in translocation negative MALT lymphomas. Leading edge analysis showed that 20 core genes accounted for the significant enrichment in translocation positive MALT lymphomas including *CCR2A, BCL2, TFEC, CD69, BCL10, TLR6, REL, LTB, IRF4, CCR7, CCR5* and *MAP4K1* (Figure 3.10A). Similarly, 70 core genes underscored the significant enrichment in translocation negative MALT lymphomas and *CXCL5, PTGIS2, NR4A3, CCL11, PTGIS, IL8, MMP3, CXCL2, CXCL1* and *CD86* were the top 10 of this biologically significant gene subset (Figure 3.10A). The differential expression of these genes was clearly seen in the heatmap illustration of Figure 3.10A.

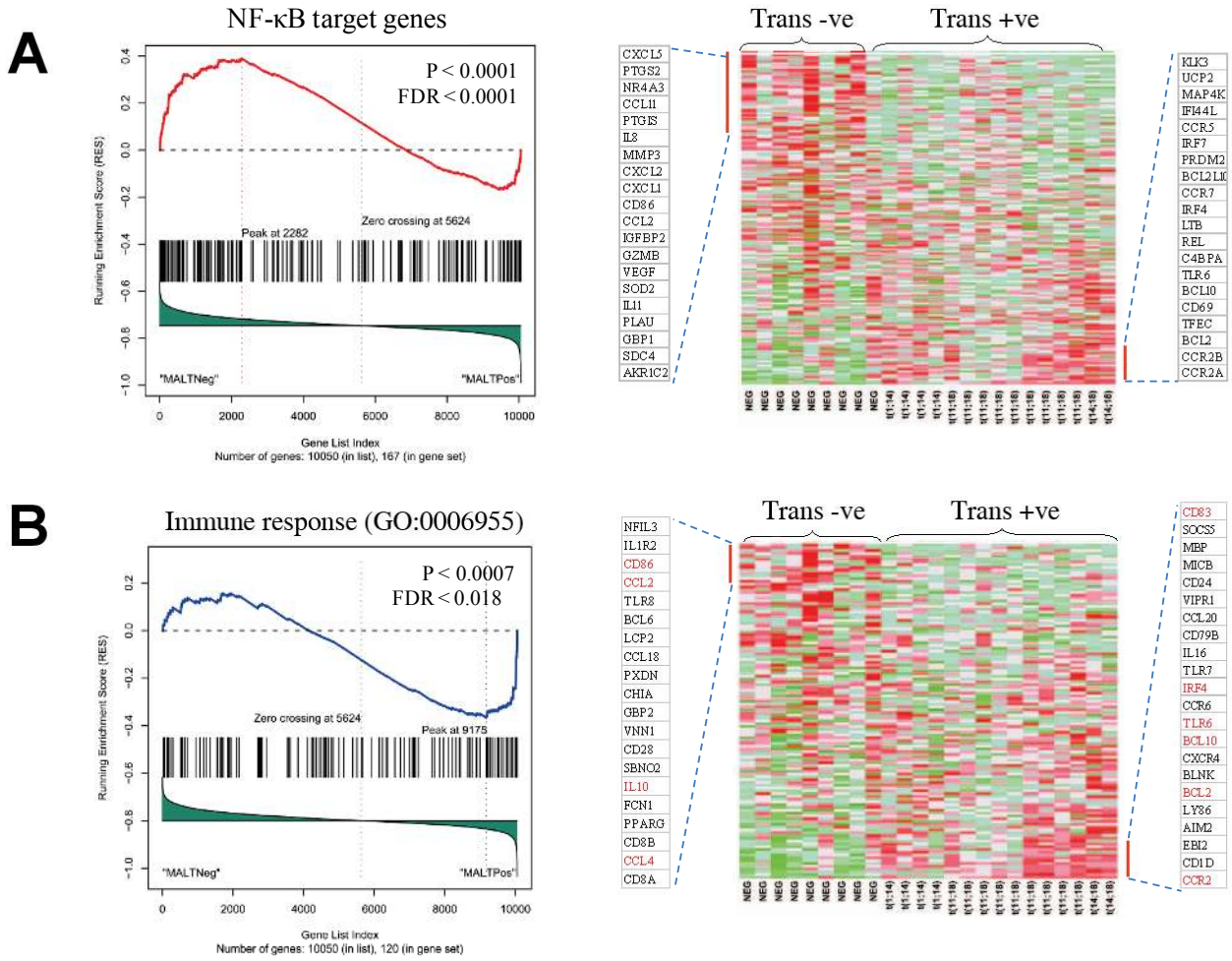


Figure 3.10 - Gene set enrichment analysis (GSEA) of NF-κB target genes in MALT lymphomas with and without chromosome translocation.
(A) NF-κB target genes and (B) Immune response set.
Left panel shows the distribution of NF-κB target genes according to their rank position. Right panel shows heatmap illustration of their expression between MALT lymphoma with and without chromosome translocation. The top 20 leading edge core genes are shown. trans -ve: translocation negative MALT lymphoma; trans +ve: translocation positive MALT lymphoma.

3.4.6.2 Other gene sets differentially enriched between MALT lymphoma with and without chromosome translocation

As there was considerable overlap among some of the gene sets that were associated with the related cellular pathways, biological processes or molecular functions, they were grouped according to their involvement in the NF-κB activation pathway, inflammation/immune responses, MAPK pathways, cell cycle and others as shown in Table 3.1. Leading edge analysis was carried out to identify the core subset genes that underscored the significant

enrichment and were thus most likely to be biologically important. The NF-κB target genes were frequently represented in each of these core subset genes, often on top end of the list particularly in the gene sets related to inflammation/immune responses (Figure 3.11).

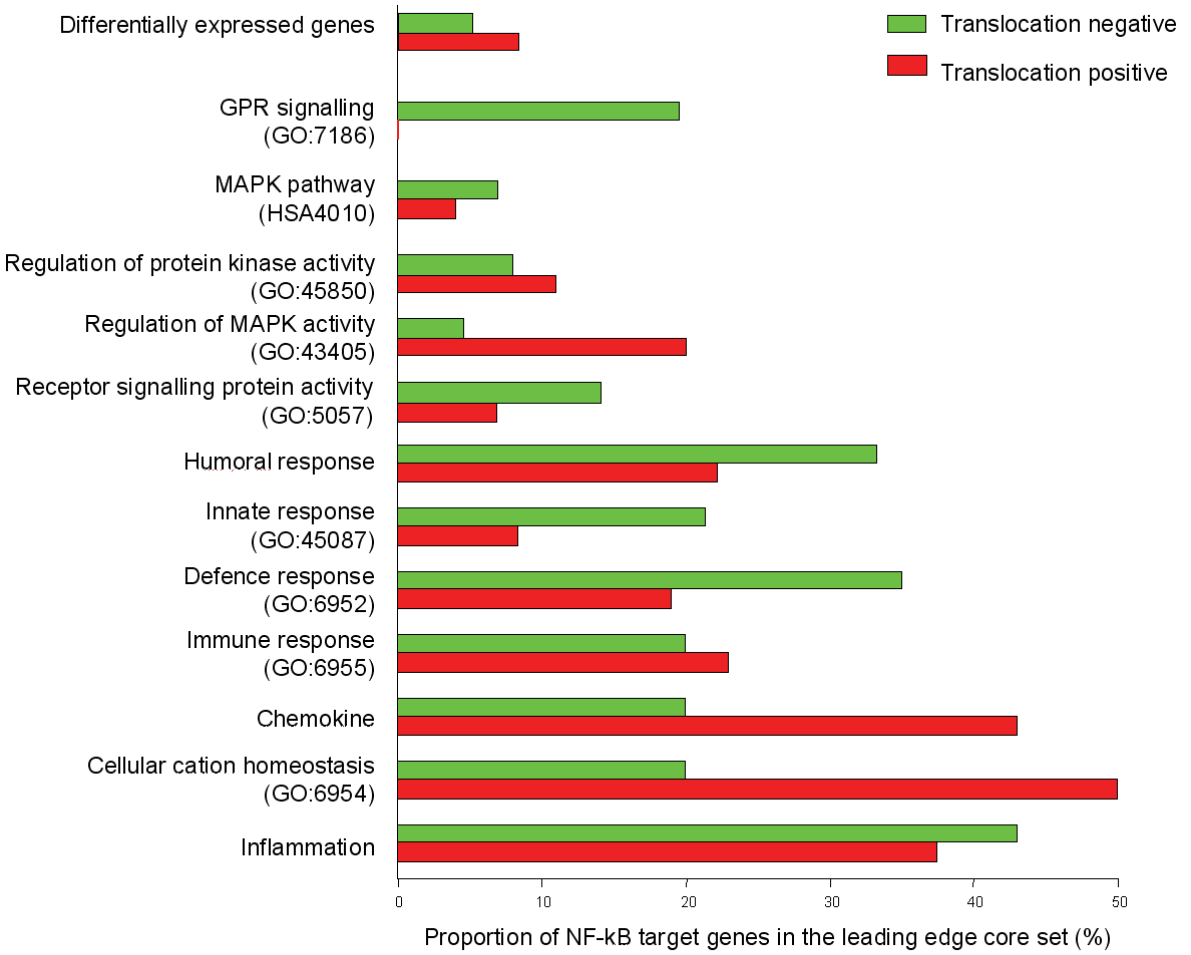


Figure 3.11 - Presence of high proportion of NF-κB target genes in the leading edge core set of various gene sets related to inflammation and immune responses.

Leading edge analysis of the positive regulation NF-κB cascade group (GO:43123) shows CD40 as one of the leading edge genes enriched in translocation positive MALT lymphoma and leading edge analysis of immune response genes (GO:6955) (Figure 3.10B) shows CD1D enriched in the translocation positive MALT lymphoma group.

3.4.6.3 Gene Ontology annotations of genes differentially expressed between MALT lymphoma with and without chromosome translocation confirms findings by GSEA

The strategy for analysis of differentially expressed genes between MALT lymphoma with and without chromosome translocation by Gene Ontology (GO) annotation is shown in Figure 3.12.

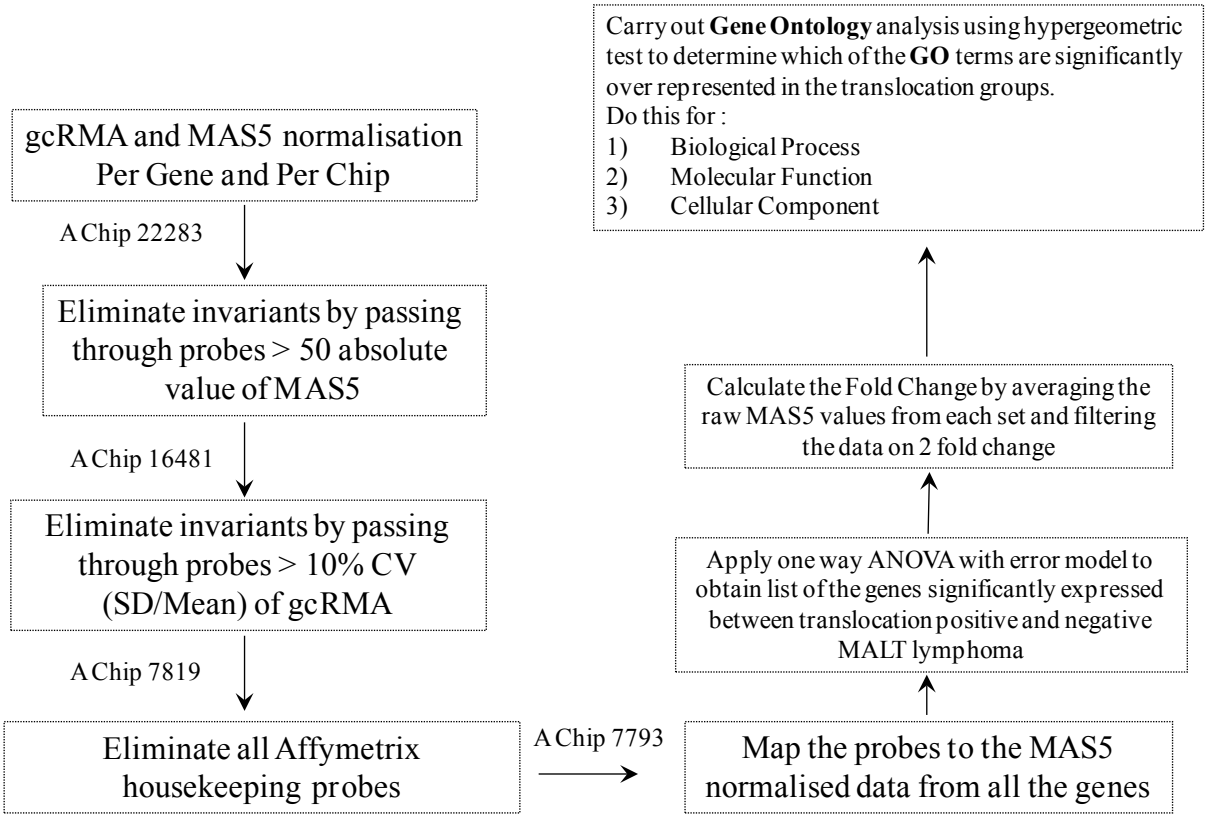


Figure 3.12 - Summary of bioinformatics strategy used for Gene Ontology analysis on MALT lymphoma with and without chromosome translocation.
After normalisation and non-specific filtering, the data is mapped to MAS5 normalised values and a one-way ANOVA was applied to it and reduced further by calculated fold change. The final probes are entered into GO to obtain any relevant pathways.

To gain further insights into the molecular pathways affected by the oncogenic products of MALT lymphoma associated chromosome translocations, genes that were significantly over or under-expressed in translocation positive MALT lymphoma in comparison with translocation negative cases were identified using the one-way ANOVA test. These

differentially expressed genes were further filtered using 2-fold change as a threshold. Ninety six genes were over-expressed in translocation positive MALT lymphoma, while 174 genes were under-expressed in these cases, i.e. over-expressed in translocation negative cases. *TLR6, CCR2, BCL2, CD1D* and *CD69* identified by GSEA were most highly expressed in translocation positive MALT lymphoma, and conversely IL8, NR4A3 and CXCL5 were on the most highly expressed in translocation negative MALT lymphoma as shown in Table (Appendix III.II).

To further assess the biological implications of the differential gene expression in MALT lymphoma with and without chromosome translocation, the representation of gene ontology (GO) terms in the above gene sets that were over or under-represented in translocation positive MALT lymphoma were measured using hypergeometric tests (Falcon *et al.*, 2007). Among the genes over-expressed in translocation positive MALT lymphoma, the GO terms relating to NF-κB pathway activation, defense/immune responses and CCR signalling were significantly over-represented (Table 3.2). While among the genes under-expressed in translocation positive MALT lymphoma, i.e. over-expressed in translocation negative cases, the GO terms relating to chemotaxis, inflammatory response and CCR signalling were significantly over-represented (Table 3.2). These findings from the analysis of differentially expressed genes between MALT lymphomas with and without chromosome translocation reinforce the GSEA results described in sections 3.2.6.1 and 3.2.6.2.

Table 3.2 - Representation of gene ontology terms in over-expressed genes in MALT lymphoma with and without chromosome translocation.

Gene ontology term	GO category	Genes in Category	% of Genes in Category	Genes in List in Category	% of Genes in List in Category	*P* value
Gene ontology term over-represented in translocation posittive MALT lymphoma						
GO:6952: defense response	Biological process	1306	8.029	17	23.61	4.18E-05
GO:9607: response to biotic stimulus	Biological process	1361	8.367	17	23.61	7.02E-05
GO:7243: protein kinase cascade	Biological process	474	2.914	8	11.11	0.00114
GO:7249: I-kappaB kinase/NF-kappaB cascade	Biological process	181	1.113	5	6.944	0.00124
GO:48522: positive regulation of cellular process	Biological process	881	5.416	11	15.28	0.00161
GO:48518: positive regulation of biological process	Biological process	1028	6.32	12	16.67	0.00173
GO:6955: immune response	Biological process	1187	7.297	13	18.06	0.00193
GO:42981: regulation of apoptosis	Biological process	525	3.227	8	11.11	0.00218
GO:43067: regulation of programmed cell death	Biological process	531	3.264	8	11.11	0.00234
GO:7250: activation of NF-kappaB-inducing kinase	Biological process	17	0.105	2	2.778	0.00252
GO:43123: positive regulation of I-kappaB kinase/NF-kappaB cascade	Biological process	133	0.818	4	5.556	0.00286
GO:43122: regulation of I-kappaB kinase/NF-kappaB cascade	Biological process	141	0.867	4	5.556	0.00353
GO:16493: C-C chemokine receptor activity	Molecular function	22	0.128	2	2.857	3.58E-03
GO:19957: C-C chemokine binding	Molecular function	22	0.128	2	2.857	3.58E-03
GO:4197: cystene-type endopeptidase activity	Molecular function	155	0.901	4	5.714	3.67E-03
Gene ontology term over-represented in translocation negative MALT lymphoma						
GO:46870: cadmium ion binding	Molecular function	10	0.0582	5	3.378	1.07E-08
GO:1664: G-protein-coupled receptor binding	Molecular function	67	0.39	7	4.73	1.71E-06
GO:5507: copper ion binding	Molecular function	79	0.459	7	4.73	5.24E-06
GO:42379: chemokine receptor binding	Molecular function	58	0.337	6	4.054	1.03E-05
GO:8009: chemokine activity	Molecular function	58	0.337	6	4.054	1.03E-05
GO:5102: receptor binding	Molecular function	902	5.246	21	14.19	3.05E-05
GO:42330: taxis	Biological process	160	0.984	8	5.882	5.96E-05
GO:6935: chemotaxis	Biological process	160	0.984	8	5.882	5.96E-05
GO:7155: cell adhesion	Biological process	978	6.012	21	15.44	6.11E-05
GO:6954: inflammatory response	Biological process	263	1.617	10	7.353	7.33E-05
GO:6817: phosphate transport	Biological process	124	0.762	7	5.147	8.17E-05
GO:9611: response to wounding	Biological process	568	3.492	15	11.03	8.17E-05
GO:7610: behaviour	Biological process	465	2.859	13	9.559	1.43E-04
GO:4295: trypsin activity	Molecular function	14	0.0814	3	2.027	2.12E-04
GO:5125: cytokine activity	Molecular function	295	1.716	10	6.757	2.41E-04

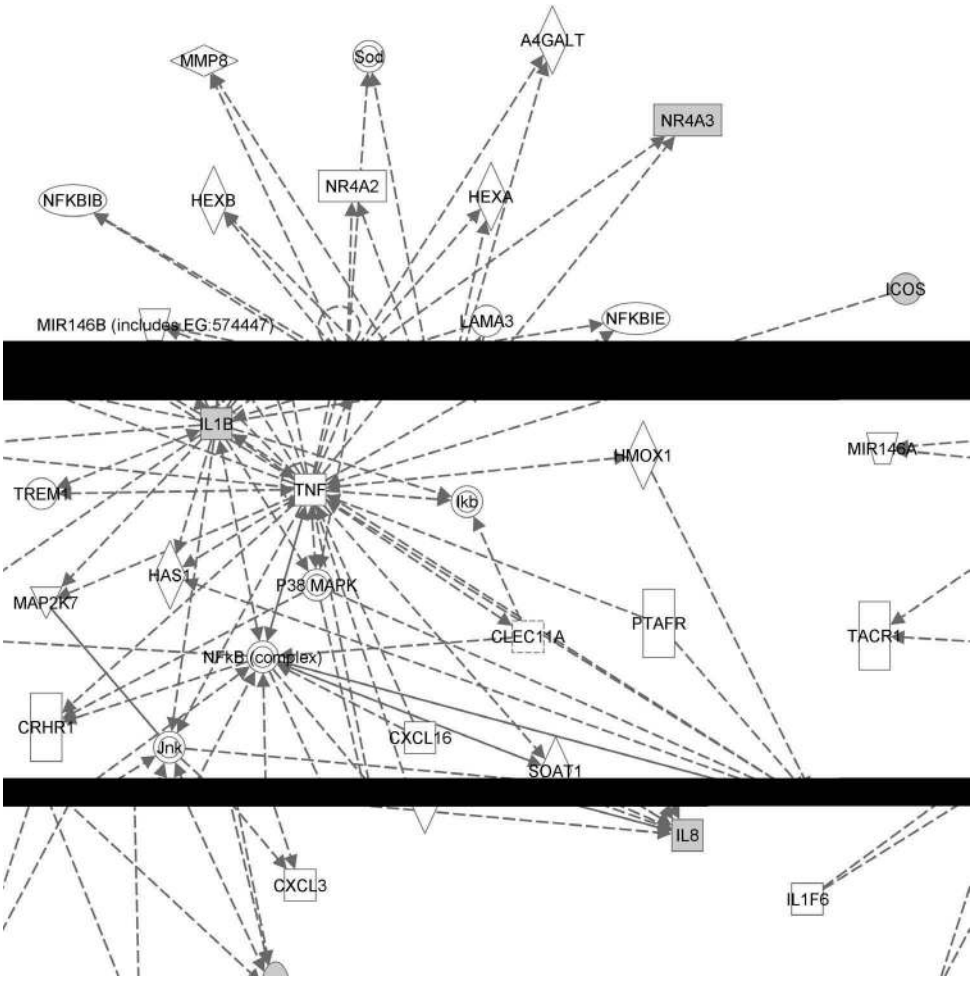
Category: the name of the category within the ontology; Genes in Category: the total number of genes in the genome that have been assigned to the category; % of Genes in Category: the percentage of genes in this category assigned to this GO term; Genes in List in Category: the total number of genes that are present both in the selected gene list and in the category; % of Genes in List in Category: the percentage of genes of this category in the selected gene list that are assigned to this GO term; P-value (hypergeometric p-value) : this is a measure of the statistical significance of the overlap. i.e. the likelihood that it is a coincidence that this many genes were in both the gene list and the category. Only the top 15 are shown.

3.4.7 Pathway analysis of molecules identified by gene expression microarray and potentially important in MALT lymphoma pathogenesis

Basic pathway analysis was carried out to investigate the pathways linked to the molecules derived from the microarray analysis.

For translocation positive MALT lymphoma, immune receptor molecules were TLR6, CD69 and CD1D, chemokine receptors were CCR2, CXCR4, CCR5, CCR6 and CCR7 and the apoptosis inhibitor BCL2. For translocation negative MALT lymphoma, pro-inflammatory cytokines were IL8 and IL1B and co-stimulatory molecules were CD86, CD28 and ICOS. MALT lymphoma with and without translocation were analysed separately using Ingenuity Pathway Analysis.

A



B

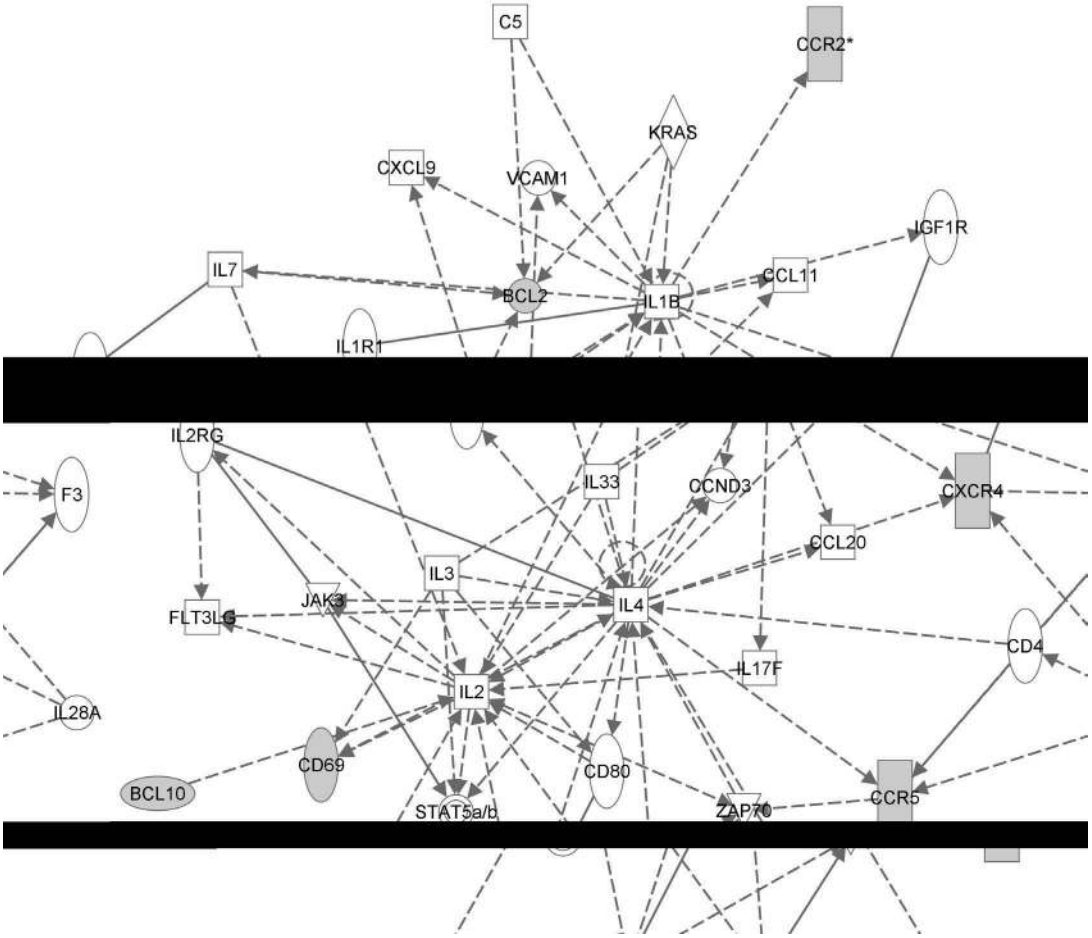


Figure 3.13 - Pathway analysis of molecules derived from MALT lymphoma microarray analysis.

Pathway analysis of molecules involved in (A) translocation negative MALT lymphoma and (B) translocation positive MALT lymphoma. The molecules highlighted with grey colour are derived from expression microarray results. A solid line indicates a direct interaction while a dashed line indicates an indirect interaction.

Pathway analysis is highly dependent on existing literature knowledge and experimental data. There is not much understood about the mechanism of translocation positive MALT lymphoma, interestingly the pathway analysis showed a possible link to the JAK3 and STAT5 pathway most likely via NF-κB inducible genes (Figure 3.13B). However, translocation negative MALT lymphoma molecules also show involvement of the NF-κB pathway especially via IL8, IL1B and CD86 as shown in (Figure 3.13A).

3.5 Discussion

By investigating the transcriptional profile, the present study demonstrated that MALT lymphoma is characterised by a distinct expression profile in comparison with FL and MCL, in line with the recent study by Chng *et al.* (Chng *et al.*, 2009). Although there was considerable overlap in the gene expression profiles between MALT lymphomas with and without chromosome translocation as demonstrated by unsupervised clustering analyses, there were important differences in the expression of NF-κB target genes between these subgroups. Systematic GSEA of various molecular pathways and biological processes, showed that the NF-κB target genes are the most differentially over-represented gene set between MALT lymphomas with and without chromosome translocation, followed by those related to inflammation, cellular homeostasis and immune responses. Importantly, several of these molecular pathways or biological processes also lead to NF-κB activation. These findings were confirmed by independent analyses of differentially expressed genes between MALT lymphomas with and without chromosome translocation using hypergeometric tests of Gene Ontology groups. Our observations provide several novel insights into the molecular mechanisms of both translocation positive and negative MALT lymphomas that potentially explain their different clinical and histological presentations.

3.5.1 Molecular mechanism of translocation positive MALT lymphoma

In comparison with translocation negative MALT lymphoma, GSEA and leading edge analysis revealed a common core subset genes that were enriched and over-expressed in translocation positive MALT lymphoma and a high proportion of these genes are NF-κB target genes involving multiple related biological processes or molecular pathways. The most

significant ones included immune receptors such as TLR6, TLR7, CD40, CD83, CD1D and CD69, chemokine receptors such as CCR2, CXCR4, CCR6 and CCR7, the apoptosis inhibitor BCL2, positive regulators of the NF-κB pathway such as REL (a component of NF-κB transcription factor), LTβ (a powerful proinflammatory cytokine) and molecules involved in MAPK pathways (Figure 3.10). All these molecules are involved in promoting tumour cell survival and proliferation either directly or indirectly. Among these, the over-expression of the above immune surface receptors is particularly interesting as this enhances the interaction between tumour cells and their microenvironment, which is known to be critical for MALT lymphoma development.

For example, TLRs are innate immune receptors critical for recognising the conserved microbial structures known as pathogen associated molecular patterns (PAMP), and are capable of activating the NF-κB pathway (Shimizu *et al.*, 2007). CD40, CD83 and CD69 are activation markers and co-stimulating molecules which are also likely to play a role in NF-κB activation. BCL2 is a classic apoptosis inhibitor, and may account for the prolonged survival of tumour cells.

The expression of these molecules was thus further investigated in independent cohorts of MALT lymphomas and discussed in the next chapter.

3.5.2 Molecular mechanism of translocation negative MALT lymphoma

In contrast to translocation positive MALT lymphoma, translocation negative cases were characterised by expression of a strong inflammatory gene signature. GSEA and leading edge analysis also revealed common core subset genes involving several related biological processes or molecular pathways, which were enriched and over-expressed in translocation negative MALT lymphoma. These included pro-inflammatory cytokines such IL8, IL1β and

LTA, molecules involved in B- and T- cell interaction such as CD86, CD28 and ICOS, several chemokine and chemokine receptors, TLR2 and NR4A3 (also known as MINOR, or NOR1) (Figure 3.10).

IL8, IL1β and LTA are the hallmark of a pro-inflammatory cytokine profile in response to *H. pylori* infection. IL8 is critical for neutrophil infiltration and activation, while IL1β and LTA induce gastrin release, inhibit acid secretion and promotes apoptosis of epithelial cells, thus affecting *H. pylori* colonisation (McNamara *et al.*, 2008). The finding of up-regulation of these pro-inflammatory cytokines in translocation negative MALT lymphomas, all from the stomach, indicates the presence of active *H. pylori* infection. In line with this, the expression of a number of chemokines and chemokine receptors was enriched in translocation negative gastric MALT lymphoma. This may reflect the trafficking and retention of various immune cells in response to an active *H. pylori* infection. In keeping with this, translocation negative gastric MALT lymphomas show more frequently an increased number of blast cells than translocation positive cases (Okabe *et al.*, 2003).

Most importantly, GSEA showed that the expression of the surface molecules involved in B- and T- cell interaction, namely CD86, CD28 and ICOS was enriched in translocation negative gastric MALT lymphoma, and this was accompanied by unregulated *IL10* expression, a well known outcome of ICOS stimulation (van Berkel *et al.*, 2006).

3.5.3 Nuclear BCL10

Both supervised and unsupervised clustering analysis showed that translocation negative MALT lymphoma cases with nuclear BCL10 staining clustered together with cases harbouring t(1;14)/BCL10-IGH (Figure 3.8). It was recently demonstrated that nuclear expression of BCL10 is significantly associated with resistance to gastric MALT lymphoma

to *H. pylori* eradication (Kuo *et al.*, 2004). In ocular adnexal MALT lymphoma, nuclear BCL10 seemed to correlate with a shorter treatment failure-free survival (Franco *et al.*, 2006), although this could not be confirmed by other groups (Vejabhuti *et al.*, 2005). Gallardo *et al.* (Gallardo *et al.*, 2006) found a significant association between nuclear BCL10 expression and a higher risk of extra-cutaneous involvement in primary cutaneous marginal zone B-cell lymphoma, whereas Li *et al.* (Li *et al.*, 2003) reported that nuclear BCL10 was associated with the development of a locally aggressive course. Based on these observations, a role for nuclear BCL10 in lymphomagenesis is suggested, although the precise pathological significance remains unclear. Based on its interaction with transcription factor IIB and its ability to activate transcription as a fusion protein linked to the Gal4-DNA-binding domain in HeLa cells, Liu *et al.* (Liu *et al.*, 2004d) suggested a role for BCL10 as a transcriptional activator, whereas Yeh *et al.* (Yeh *et al.*, 2006) found nuclear BCL10 to be involved in the transcriptional activity of NF-κB following TNF-α signalling in MCF7 cells. The current microarray analysis further enforces a role for nuclear BCL10 in the development of MALT lymphoma. However, further functional studies on the role of BCL10 in the nucleus are needed to determine which pathways and interacting molecules are involved.

3.5.4 Summary and conclusion

In summary, gene expression microarray studies showed that MALT lymphoma is a distinct entity, but with overlapping gene expression signatures between MALT lymphoma with and without chromosomal translocation. In both supervised and unsupervised clustering analysis, translocation negative MALT lymphoma with nuclear BCL10 expression clustered with translocation positive MALT lymphoma cases suggesting a role of nuclear BCL10 in MALT lymphomagenesis. Gene set enrichment analysis and Gene Ontology hypergeometric

analysis, showed differences in the molecular pathways involved in MALT lymphoma with and without chromosome translocation. Many of these differences were attributed to different involvement of the of NF-κB target genes. Leading edge analysis identified a number of important molecules that are potentially important for the pathogenesis of MALT lymphoma with or without chromosome translocation which will be investigated and discussed in the next chapter.

CHAPTER 4 – Validation of the genes identified by expression microarray of MALT lymphoma with and without chromosome translocation using qRT-PCR and immunohistochemistry

4.1 Introduction

Gene expression microarray study of MALT lymphoma with and without chromosome translocation identified a number of genes preferentially over-expressed in either subset (Chapter 3). Translocation positive MALT lymphomas were highly enriched in the expression of *CD69*, *CCR2A*, *CCR5*, *TLR6*, *BCL2* and *IRF4* (also known as *MUM1*) in addition to the already characterised genes *BCL10*, specific to t(1;14), and *MALT1* specific to t(11;18) and t(14;18). In contrast, translocation negative MALT lymphomas were highly enriched in the expression of *CD86* and *NR4A3*. The differential expression of these molecules was thought to play an important role in the pathogenesis of the respective MALT lymphoma subgroups. Thus, it was necessary to validate further their differential expression in MALT lymphomas with and without chromosome translocation in independent cohorts by qRT-PCR and immunohistochemistry and Western blotting.

4.2 Aims of the study

To validate the molecules derived from gene expression microarray studies in a large cohort of MALT lymphoma specimens using qRT-PCR and immunohistochemistry or Western blotting and to correlate the transcript and protein expression with the chromosomal translocation status.

4.3 Experimental design

4.3.1 Case selection

A total of 73 cases of MALT lymphoma with known translocation status were investigated. These included 8 cases with t(1;14), 18 cases with t(11;18), 9 cases with t(14;18), 38 cases without MALT lymphoma associated chromosomal translocations including 10 with nuclear and 28 with cytoplasmic BCL10 expression. These MALT lymphomas originated from the stomach (48), liver (2), ocular adnexa (4), small intestine (4) and lung (15).

4.3.2 Validation of gene expression by qRT-PCR

The mRNA expression level of the genes differentially expressed between MALT lymphomas with and without chromosome translocation (*CD69, CCR2A, CCR5, TLR6, BCL2, IRF4, CD86, NR4A3, BCL10* and N-terminal part of *MALT1*) was investigated by qRT-PCR. RNA was extracted from microdissected tumour cells of FFPE MALT lymphoma specimens and treated with Turbo DNAse to remove genomic DNA (Section 2.2.3.1). Where possible, primer pairs were designed to span exons to prevent amplification of any residual genomic DNA and to target up to 150bp (Table 2.2) (Section 2.2.6.1), and were thus suitable for FFPE tissues. This was the case for all genes except TLR6 which had only one coding exon and CCR2A which shared the sequence with CCR2B except for a small region. For those two primer pairs within exons, the negative control of the RNA that has undergone Turbo DNAase was checked to confirm the amplification was that of the cDNA rather than genomic DNA. All primers were checked on tonsillar RNA to ensure that they gave specific PCR products and ran on gels to ensure they amplified the expected size. The results are presented as ΔCT values calculated as ($\Delta CT = T_{Reference\ Gene} - T_{Test\ Gene}$), so the higher the value, the lower the transcript expression and *vice versa*. Unsupervised clustering on subset

of the genes across all samples using Ward similarity measure and average linkage was carried out to determine the discriminatory power of those genes on the MALT lymphoma cases. Both BCL2 and IRF4 were excluded from clustering as their qRT-PCR was carried out on a smaller cohort of MALT lymphomas (25 and 24 cases respectively) due to lack of sufficient tissue in the remaining MALT lymphomas.

The results of the qRT-PCR were correlated with the known chromosomal translocation established by conventional cytogenetics, interphase FISH and RT-PCR, as described in Chapter 3. The Mann-Whitney U Test determined whether there were statistically significant differences in the expression of particular transcripts between groups of cases with different chromosome translocation status.

4.3.3 BCL2, CD69, IRF4, MALT1, BCL10 and CD86 immunohistochemistry

Immunohistochemistry was carried out by Dr. Hongtao Ye on all cases using the BCL2, CD69, IRF4 and CD86 antibodies. Data on BCL10 and MALT1 expression from same cases of MALT lymphoma subgroups was obtained from a previous immunohistochemistry based study (Ye *et al.*, 2005) (Figure 1.6). The immunohistochemical conditions, including antigen retrieval, antibody dilution and incubation times, were systematically optimised as described in Chapter 2 (Table 2.3). The immunostaining was evaluated independently by two assessors (Professor Ming Du and Dr. Hongtao Ye) and scored according to the percentage of positive cells in a section (<30%, 30-70%, >70%) and the intensity of staining (weak, moderate, strong). Mr. Rifat Hamoudi helped in section cutting, slide preparation, scoring of immunohistochemistry under the guidance of Dr. Hongtao Ye, collation of the immunohistochemical data and analysis. Cases were considered positive if 30% or more tumour cells were stained. Cases with scoring discrepancies between the assessors were

reviewed. The results of the immunostaining were then correlated with the known chromosome translocation status as determined by qRT-PCR and interphase FISH (detailed in Chapter 3). Differences in the proportion of cases staining positive for each of the six proteins across the different chromosome translocation groups were assessed by the Fisher's exact test.

4.3.4 Validation of TLR6 using Western blotting

Imunohistochemistry of TLR6 antibody did not yield satisfactory results thus, western blotting was carried out using the TLR6 antibody on 5 fresh frozen t(11;18) positive and 9 translocation negative MALT lymphoma cases with one FL case and one Jurkat cell line used as control. Protein extraction and Western blotting were carried out as detailed in Chapter 2. The filter was stripped and re-probed with β-actin used as a loading control, followed by stripping and re-probing with MALT1 (97 kD) as an integrity control to ensure all archival samples used were adequate for investigation using TLR6 (92 kD). TLR6 and β-actin bands were quantified using AIDA (Advanced Image Data Analyzer, version 4.18) (Raytest, Straubenhardt, Germany), and normalised TLR6 expression was calculated as the ratio of TLR6 / β-actin. Differences in the cases with different TLR6 expression across the various chromosomal translocation groups were assessed by Fisher's exact test.

4.4 Results

4.4.1 Correlation of mRNA expression of *CD69*, *CCR2A*, *CCR5*, *TLR6*, *BCL2*, *IRF4*, *CD86*, *NR4A3*, *BCL10* and *MALT1* genes in MALT lymphoma with and without chromosomal translocation

BCL10 transcript expression was highest in the t(1;14) group (p = 0.001) whereas 5‘ end of *MALT1* (encoding the N-terminus of MALT1) was highest in the t(14;18) group (p = 0.02) reflecting the pattern seen in the gene expression microarray data and confirming the validity of the qRT-PCR methodology (Figure 4.1). In keeping with the results of the gene expression microarray study, *CCR5* was highly expressed in t(1;14) and *TLR6, CCR2A, BCL2, CD69* and *IRF4* were significantly highly expressed in t(1;14) and t(11;18) positive cases in comparison with translocation negative cases (Figure 4.1), whereas *CD86* and *NR4A3* were significantly highly expressed in translocation negative cases in comparison with translocation postive cases (Figure 4.1).

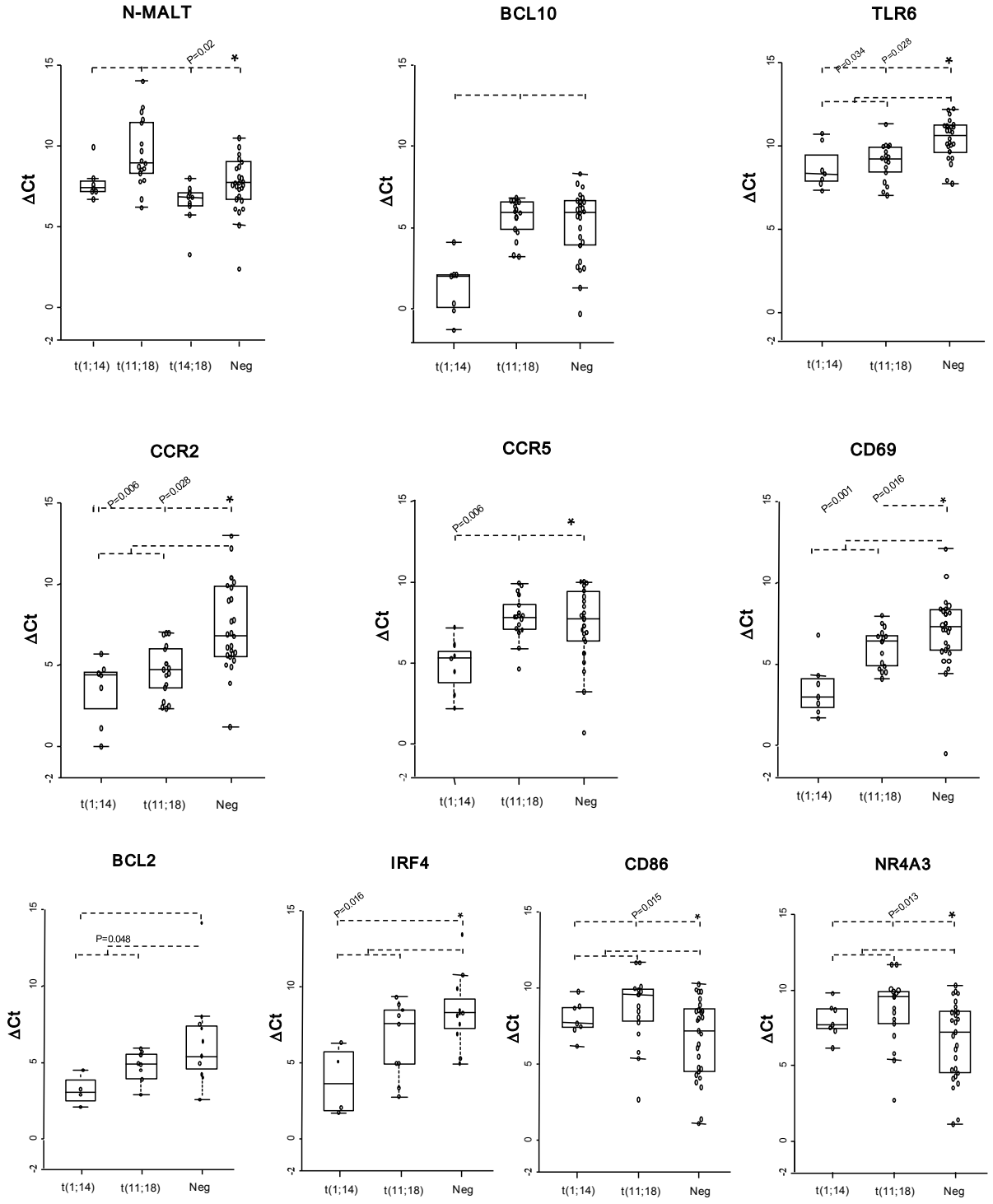


Figure 4.1 - Validation of gene expression in MALT lymphoma with and without chromosome translocation by real-time quantitative RT-PCR.

This was performed in triplicate using RNA samples extracted from tumour cells microdissected from paraffin-embedded tissue sections. Asterisk indicates statistically significant differences between various translocation positive groups and translocation negative group by Mann-Whitney non-parametric statistical test. The medians are indicated by horizontal bars in the rectangular boxes. Error bars show the standard deviation of the results in each group. Neg: translocation negative MALT lymphoma. High ΔCt values reflect low transcript expression and vice versa.

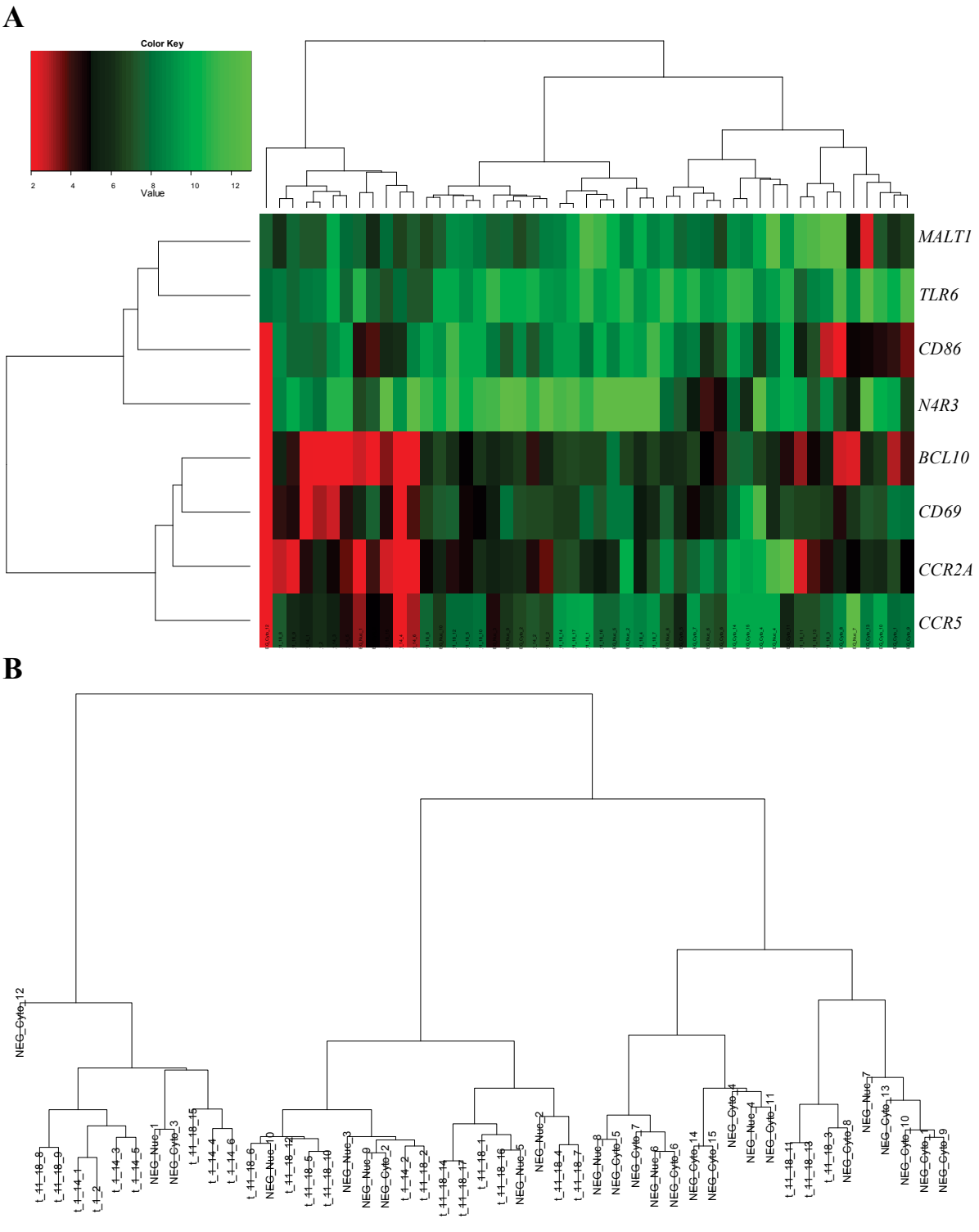


Figure 4.2 - Unsupervised hierarchical clustering of qRT-PCR data on MALT lymphoma cases with various translocation status.

(A) Unsupervised clustering heatmap of the qRT-PCR genes that are differentially expressed between translocation positive and negative MALT lymphoma. Red colour indicates high expression and green low expression. (B) dendrogram of the MALT lymphoma cases shown in the heatmap of part A.

The unsupervised clustering analysis did not show clear segregation between MALT lymphoma with and without translocation. Nevertheless, the dendrogram (Figure 4.2 B) showed 3 main groups; the far right had mainly translocation negative MALT lymphomas (16 negative and 3 t(11;18) positive), the middle group contained a mixture of translocation positive and negative MALT lymphomas but with nuclear BCL10 expression (12 positive, 5 negative with nuclear and 1 cytoplasmic BCL10 expression), and the far left contained mainly translocation positive MALT lymphomas (9 positive and 2 negative one with nuclear and the other with cytoplasmic BCL10 expression) (Figure 4.2B). Both the heatmap and dendrogram, revealed on the left, one MALT lymphoma negative case with cytoplasmic BCL10 expression labelled "NEG_Cyto_12" had high expression for all 8 genes but low expression for TLR6 (Figure 4.2 A and B).

4.4.2 Comparison of protein expression of CD69, BCL2, CD86 and IRF4, MALT1 and BCL10 in MALT lymphoma with and without chromosomal translocation using immunohistochemistry

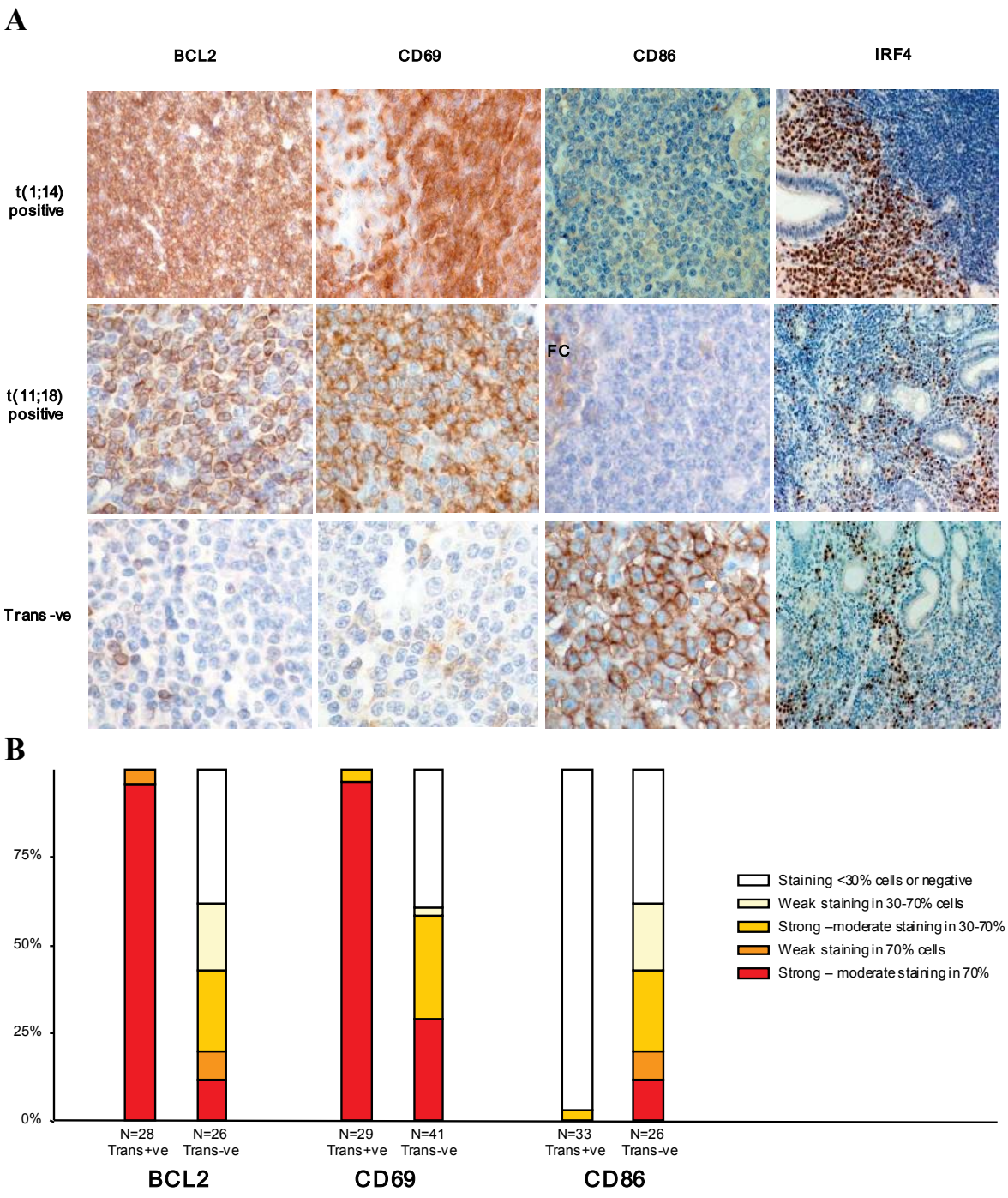


Figure 4.3 - Immunohistochemistry of BCL2, CD69, CD86 and IRF4.
(A) BCL2, CD69 and CD86 immunohistochemistry in MALT lymphomas with and without chromosome translocation.
(B) Summaries of immunohistochemistry showing that BCL2 and CD69 are more strongly and homogeneously expressed in translocation positive than translocation negative MALT lymphoma ($p < 6.9 \times 10^{-5}$, $p < 2.2 \times 10^{-4}$ respectively by Fisher's exact test), while CD86 is more strongly expressed in translocation negative than translocation positive MALT lymphoma ($p < 6.4 \times 10^{-7}$ by Fisher's exact test). IRF4 is typically expressed in activated B cells and cells showing plasma cell differentiation.
FC: follicle centre; Trans-ve: translocation negative.

Table 4.1 - Correlation of BCL2, CD69 and CD86 expression in MALT lymphoma with and without chromosome translocation.

Marker	MALT lymphoma translocation	No. of cases	Expression in >70% cells		Expression in 30-70% cells		Negative or expression in <30%
			Strong or moderate staining	Weak staining	Strong or moderate staining	Weak staining	
BCL2	Positive	28	27 (96%)	1 (4%)	-	-	-
	Negative	26	3 (12%)	2 (8%)	6 (23%)	5 (19%)	10 (38%)
CD69	Positive	29	28 (97%)	-	1 (3%)	-	-
	Negative	41	12 (29%)	-	12 (29%)	1 (2%)	16 (39%)
CD86	Positive	33	-	-	1 (3%)	-	32 (97%)
	Negative	26	3 (12%)	2 (8%)	6 (23%)	5 (19%)	10 (38%)

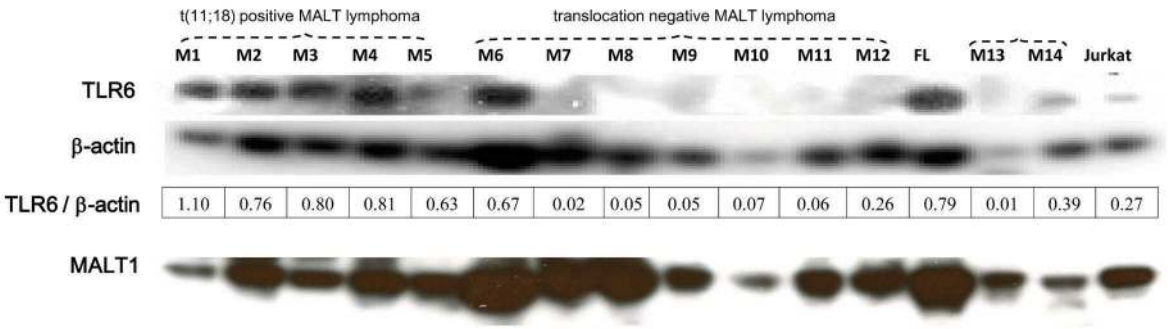
BCL10 and MALT1 staining were carried out as part of the study by Ye *et al.* (Ye *et al.*, 2005) and discussed in section 1.3.1.4. CD69, BCL2, CD86 and IRF4 protein expressions were further investigated by immunohistochemistry in MALT lymphomas (Table 4.1). IRF4, known to be expressed in activated B cells and plasma cells, was highly expressed in the neoplastic plasma cells (confirmed by immunoglobulin light chain staining) of t(1;14) positive MALT lymphoma, which were prominent in the lamina propria. Since BL10, MALT1 and IRF4 gave staining patterns similar to that reported in the literature, Both BCL2 and CD69 showed homogeneous and strong expression in 97% (28/29) of both t(11;18) and t(1;14) translocation positive MALT lymphoma cases, whereas their expression was heterogenous and much weaker expression in 29% (12/41) of translocation negative cases (Figure 4.3 and Table 4.1). For example, BCL2 was strongly expressed in at least 70% tumour cells in 96% (27/28) of translocation positive cases but only in 12% (3/26) translocation negative cases. In contrast, CD86 showed heterogeneous and strong expression in translocation negative MALT lymphomas (Figure 4.2) but weak or negative staining in

most (97% or 32/33) translocation positive MALT lymphomas. Statistical analysis using the Fisher's exact test showed that BCL2, CD69 and CD86 were significantly different between translocation positive and negative MALT lymphoma.

4.4.3 Comparison of protein expression of TLR6 in MALT lymphoma with and without chromosome translocation using Western blotting

All MALT lymphoma cases expressed a MALT1 protein of the expected molecular weight (approximately 90 kDa) indicating that the archival protein extracts are adequate for Western blot analysis (Figure 4.4A). TLR6 was highly expressed in translocation positive MALT lymphoma, but at low levels in translocation negative cases (Figure 4.4). Interestingly, case M6 which is a translocation negative MALT lymphoma with nuclear BCL10 showed relatively high expression of TLR6 (ratio of TLR6/actin = 0.67) comparable to t(11;18) positive cases (Figure 4.4B).

A



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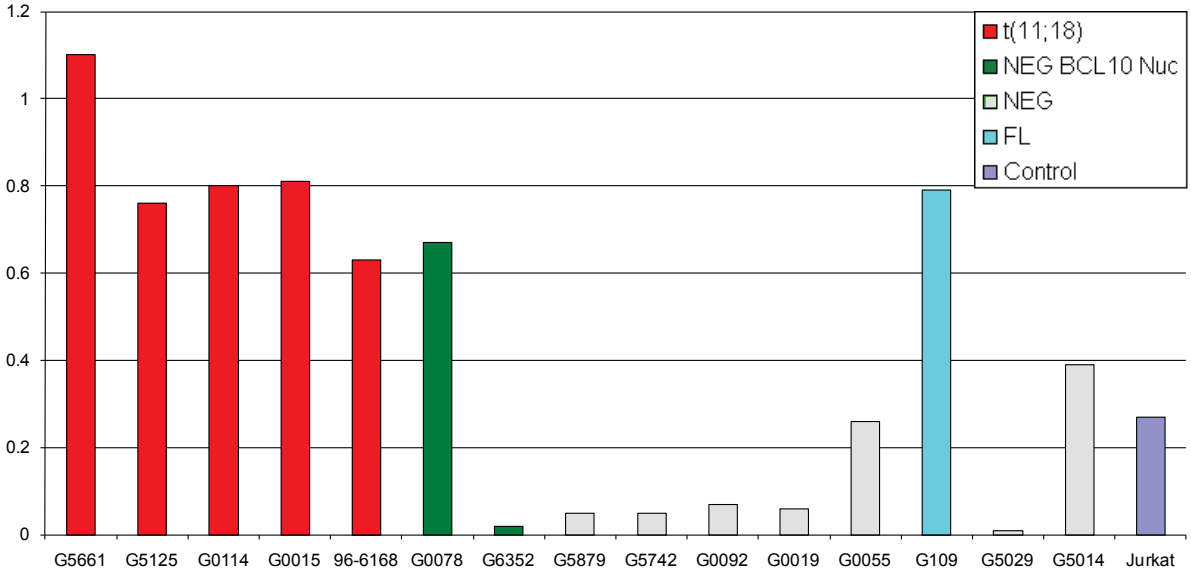


Figure 4.4 - TLR6 expression in MALT lymphoma with and without chromosomal translocation using Western blot analysis.
(A) Western blot showing that TLR6 is highly expressed in translocation positive MALT lymphoma, but at low levels in translocation negative cases. M: MALT lymphoma; M6 and M7 are translocation negative MALT lymphoma with nuclear BCL10 staining: FL: follicular lymphoma. Jurkat cell line used as control.
(B) Graphical representation of the ratio of TLR6 expression across MALT lymphoma cases with and without chromosomal translocation.

4.5 Discussion

MALT1 was highly expressed in cases with t(11;18) and t(14;18) translocation. *TLR6*, *CCR2A*, *CD69*, *BCL2* and *IRF4* were highly expressed in cases with t(11;18) translocation. *BCL10* and *CCR5* were highly expressed in cases with t(1;14) translocation. *CD86* and *NR4A3* were highly expressed in translocation negative MALT lymphomas. This was the case for 20 MALT lymphoma cases that were subjected to microarray analysis, qRT-PCR and immunohistochemistry for MALT1, BCL10, BCL2, CD69 and CD86. Thus this shows that mRNA correlates with protein expression for the above proteins and validates both methodologies as well as providing further evidence of the reliability of the gene expression microarray results in this thesis.

Translocation negative MALT lymphomas were characterised by high expression of CD86 and N4RA3. CD86 is a surface molecule involved in B- and T- cell interaction and thus might play a role in response to *H. pylori* eradication. In line with this finding, a previous study showed significantly higher CD86 expression in gastric MALT lymphomas that responded to *H. pylori* eradication when compared with those resistant to the therapy (66% VS 10%) (de Jong *et al.*, 2001). Though the chromosome translocation status in these cases is not available, it is most likely that the cases that responded to *H. pylori* were translocation-negative (Liu *et al.*, 2002b). Although residual reactive follicles may be present and contribute to the high CD86 expression in translocation negative cases, the over-expression of CD86 in tumour cells was clearly demonstrated by microdissection, qRT-PCR and immunohistochemistry. Taken together, these findings suggest that there is an active immune response to *H. pylori* infection in translocation-negative gastric MALT lymphoma, and this most likely underscores the tumour cell survival and expansion and thus determines their response to *H. pylori* eradication.

NR4A3 is another molecule significantly enriched and over-expressed in translocation negative MALT lymphoma. NR4A3 is a member of the nerve growth factor-1B (NGF1B, or NR4A1 or Nur77) subfamily of nuclear orphan receptors. In T cells, NGF1B and NR4A3 are involved in TCR mediated cell death and thymocyte negative selection (He, 2002). These nuclear orphan receptors are also involved in the apoptotic process of other cell types in response to external signals (Hashida *et al.*, 2007). The function of NR4A3 in B cells is currently unclear. Nonetheless, NR4A3 is one of the top over-expressed genes in curable, as opposed to fatal/refractory, DLBCL (Shipp *et al.*, 2002). It is possible that over-expression of NR4A3 in lymphoma cells might sensitise their response to pro-apoptotic signals following *H. pylori* eradication and elimination of the microbial mediated immune stimulations.

The over-expression of IRF4, BCL2, CD69, CCR2A, CCR5 and TLR6 in translocation positive cases was further confirmed in a separate cohort of MALT lymphomas by qRT-PCR and immunohistochemistry or Western blot analysis.

IRF4 encodes a transcriptional factor and is expressed in activated B cells and cells showing plasma cell differentiation (Pernis, 2002). In line with its known expression pattern, IRF4 is highly expressed in the plasma cell component of MALT lymphoma. Among different subgroups of MALT lymphoma, plasma cell differentiation of neoplastic B cells is most prominent in those with t(1;14) translocation. IRF4 is transcriptionally activated by t(6;14)(p25;q32) in multiple myeloma and strong IRF4 expression has also been found in several lymphoma subtypes including lymphoplasmacytic lymphoma and 75% of diffuse large B-cell lymphoma and primary effusion lymphoma, which are not associated with t(6;14) (Falini *et al.*, 2002). IRF4 was one of the molecules found in the activated DLBCL signature profile using cDNA microarrays (Alizadeh *et al.*, 2000). The oncogenic activity of IRF4 is thought to be related to its transcriptional repression of interferon (IFN) inducible

genes and thus suppression of the anti-proliferative effects of IFN (Hrdlickova *et al.*, 2001; Pernis, 2002). Interestingly, NF-κB transactivates IFN and a simultaneous up-regulation of IRF4 may block the effect of NF-κB on IFN activation.

BCL2 is an apoptosis inhibitor protein; its over-expression in lymphocytes alone was shown to be insufficient for malignant transformation, but simultaneous over-expression of BCL2 and the proto-oncogene MYC may produce aggressive B-cell malignancies (Otake *et al.*, 2007). Hence in MALT lymphomas, over-expression of BCL2 may lead to oncogenesis by reducing cell death. Currently, the exact anti-apoptotic pathways through which BCL2 exerts its role are only partially understood, involving decreased mitochondrial release of cytochrome C, which in turn is required for the activation of procaspase-9 and the subsequent initiation of the apoptotic cascade (Adams *et al.*, 1998).

CD69, a type II transmembrane glycoprotein with an extracellular C-type lectin binding domain, is another potential co-stimulatory receptor and may also have an immunoregulatory role (Sancho *et al.*, 2005). Although the precise function of CD69 in B cells is largely unknown, it is a well described activation marker in a number of cell types. CD69 is frequently expressed in low grade B-cell non-Hodgkin lymphomas and in follicular lymphoma, its expression on tumour cells is associated with poor treatment outcome (de Jong *et al.*, 2009; Erlanson *et al.*, 1998). The finding of enriched expression of CD69 in translocation positive MALT lymphoma in the present study further implicates its role in lymphoma pathogenesis.

Other types of molecule enriched in translocation positive MALT lymphomas are chemokine receptors such as CCR2 and CCR5. These are G protein coupled receptors (GPR) whose major function is to regulate leukocyte trafficking and mediate immune cell migration and their retention in inflammatory sites (Murphy *et al.*, 2000). Other functions include

angiogenic activity, apoptosis, T-cell differentiation and phagocyte activation. Following interaction with their specific chemokine ligands, chemokine receptors trigger a flux of intracellular calcium (Ca^{2+}) ions (calcium signalling). This causes cellular responses, including the onset of a process known as chemotaxis that triggers cells to migrate to specific anatomical sites. Several homeostatic chemokines have been shown to play an important role in mucosal immunology including germinal centre formation, homing mechanisms, migration and retention of lymphocytes to the sites of inflammation. Recently a study showed that CCR2 is expressed in several haematopoetic cell lineages and is critical for migration of haematopoetic stem and progenitor cells to sites of inflammation (Si *et al.*, 2010). Although the specific role of CCR2 in B-cell trafficking and homing is unclear, it forms a heterodimer with CXCR4 that is critical for B-cell homing to the Peyer's patches and splenic marginal zone (Springael *et al.*, 2005), thus potentially playing a role in mature B-cell homing processes. CCR5 is a receptor for a number of inflammatory CC-chemokines including MIP-1-alpha, MIP-1-beta and RANTES, which may prove to be important in translocation positive MALT lymphoma.

Another interesting molecule highly expressed in translocation positive MALT lymphoma is Toll-like receptor 6 (TLR6). Toll-like receptors play a role in innate immunity by recognising conserved microbial structures known as pathogen associated molecular patterns (PAMPs). TLR6 is activated by bacterial lipopolysaccharide (LPS) and signals through MyD88 to activate NFκB; the signalling pathway of TLR6 is similar to that of the IL-1 receptor upon activation by the cytokine IL-1. In a mouse model, it has been shown that TLR signalling promotes marginal zone B-cell activation and migration (Rubtsov *et al.*, 2008). TLR6 typically forms heterodimers with TLR2 on the cell surface to recognize bacterial antigens (Gomariz *et al.*, 2007). TLR2/TLR6 signalling activates not only IKK complex that leads to

activation of the NF-κB transcriptional factor, but also the MAP kinase p38 and Jun amino-terminal kinase (JNK) that leads to activation of the AP-1 transcriptional factor (Akira *et al.*, 2004). Hence, over-expression of TLR6 in translocation-positive MALT lymphoma could potentially augment the NF-κB activity mediated by MALT lymphoma associated oncogenic products and also activate the MAP kinase pathways. In order to test the former hypothesis, functional studies involving the expression of TLR6, in the presence of TLR2, were carried out to investigate whether they could enhance both BCL10 and API2-MALT1 mediated NF-κB activation *in vitro* and whether this effect was particularly significant upon LPS stimulation.

In summary, all of the key NF-κB target genes, found to be over-expressed by expression microarrays, were confirmed by qRT-PCR and immunohistochemistry. Those genes are involved in promoting tumour cell survival and proliferation either directly or indirectly and their over-expression may enhance the interaction between tumour cells and their microenvironment, which is known to be critical for MALT lymphoma development. Thus further investigation of the role of some of those genes in MALT lymphogenesis is warranted and the effect of some of the identified genes (such as TLR6) on NF-κB activation, will be investigated using *in vitro* model as described in the next chapter.

CHAPTER 5 – Cooperation between MALT lymphoma oncogenes and immunological stimulation in activating the NF-κB pathway

5.1 Introduction

MALT lymphoma commonly occurs in sites that are normally devoid of organised lymphoid tissues where the lymphoma is preceded by the accumulation of reactive lymphoid tissue, suggesting that the tumours arise during a chronic immune response. Several previous studies suggest that surface receptor stimulation may play an important role in MALT lymphoma pathogenesis. MALT lymphomas invariably express surface Ig. Stimulation by the anti-idiotype antibody has been shown to enhance MALT lymphoma cell proliferation and this synergises with mitogenic stimulation *in vitro* (Hussell *et al.*, 1993b). In line with this, expression of either *API2-MALT1* or *MALT1* in BJAB B-cells enhances the activation of IKK and NF-κB by CD40/CD40L stimulation (Ho *et al.*, 2005). Both API2-MALT1 and BCL10 transgenic mice acquired expansion of the white pulp of the spleen but not lymphoma (Baens *et al.*, 2006). However, treatment of these mice with the Freund's complete adjuvant led to the development of marginal zone hyperplasia reminiscent of human MALT lymphoma (Baens *et al.*, 2006).

Thus antigen stimulation may play a critical role in the clonal expansion and survival of MALT lymphoma cells. However, the surface receptors involved and the molecular mechanisms underlying the proliferation and survival of MALT lymphoma cells remain to be investigated. Also, TLR signalling activates the IKK complex that leads to activation of the NF-κB transcriptional factor, thus the effect of TLR6/2 on MALT lymphoma associated oncogenes mediated NF-κB activation was investigated. In addition, the potential cooperation

between MALT lymphoma associated oncogenes and immune receptor signalling such as those from TLR, B- and T-cell antigen receptors and CD40 was investigated. The possible molecular mechanisms underlying such cooperation were explored.

In view of the finding of enriched *TLR6* expression in translocation positive MALT lymphoma as described in chapter 4, cooperation between *TLR6* and MALT lymphoma associated oncogenes in NF-κB activation *in vivo* was first investigated.

5.2 Aims of the study

1) To test the hypothesis that there is cooperation between BCL10, MALT1 and API2-MALT1 and TLR6 on NF-κB activation;
2) To investigate whether there is a synergistic effect between expression of *MALT1*, *BCL10* and *API2-MALT1* and antigen receptor stimulation on NF-κB activation;
3) To explore the effect of expression of *MALT1*, *BCL10* and *API2-MALT1* on key regulators of canonical NF-κB activation pathway in B- and T- cell lines;
4) To determine BCL10 and MALT1 sub-cellular localisation and the effect of MALT1 on BCL10 sub-cellular localisation.

5.3 Experimental design

5.3.1 Cell lines

Murine BaF3 and WEHI cell lines were selected to investigate the effect of expression of *BCL10*, *MALT1* and *API2-MALT1* on NF-κB with and without stimulation by LPS, anti-IgM and CD40L, as they are responsive to such stimulation. The BaF3 clone used was IL-3 independent. Jurkat human T-cell line was selected to investigate the effect of expression of *BCL10*, *MALT1* and *API2-MALT1* on NF-κB with and without stimulation using anti-CD3 and anti-CD28. BJAB human B-cell line was used to investigate the effect of expression of *BCL10*, *MALT1* and *API2-MALT1* on their sub-cellular localisation. It was not possible to use the BJAB B-cell line for NF-κB luciferase reporter assays as no increased activity has been observed in response to known NF-κB activators such as LPS, anti-IgM and CD40L.

5.3.2 Investigation of the cooperation between *BCL10*, *MALT1* and *API2-MALT1* and *TLR6* on NF-κB activation with and without LPS stimulation

BCL10, *MALT1* and *API2-MALT1* together with *TLR2* and *TLR6* or *TLR1* and *TLR6* expression constructs along with a control vector *pIRES* were transiently transfected into human Jurkat T cells, which are known to be non-responsive to LPS stimulation and thus are a good model to investigate LPS mediated NF-κB activation. The transfected cells were seeded in multi-well plates, cultured for 18 hours and then treated with LPS or vehicle alone for 6 hours. Twenty four hours after transfection, the cells were harvested and measured for NF-κB activity using a luciferase assay (Section 2.2.11.1). NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. The experimental work in this section was carried out with the help of Dr. Alex Appert.

5.3.3 Investigation of the effect of *BCL10*, *MALT1* and *API2-MALT1* expression and antigen receptor stimulation on NF-κB activation

Murine B-cell lines BaF3, WEHI and Jurkat human T cells were transfected with *BCL10*, *MALT1* and *API2-MALT1* vectors and a control vector independently (Section 2.2.11.1). The transfected cells were seeded in multi-well plates, cultured for 18 hours. Prior to harvest for NF-κB luciferase assay, BaF3 cells were stimulated for 6 hours with LPS, WEHI cells were stimulated for 6 hours with LPS, anti-IgM or CD40L and Jurkat cells were stimulated for 6 hours with anti-CD3 and anti-CD28. Each of these experiments was performed three times independently. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. The experimental work in this section was carried out with the help of Dr. Alex Appert.

5.3.4 Investigation of the effect of MALT lymphoma associated oncogenes expression on canonical NF-κB pathway activation

This was carried out using *BCL10*, *MALT1*, *API2-MALT1* and *BCL10/MALT1* double inducible BJAB cells and *BCL10* stably expressed BJAB cells. BJAB cells transfected with *pIRES vector* were used as control (Section 2.2.9.5).

The following proteins were investigated by Western blotting 3 NF-κB negative regulators, IκBα, IκBβ and IκBε; 3 central molecules, IKKα, IKKβ and NEMO; and 5 NF-κB subunits including c-Rel, RelB, p65, p50/p105 and p52/p100. IκBα and IκBβ are phosphorylated and degraded during activation of the classical NF-κB pathway. p105 is cleaved into p50 and p100 is cleaved into p52 during activation of the canonical and non-canonical NF-κB pathway respectively. β-actin was used as loading control. Western blotting results were quantified (Section 4.3.4) and ratio of each molecule over β-actin was calculated.

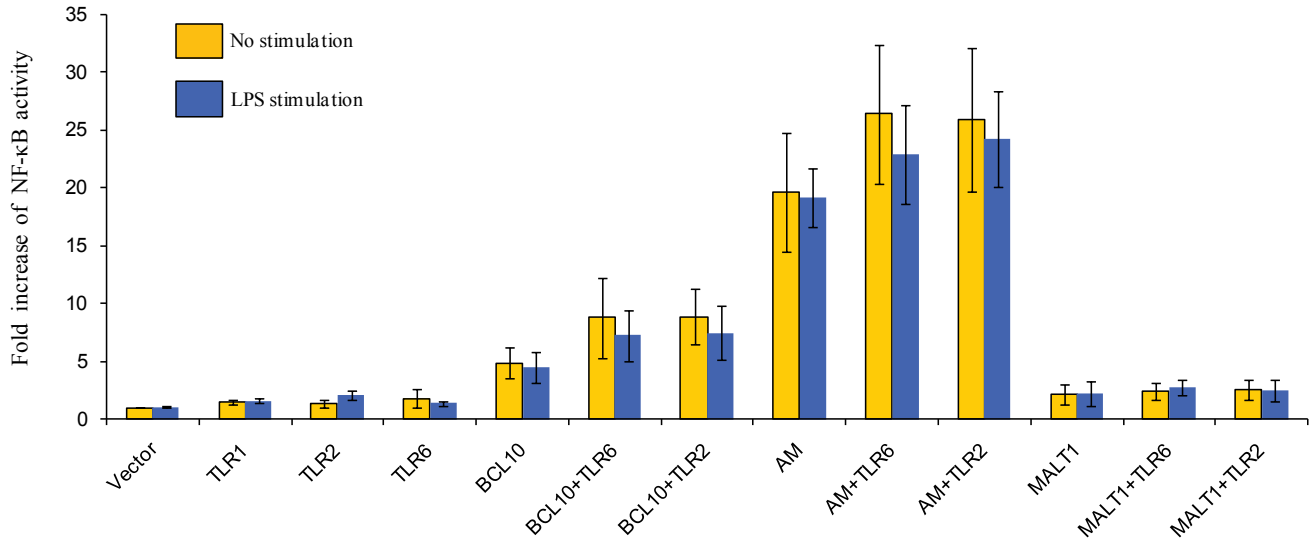
5.3.5 Investigation of the effect of *BCL10* and *MALT1* expression on their sub-cellular localisation

This was carried out using *BCL10*, *MALT1*, *API2-MALT1* and *BCL10/MALT1* double inducible BJAB cells and *BCL10* stably expressed BJAB cells. BJAB cells transfected with *pIRES vector* were used as control. The expression of each of the oncogenes mentioned above in the BJAB cells was investigated at 9, 16 and 24 hours following induction by the addition of doxycycline to the culture medium. Cell clots were prepared (section 2.2.9.3) and the expression of these oncogenes was investigated by immunocytochemistry using the BCL10 and C-MALT1 antibodies. In addition, co-immunoprecipitation was carried out on BCL10 and MALT1-expressing BJAB cells (section 2.2.10.8). Western blots were probed with BCL10 and MALT1 antibodies. The immunostaining with BCL10 and C-MALT1 was carried out by Dr. Hongtao Ye.

5.4 Results

5.4.1 TLR6 (in the presence of TLR2), enhances BCL10 and API2-MALT1 mediated NF-κB activation in Jurkat T cells

A



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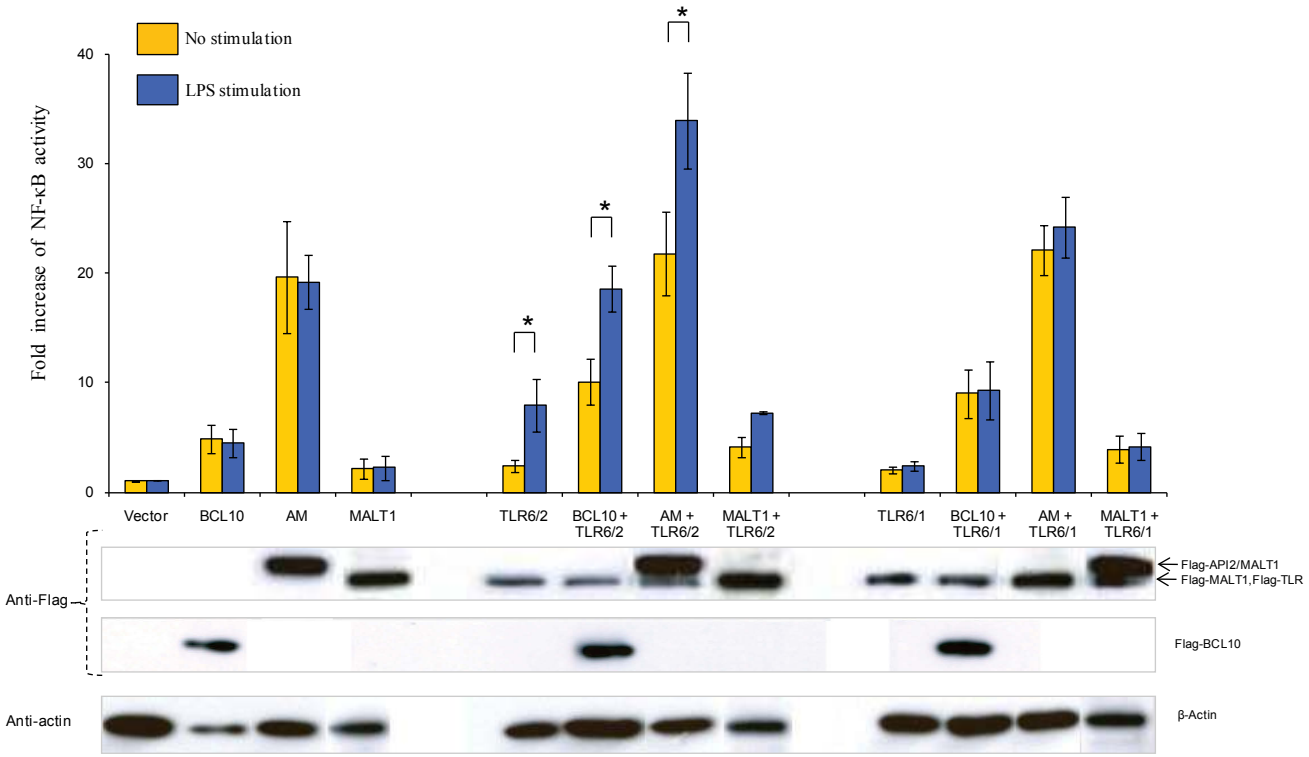


Figure 5.1 - TLR6 enhances BCL10 and API2-MALT1 mediated NF-κB activation, in the presence of TLR2 but not TLR1, in Jurkat T cells.
Jurkat T cells were co-transfected with vector (pIRESpuro2) or plasmids containing FLAG-tagged *BCL10*, *API2-MALT1* (AM), *MALT1, TLR6*, *TLR2* and *TLR1* as indicated, together with NF-κB luciferase reporter gene. (A) NF-κB luciferase reporter assay data for each oncogene alone and in combination with TLR6 or TLR2 with and without LPS stimulation.
(B) NF-κB luciferase reporter assay data for each oncogene alone and in combination with either TLR6/2 or TLR6/1 with and without LPS stimulation.
The transfected cells were seeded in multi-well plate, cultured for 18 hours and then treated with 10μg/ml LPS or vehicle alone for 6 hours. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blots in (B) show the appropriate expression of the various constructs. *P<0.01 by Student's t-test. Arrows show that *API2-MALT1*, *MALT1* and *TLRs* expression were detected using the anti-FLAG antibody with MALT1 and TLRs having approximately 90 kDa (which is the expected molecular weight) and API2-MALT1 have slightly higher molecular weight of approximately 125 kDa (which is the expected molecular weight).

Expression of *TLR1*, *TLR2*, *TLR6* alone was insufficient to induce NF-κB reporter activity (Figure 5.1 A). TLR6 alone and TLR2 alone, both failed to enhance BCL10 and API2-MALT1 mediated NF-κB activation in Jurkat cells even in the presence of LPS stimulation (Figure 5.1 A). However, the expression of both *TLR6* and *TLR2* significantly enhanced BCL10 (4.9 fold increase) and API2-MALT1 (19.6 fold increase), but not MALT1, mediated

NF-κB activation, and this effect was much potent in the presence of LPS stimulation. Co-expression of *TLR6/2* and *BCL10* or *API2-MALT1* appeared to be synergistic in NF-κB activation as shown by the reporter assay (Figure 5.1 B), with API2-MALT1 showing the highest synergistic effect (Figure 5.1 B). In contrast, there was no co-operation between co-expression of *TLR6/1* and MALT lymphoma associated oncogenes. These results are consistent with the previous finding that TLR6 typically forms a heterodimer with TLR2 in response to stimulation by bacterial antigens (Shimizu *et al.*, 2007; Takeuchi *et al.*, 2001).

5.4.2 Cooperation of BCL10, MALT1, API2-MALT1 and immune receptor signalling in NF-κB activation in B-cells

To further understand the potential co-operation between MALT lymphoma associated oncogenes and immunological stimulation in NF-κB activation, NF-κB reporter assays in B- and T- cell lines were performed in the presence of stimulation to TLR, antigen receptors and CD40.

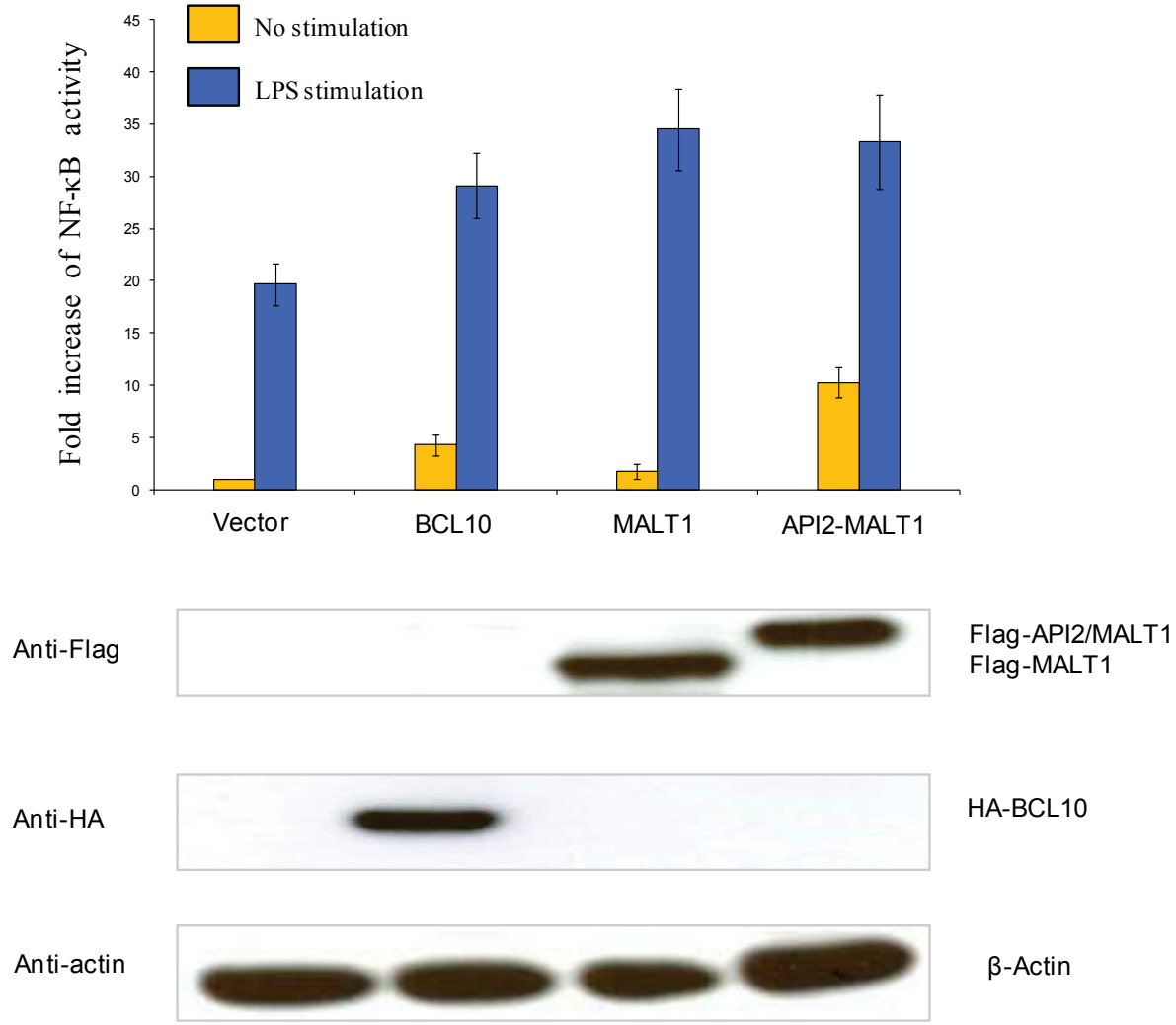


Figure 5.2. BCL10, MALT1 and API2-MALT1 mediated NF-κB activation, with and without LPS stimulation in BaF3 murine B-cells.
BaF3 B-cells were co-transfected with vector (pIRESpuro2) or plasmids containing HA-tagged *BCL10*, FLAG-tagged *MALT1* and *API2-MALT1* (AM) as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multi-well plate, cultured for 18 hours and then treated with 10μg/ml LPS or vehicle alone for 6 hours. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blot in the lower panel shows appropriate expression of the various constructs.

In BaF3 B-cells, expression of each of the three MALT lymphoma associated oncogenes yielded moderate increase of NF-κB activity with API2-MALT1 showing the greatest NF-κB activity in comparison with vector control (Figure 5.2). LPS stimulation of BaF3 B-cells transfected with the *pIRES* control vector produced a mean of 19.7s fold increase of NF-κB activity over un-stimulated cells (Figure 5.2). The expression of the three oncogenes in BaF3 cells was comparable as shown in the Western blots (Figure 5.2). Thus, it can be concluded that the expression of each of the three oncogenes enhanced the LPS mediated activation of NF-κB in BaF3 B-cells in a synergistic manner with fold increase of 29.1, 34.5, and 33.3 for BCL10, MALT1 and API2-MALT1 respectively (Figure 5.2). Similar results were also seen in WEHI cells (Figure 5.3).

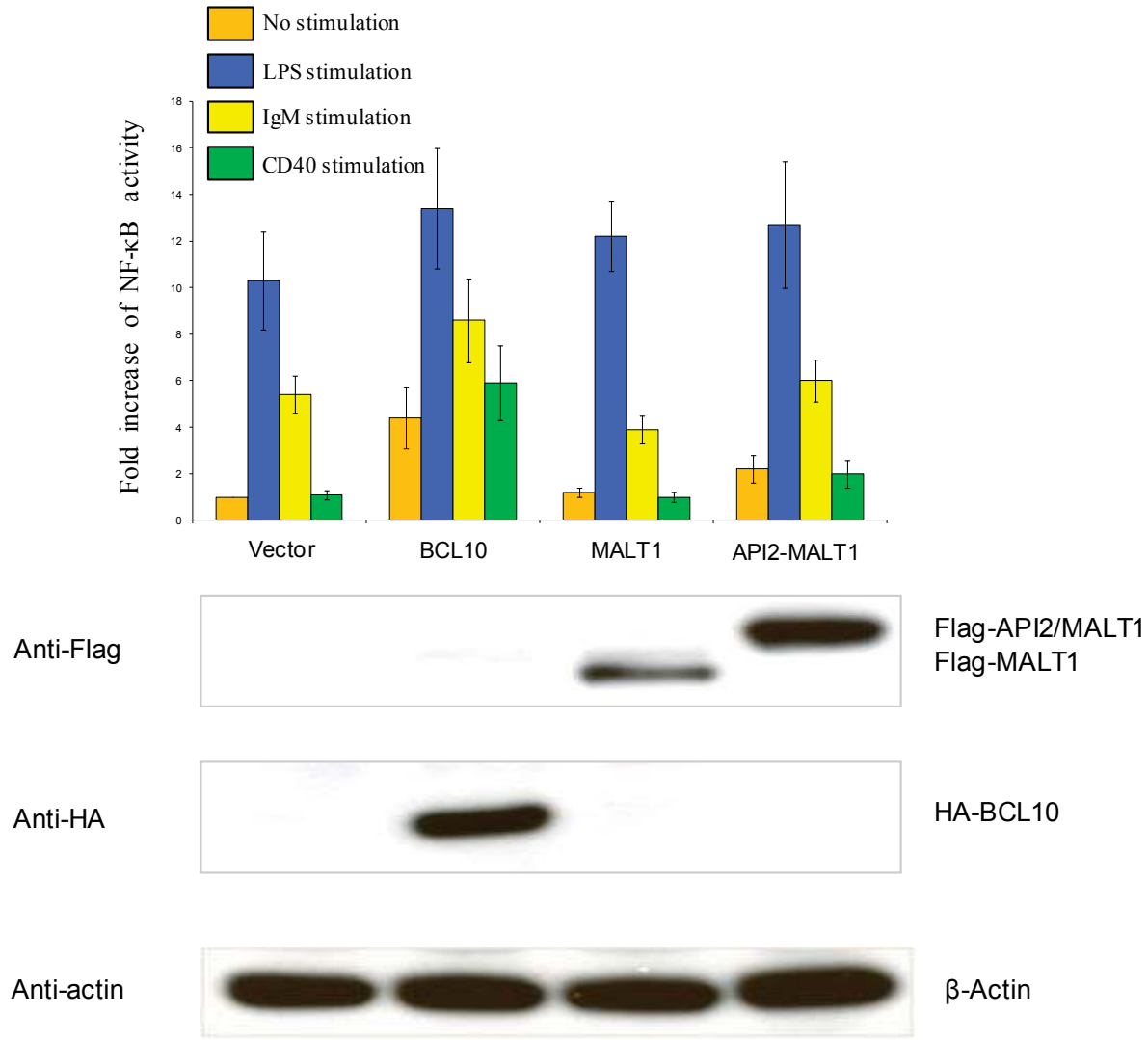


Figure 5.3 - BCL10, MALT1 and API2-MALT1 mediated NF-κB activation, with and without 6 hours of LPS, anti-IgM and CD40-L stimulation in WEHI murine B-cells.
WEHI B-cells were co-transfected with vector (pIRESpuro2) or plasmids containing HA-tagged *BCL10*, FLAG-tagged *MALT1* and *API2-MALT1* (AM) as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multi-well plates, cultured for 18 hours and then treated with 10μg/ml LPS, 0.1μg/ml CD40 ligand (with 1μg/ml CD40 enhancer), 10μg/ml anti-IgM or vehicle alone for 6 hours where indicated. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blot in the lower panel shows appropriate expression of the various constructs.

The expression of the three oncogenes in WEHI cells was comparable as shown in the Western blots (Figure 5.3). However, unlike LPS stimulation, antigen receptor stimulation by anti-IgM showed variable co-operation with the expression of MALT lymphoma associated oncogenes in NF-κB activation in WEHI cells (Figure 5.3). *BCL10* expression slightly enhanced anti-IgM mediated NF-κB activation in an additive manner (Figure 5.3). However

both *MALT1* and *API2-MALT1* expression failed to enhance anti-IgM mediated NF-κB activation. Similarly, stimulation using CD40L enhanced NF-κB activation mediated by the expression of *BCL10* (but not *MALT1* or *API2-MALT1*) in an additive manner (Figure 5.3).

5.4.3 Cooperation between BCL10, MALT1, API2-MALT1 immune receptor signalling on NF-κB activation in Jurkat T cells

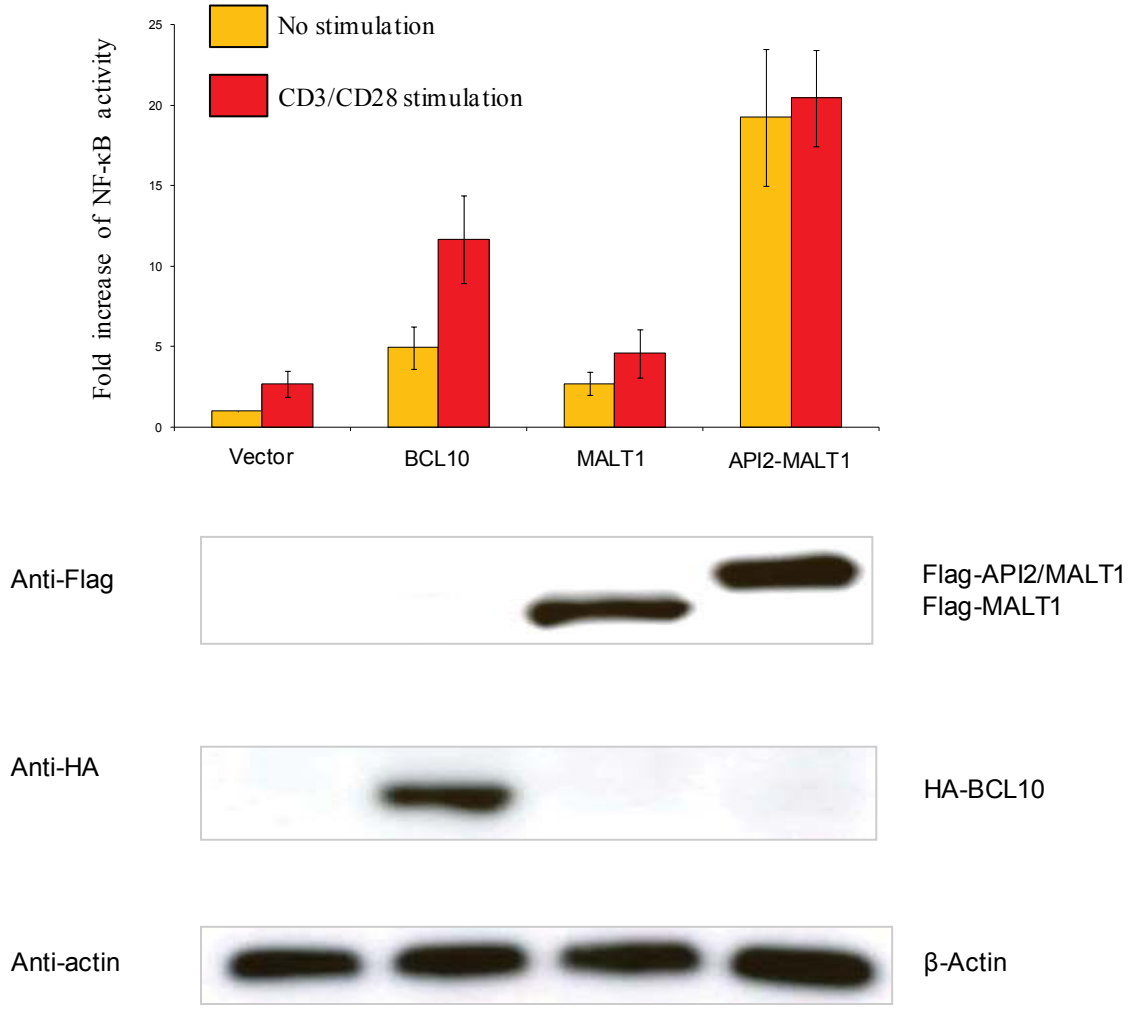


Figure 5.4 - BCL10, MALT1 and API2-MALT1 mediated NF-κB activation, with and without 6 hours of CD3/CD28 stimulation in Jurkat T cells.
Jurkat T cells were co-transfected with vector (pIRESpuro2) or plasmids containing HA-tagged *BCL10*, FLAG-tagged *MALT1* and *API2-MALT1* (AM) as indicated, together with NF-κB luciferase reporter gene. The transfected cells were seeded in multi-well plates, cultured for 18 hours and then treated with 1μg/ml anti-CD3 and 1μg/ml anti-CD28 or vehicle alone for 6 hours where indicated. NF-κB activities were measured in triplicate experiments and recorded as fold increase of the vector control. Western blot in the lower panel shows appropriate expression of the various constructs.

The expression of the three oncogenes in Jurkat T cells was comparable as shown in the Western blots (Figure 5.4). Thus it can be concluded that the expression of *BCL10*, *MALT1* and *API2-MALT1* alone showed 5.0, 2.7 and 19.2 fold increase of NF-κB activity respectively in Jurkat T cells (Figure 5.4). Co-stimulation of CD3 and CD28 produced a mean of 2.7 fold increase of NF-κB activity over un-stimulated cells (Figure 5.4). In comparison with the control, CD3/CD28 co-stimulation clearly enhanced BCL10 but not MALT1 or API2-MALT1 mediated NF-κB activation (Figure 5.4).

5.4.4 Investigation of the effect of BCL10 and MALT1 expression on their sub-cellular localisation

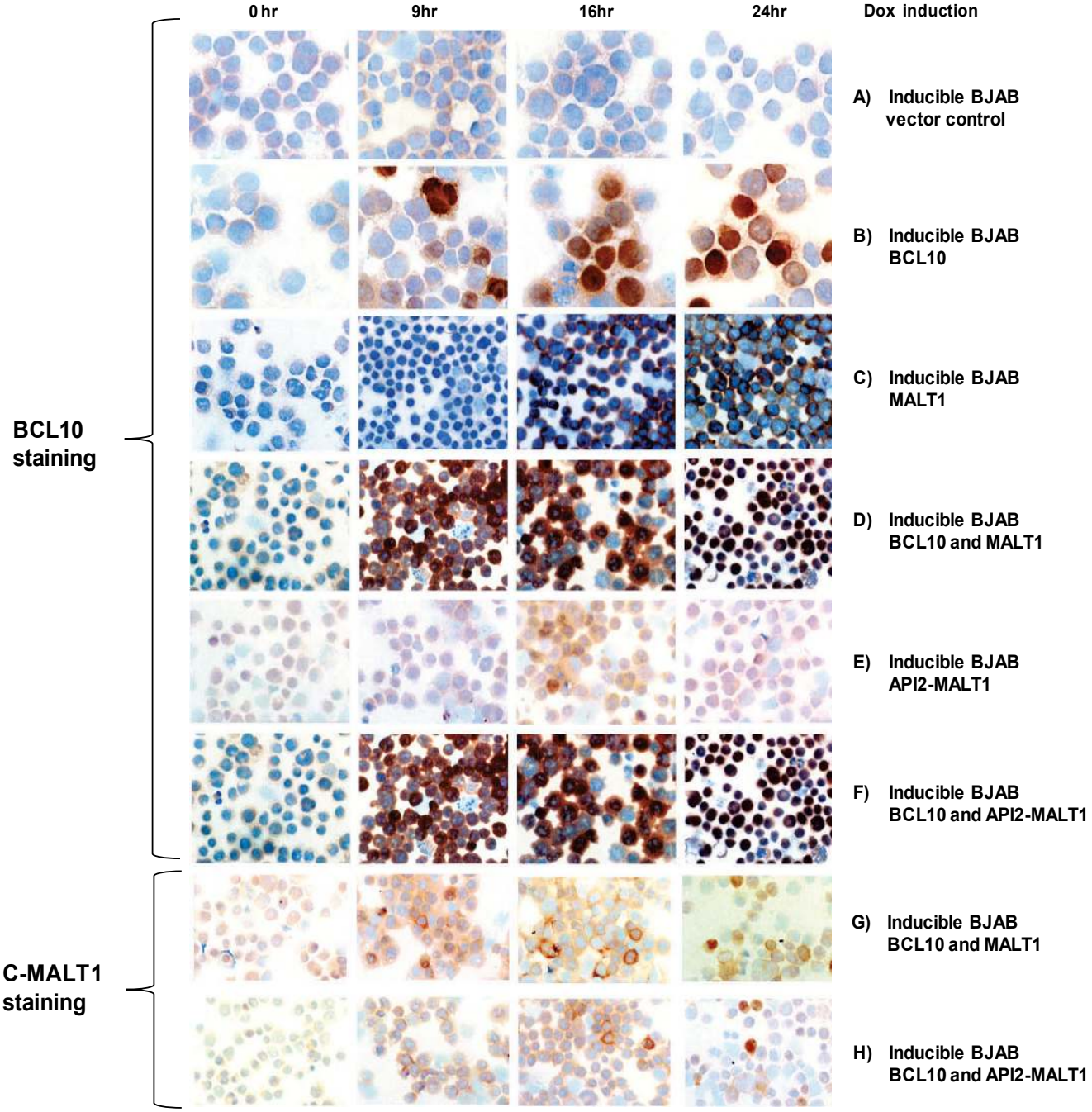


Figure 5.5 - Immunohistochemistry on cell clots expressing various MALT lymphoma associated oncogenes.
BJAB B-cell lines that express various inducible MALT lymphoma associated oncogenes were generated and used for investigation of their subcellular localisation. The expression of MALT lymphoma associated oncogenes were induced by doxycycline (Dox) and cells were harvested at 9, 16 and 24 hours followed by preparation of cell clots and immunocytochmistry with antibodies against BCL10 or C-terminal MALT1 where indicated. For each cell line, a control of cells without doxycycline treatment was used as reference.

BJAB cells transfected with vector only showed weak cytoplasmic BCL10 and MALT1 expression (Figure 5.5 A). In contrast, BJAB cells transfected with inducible BCL10 expression construct showed both strong nuclear and cytoplasmic expression in a proportion (10 - 15%) of cells after 9 hours induction by doxycycline, which increased to around 60% of cells at 24 hours induction (Figure 5.5 B). Interestingly, in BJAB with inducible MALT1 expression, immunostaining showed strong BCL10 in the cytoplasm, suggesting that MALT1 resulted in BCL10 accumulation and retainment in the cytoplasm (Figure 5.5 C). In BJAB cells with both BCL10 and MALT1 inducible expression, BCL10 was largely in the cytoplasm at 9 and 16 hours, but was predominantly in the nuclei at 24 hours after doxycycline induction (Figure 5.5 G and D). In contrast, co-expression of API2-MALT1 did not have any apparent effect on BCL10 sub-cellular localisation (Figure 5.5 E, F and H). Based on the co-localisation of BCL10 and MALT1 upon doxycycline induction, it can be hypothesised that these two molecules may interact. To investigate this further, co-immunoprecipitation investigating the interaction of BCL10 with MALT1 was carried out next.

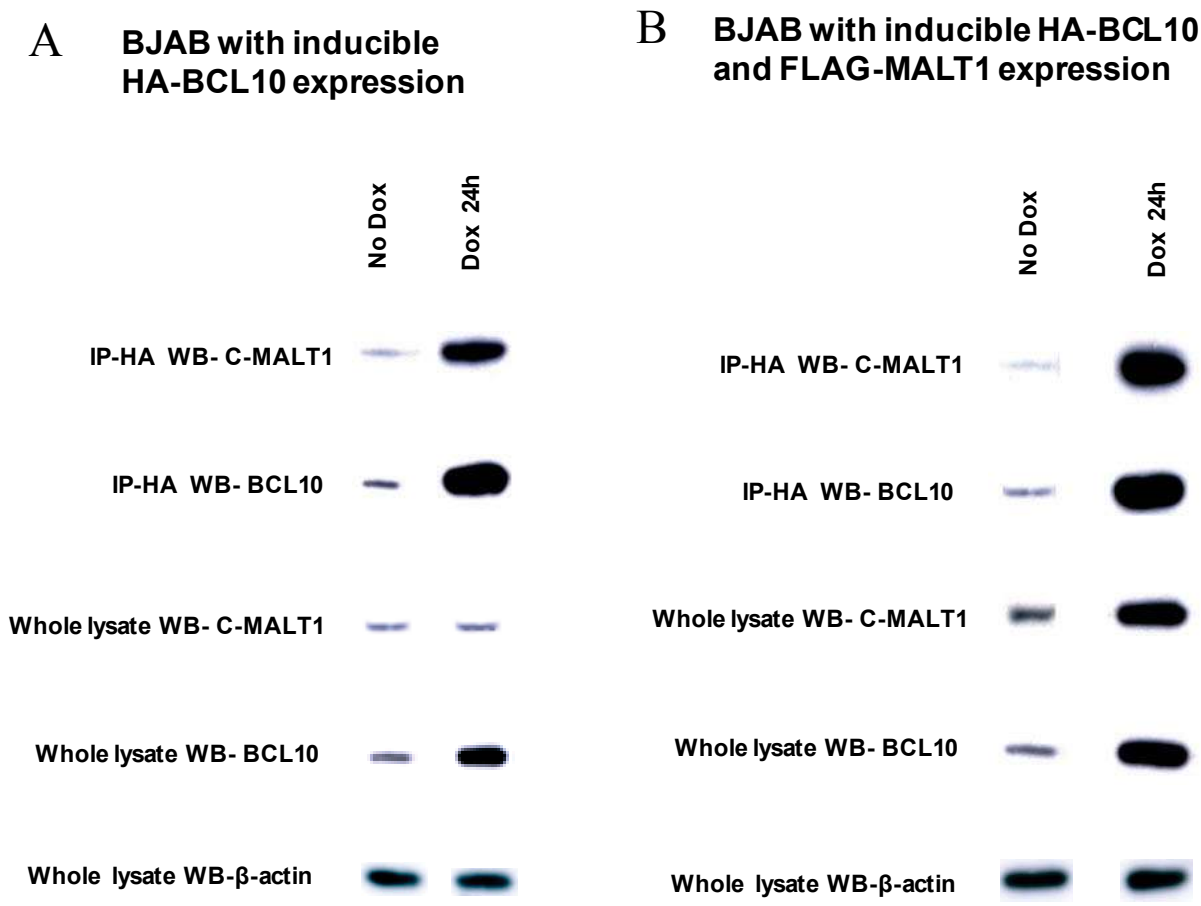


Figure 5.6 - Co-immunoprecipitation showing that expression of BCL10 leads to increased interaction with MALT1.
BJAB B-cells expressing inducible HA-tagged BCL10 or both HA-tagged BCL10 and FLAG-tagged MALT1 were treated with doxycycline (dox) for 24 hours. The protein extracts were subjected to co-immunopreciptation with HA antibody. Both co-immunoprecipitated products and whole cell lysates were analysed by Western blotting with antibodies against BCL10 or C-MALT1 and β-actin where indicated.

It is known that BCL10 interacts with MALT1 and mediates MALT1 oligomerisation in response to upstream signalling. In line with this, co-immunoprecipitation experiments showed that BCL10 was capable of interacting with both endogenous and exogenous MALT1 (Figure 5.6 A and B). It is most likely that through such direct interaction, MALT1 affects the subcellular localization of BCL10 especially in the cytoplasm where over-expression of BCL10 leads to strong nuclear and cytoplasmic accumulation of BCL10 initially (Figure 5.5 B). In addition, subcellular localization data (Figure 5.5 D) and co-IP data (Figure 5.6 B)

showed more BCL10 in the presence of MALT1 indicating the possibility that increased MALT1-BCL10 interaction might lead to more BCL10 expression.

5.4.5 Effect of *BCL10*, *MALT1* and *API2-MALT1* expression on canonical NF-κB pathway activation

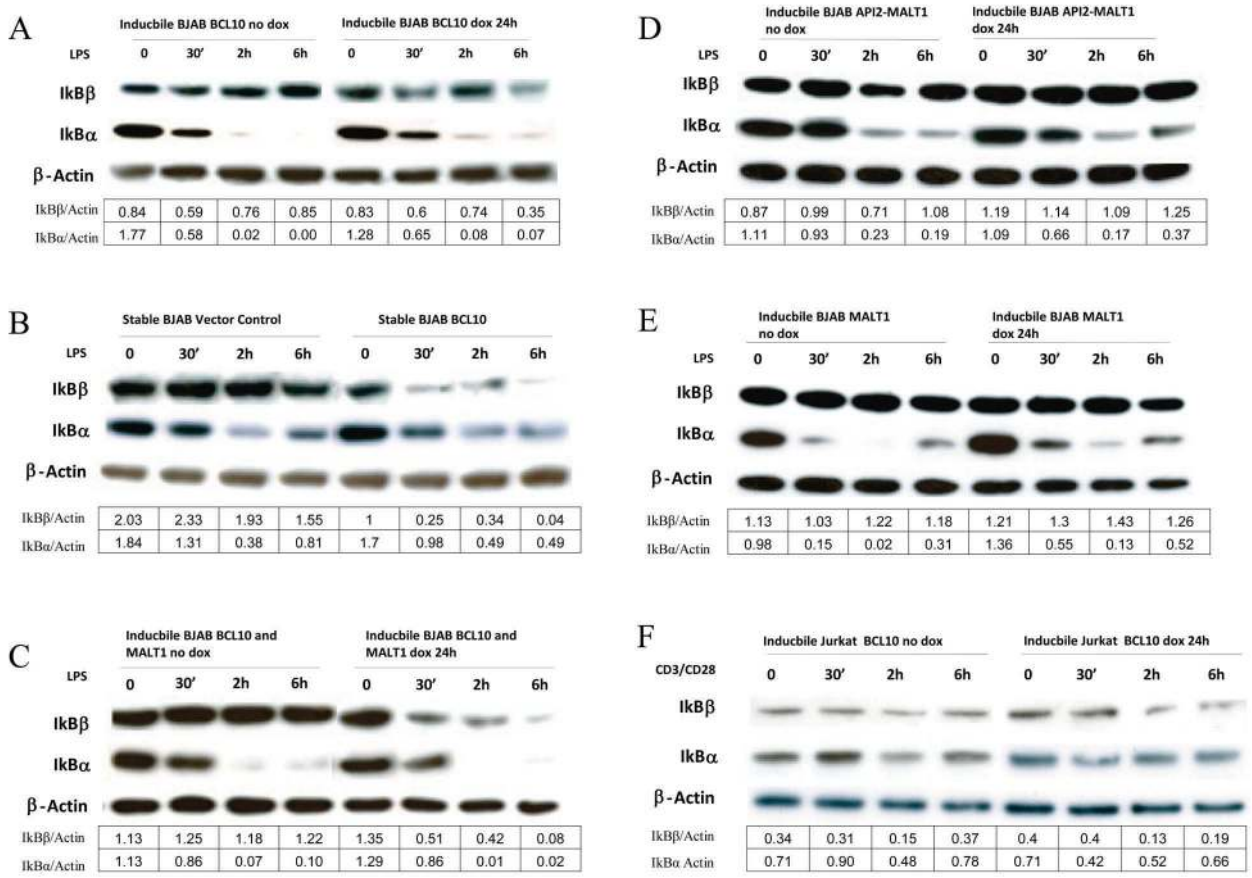


Figure 5.7 - Activation of canonical NF-κB pathway by BCL10, MALT1 and API2-MALT1 and association of IκBβ degradation with BCL10 expression.
Various BJAB B-cells and Jurkat T cells expressing inducible BCL10, MALT1, API2-MALT1 were subjected to doxycycline (dox) treatment for 18 hours, followed by stimulation with 10μg/ml LPS for BJAB and 1μg/ml anti-CD3/anti-CD28 for Jurkat for 30 minutes, 2, 6 hours where indicated. Protein extracts from each experiment were subjected to Western blotting with IκBα and IκBβ and β-actin as the loading control. The bands were quantified and the ratio of each over β-actin is calculated in the tables accompanying each figure.

The effect of over-expression of *BCL10*, *MALT1* and *API2-MALT1* on canonical NF-κB pathway was investigated using various BJAB B-cells and Jurkat T-cell lines that inducibly

or stably expressed these proteins. As expected, LPS stimulation, induced prominent IκBα degradation in both B- and T- cells (Figure 5.7 A-E). Very interestingly, expression of *BCL10* together with LPS stimulation, also caused IκBβ degradation in BJAB B-cells (Figure 5.7 A-C). IκBβ degradation was strongest with stable BJAB *BCL10* cell lines (Figure 5.7 B), possibly due to the fact that the inducible cell lines have the Tet element which may slightly affect the expression of the transgene.

However, neither LPS stimulation alone nor its combination with *MALT1* or *API2-MALT1* expression induced IκBβ degradation (Figure 5.7 D and E). In Jurkat T cells, expression of *BCL10*, together with CD3/CD28 stimulation, also induced IκBβ degradation (Figure 5.7 F). All other regulators of the canonical NF-κB pathway including the NF-κB negative regulators, IκBε; 3 central molecules, IKKα, IKKβ and NEMO; and 5 NF-κB subunits including c-Rel, RelB, p65, p50/p105 and p52/p100 did not show any significant difference among the three MALT lymphoma associated oncogenes when they were over-expressed (data not shown).

Similar experiments were carried out with anti-IgM stimulation in BJAB cells but no IκBβ degradation was seen in presence or absence of expression of the above MALT lymphoma associated oncogenes (data not shown).

Taken together, these results suggest that under these conditions, expression of *BCL10* may trigger the release of NF-κB dimer inactivated by IκBβ. In this context, BCL10 may play a more significant role than MALT1 or API2-MALT1.

5.5 Discussion

In this chapter, the potential cooperation between MALT lymphoma associated oncogenes and immune receptors stimulation in NF-κB activation was investigated and the common and distinct features of MALT lymphoma associated oncogenes in the activation of the canonical NF-κB pathway were explored.

5.5.1 Cooperation between the expression of MALT lymphoma associated oncogenes and TLR stimulation

Reporter assay results in Jurkat T cells showed a synergy between MALT lymphoma associated oncogenes, in particular BCL10 and API2-MALT1, and TLR6/2 expression on NF-κB activation upon LPS stimulation. Similarly, in BaF3 and WEHI B-cells, co-expression of TLR6/2 enhanced BCL10 and API2-MALT1 mediated NF-κB activation in the presence of LPS stimulation.

Based on the results of *in vitro* assays and expression microarray data, it is pertinent to speculate that TLR signalling may play a role in the pathogenesis of translocation positive MALT lymphoma. This is further supported by the following observations: 1) *H. pylori* activates NF-κB via both the classical and alternative pathway in B lymphocytes and this effect is dependent on LPS but not the Cag pathogenecity island (Ohmae *et al.*, 2005); and 2) *H. pylori* associated LPS induced NF-κB activation requires TLR2/TLR6 or TLR2/TLR1 complex but not those containing TLR4 (Yokota *et al.*, 2007), which typically recognises LPS from other Gram-negative bacteria (Akira *et al.*, 2004).

5.5.2 Effect of antigen receptor stimulation on MALT lymphomagenesis

The three recurrent translocations identified in MALT lymphoma all induce lymphomagenesis through constitutive activation of the NF-κB pathway (Zhou *et al.*, 2005). Functional analysis presented here showed that expression of BCL10, MALT1 and API2-MALT1 alone was capable of activating NF-κB in both B- and T- cells which are in line with the literature findings. In addition, the *in vitro* experiments showed that the expression of BCL10 enhanced the antigen receptor CD40 mediated NF-κB activation in B-cells. This finding is important in the context of MALT lymphoma as the lymphoma cells express functional BCRs and remain responsive to antigen, at least in the earlier stages of the disease (Hussell *et al.*, 1993b). For example, in an early study by Hussell *et al.,* gastric MALT lymphoma cells are stimulated to proliferate through CD40-CD40L interactions with T cells that recognise *H. pylori* antigens (Hussell *et al.*, 1993b). If these gastric MALT lymphoma cells already over-express BCL10, API2-MALT1 or MALT1, then the activation of NF-κB by CD40 stimulation will be most likely enhanced. In addition, the only case that responded to anti-idiotype antibody stimulation and showed enhanced MALT lymphoma cell proliferation was a t(1;14) positive case (Hussell *et al.*, 1993b) suggesting that a combination of high BCL10 protein expression and surface immune receptor stimulation may play an important role in MALT lymphoma development.

Recent study showed that CD40 signalling can enhance both API2-MALT1 and MALT1 mediated NF-κB activation in B-cells *in vitro* (Ho *et al.*, 2005), however the results in this thesis showed that out of the three MALT lymphoma oncogenes, only BCL10 over-expression moderately enhanced CD40 mediated NF-κB activation. This discrepancy might be due to the fact that the study by Ho *et al.* used a different strain of the BJAB cell line to

the one used in this thesis which had high constitutive NF-κB activity. Another possible explanation might be that the NF-κB activation was assessed indirectly by measuring the degradation of IκBα (which is transiently degraded) (Ho *et al.*, 2005) rather than by measuring the level of NF-κB transcriptional activity as done in the present study.

Taken together, it is tempting to speculate that the microbe mediated immune responses including help from T cells may also play a role in the pathogenesis of translocation positive MALT lymphoma. The potential involvement of TLR, CD40 and IgM signalling in translocation positive MALT lymphoma may explain the finding that rare cases of translocation positive gastric MALT lymphoma responded to *H. pylori* eradication (Liu *et al.*, 2002b; Wundisch *et al.*, 2005). The finding that API2-MALT1 and BCL10 mediated the strongest NF-κB activity is interesting considering the fact that both are highly expressed in translocation positive MALT lymphoma cases. Thus, the constitutive NF-κB activation seen in translocation positive MALT lymphoma may be, at least in part, due to preferential expression of the API2-MALT1 or BCL10 oncogenic products.

5.5.3 BCL10 expression associated with IκBβ degradation

All cell lines showed an intact IkBα mechanism, however unlike *MALT1* and *API2-MALT1*, the expression of *BCL10* also induced IκBβ degradation in the presence of CD3/CD28 and LPS stimulation in both Jurkat T- and BJAB B-cells respectively. IκBα and IκBβ interact with various NF-κB/Rel dimers with similar affinity, but recent studies demonstrated that they have slightly different mechanisms and operate through distinct properties. IκBα can shuttle NF-κB/Rel complexes in and out of the nucleus, whereas IκBβ is more efficient at sequestering NF-κB/Rel complexes in the cytoplasm. IκBβ causes cytoplasmic retention of NF-κB due to the masking of two NLSs on the NF-κB dimers. Interaction between the NF-

κB/IκBβ complex and the small guanosine triphosphatases κB-Ras-1, -2 also contributes to the NF-κB activation. When binding to κB-Ras, IκBβ cannot be phosphorylated by IKK, thus blocking the NF-κB activation signal from IKK (Fenwick *et al.*, 2000). The differential control between IκBα and IκBβ may lead to biphasic activation of NF-κB. IκBα is promptly upregulated upon NF-κB activation and thus controls the fast transient activation of NF-κB, whereas IκBβ controls the persistent activation of NF-κB (Ladner *et al.*, 2003). It can be speculated that BCL10 may affect the regulation of this biphasic activation of NF-κB, and thus lead to further NF-κB activation and perhaps the expression of a different set of NF-κB target genes.

5.5.4 BCL10 sub-cellular localisation and its relationship with MALT1 expression

The temporal interplay between API2-MALT1, MALT1 and BCL10 in cellular localisation *in vivo* showed that over-expression of BCL10 leads to the movement of excess BCL10 to the nucleus. This confirms the observation that tumour cells with t(1;14)(p22;q32)/*BCL10-IGH* show strong BCL10 nuclear expression (Liu *et al.*, 2001b), but interestingly the results also showed that over-expression of *BCL10* led to the presence of BCL10 in both the cytoplasmic and nuclear compartments. Over-expression of *MALT1* resulted in strong homogeneous cytoplasmic localisation of both *BCL10* and *MALT1*. But over-expression of API2-MALT1 had no effect on BCL10 localisation probably because *MALT1* expression is reduced by half due to the API2-MALT1 fusion, hence the expected reduced efficiency of the nuclear export of BCL10. In cells with t(1;14)(p22;q32)/*BCL10-IGH*, *MALT1* endogenous expression may not be sufficient for the export of over-expressed *BCL10*, resulting in BCL10 nuclear retention. Finally, in cells with t(14;18)(q32;q21)/*IGH-MALT1*, the over expression of *MALT1* results in an increased retention of BCL10 in the cytoplasm, mimicking the

phenomenon seen in t(14;18) MALT lymphomas, where both proteins are thus strongly expressed in the cytoplasm (Ye *et al.*, 2005). In addition, subcellular localization and co-IP data showed more BCL10 in the presence of MALT1 reflecting the possibility that increased MALT1-BCL10 interaction might lead to increase in BCL10 expression.

The phenomenon of nuclear BCL10 warrants further investigation, as it may have an unidentified role in the deregulation of NF-κB or other cellular pathways that may be linked to MALT lymphomagenesis. Also, BCL10 does not contain NLS, so the mechanism by which it moves to the nucleus remains to be investigated. Co-immunoprecipitation results showed that over-expression of BCL10 protein was able to interact with both endogenous and exogenous MALT1.

Taken together, it can be suggested that MALT1 and BCL10 may play an important role in the generation of the CBM complex, facilitating the initial surface receptors' signal transduction to NF-κB. Following stimulation of surface receptors such as LPS, IgM or CD40 in B- cells or CD3/CD28 in T- cells, over-expression of BCL10 also leads to the degradation of IκBβ, facilitating the activation of NF-κB pathway. Thus, BCL10 probably plays a dual role in the NF-κB pathway by transducing the signals from B- and T- cells to the NF-κB subunits as well as facilitating NF-κB activation partly via IkBβ degradation.

In addition to the mechanistic details of how MALT lymphoma associated oncogenes affects NF-κB activation, the data also showed the heterogeneity of MALT lymphoma in that translocation positive MALT lymphoma has four recurrent translocations, each having different mechanisms of NF-κB activation. Also, other translocation positive MALT lymphoma rearrangements were recently found such as t(1;14)(p22;q32)/CNN3-IGH, t(5;14)(q34;q32)/ODZ2-IGH and t(9;14)(p24;q32)/JMJD2C-IGH (Vinatzer *et al.*, 2008) which so far do not seem to be directly affecting the NF-κB pathway. Some of the

translocation negative MALT lymphomas have recently been shown to be associated with negative regulators of NF-κB such as A20 deletion (Chanudet *et al.*, 2010; Kato *et al.*, 2009). Taken together, it can be concluded that at the molecular level, different molecules and pathways play a role in the pathogenesis of MALT lymphoma. Thus it would be useful to use the expression microarray data to identify putative phenotypic markers that are specifically highly expressed in both translocation positive and negative MALT lymphoma. This will aid in the diagnosis and prognosis of MALT lymphoma and may provide further insights into the molecular pathogenesis of MALT lymphoma.

CHAPTER 6 – Identification of MALT lymphoma specific phenotypic markers using gene expression microarray

6.1 Introduction

Currently, there is no MALT lymphoma specific phenotypic marker. The differential diagnosis of MALT lymphoma includes the reactive inflammatory processes that typically precede the lymphoma including *H. pylori* gastritis, lymphoepithelial sialadentis, Hashimoto thyroiditis and other small B-cell lymphomas such as follicular lymphoma, mantle cell lymphoma and small lymphocytic lymphoma. Distinction from reactive processes is based mainly on the presence of destructive infiltrates of extra-follicular B cells, typically with the morphology of marginal zone cells (Wotherspoon *et al.*, 1993). Immunophenotyping and molecular genetics analysis is used in borderline cases to assess B-cell clonality to help establish or exclude a diagnosis of MALT lymphoma, however molecular analysis may also demonstrate clonal B cells in some non-neoplastic MALT proliferations or persistant clonal population in gastric MALT lymphomas even after histologic complete remissions (Wundisch *et al.*, 2003). Distinction from other small B-cell lymphomas is based on a combination of the characteristic morphologic and immunophenotypic features such as the presence of a diffuse infiltrate of $CD20^{+}$, IgM^{+}, IgD^{-} B cells beyond the reactive follicles outside the mantle zone. Once the marginal zone phenotype is established, light chain restriction in this marginal zone population or if present, within the plasma cells, would define the diagnosis. Thus, MALT lymphoma diagnosis is rather difficult as well as prone to errors (Pongpruttipan *et al.*, 2007) and having a marker specific for MALT lymphoma would

lead to more accurate MALT lymphoma diagnosis and would help both haemtopathologists and non-haemtopathologists to make a correct diagnosis.

6.2 Aims of the study

1) To identify genes highly and specifically expressed in MALT lymphoma by comparing the gene expression microarray data of MALT lymphoma with those of FL, MCL, SMZL and CLL;
2) To validate the putative phenotypic markers identified by the gene expression microarray study in a large cohort of FFPE MALT lymphomas, FL, MCL and SMZL specimens using qRT-PCR and immunohistochemistry.

6.3 Experimental design

6.3.1 Case selection

A total of 77 cases from five different lymphoma subtypes with expression microarray data were included in this study. These were composed of 26 cases of MALT lymphomas (21 from stomach, 3 from lung, 1 from ocular adnexa and 1 from liver) with Affymetrix HG-U133A/B GeneChips; 7 cases of nodal FL; 8 cases of nodal MCL; 14 cases of SMZL and 22 cases of CLL with Affymetrix HG-U133plus2 GeneChip. The CLL expression microarrays were from a previous study carried out by Calin *et al.* (Calin *et al.*, 2008), while all the other expression microarray data were obtained as part of this study.

6.3.2 Identification of genes highly and specifically expressed in MALT lymphoma by gene expression microarray analysis

After normalisation using RMA, the MALT lymphoma, SMZL, CLL, FL and MCL gene expression microarrays were subjected to the following two different analyses: Firstly, One-way ANOVA with Bonferroni multiple testing correction using Genespring GX 7.3.1 and secondly, Baysian statistics using in house programs written in R (version 2.8.0). Genes that had $p < 0.05$ were considered significant. Probes that were common in the two analyses were further filtered using SOM and Volcano plot and finally narrowed down using fold change calculation on the raw expression data as well as literature search of the genes preliminarily identified. Supervised clustering was carried out using Pearson correlation and average linkage as the similarity measure and clustering algorithm respectively within Genespring GX 7.3.1 (Figure 6.1).

6.3.3 Validation of genes highly expressed in MALT lymphoma by qRT-PCR and immunohistochemistry

The genes that were found to be specifically expressed in MALT lymphoma were validated by qRT-PCR and immunohistochemistry. mRNA expression was measured using qRT-PCR on a total of 79 cases of lymphoma including 44 MALT lymphomas (38 from stomach, 4 from lung, 1 from ocular adnexa and 1 from liver); 13 nodal FL; 11 nodal MCL and 11 SMZL. No CLL samples were available thus no CLL cases were included in the downstream validation. 18s rRNA was used as the housekeeping gene. RNA was extracted from microdissected tumour cells of FFPE tissue specimens of MALT lymphoma cases and qRT-PCR was carried out as described in Chapter 2. Where possible, primer pairs were designed to span exons to prevent any amplification of genomic DNA and to target up to 150bp (Table 3 in section 2.2.6), and were thus suitable for FFPE tissues. The results were presented as ΔC_t

values, so the higher the value, the lower the transcript expression, and *vice versa*. The Mann-Whitney U Test was used to determine whether there were statistically significant differences in the expression of a particular transcript between the various lymphoma groups. Immunohistochemistry using Lactoferrin antibody was carried out by Dr. Hongtao Ye on 5 gastric MALT lymphoma, 2 FL and 2 MCL specimens using 1:200 dilution of rabbit polyclonal antibody (product no. ab15811, Abcam) as based on the information given, seemed suited for immunohistochemistry on FFPE tissue.

6.4 Results

6.4.1 Identification of genes highly and specifically expressed in MALT lymphoma

The strategy used to combine HG-U133A&B microarray data of MALT lymphoma with HG-U133plus2 microarray data of FL, MCL, SMZL and CLL is summarised in Figure 6.1.

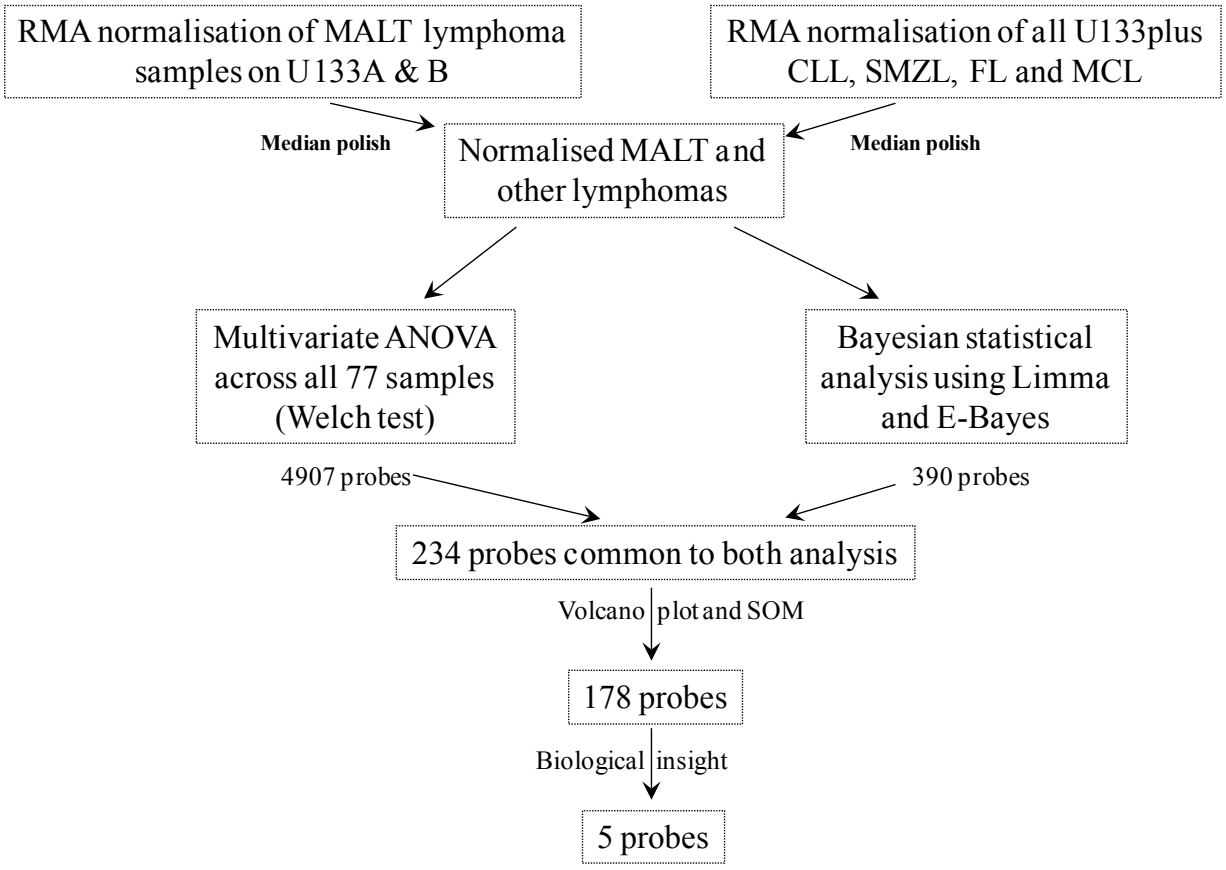
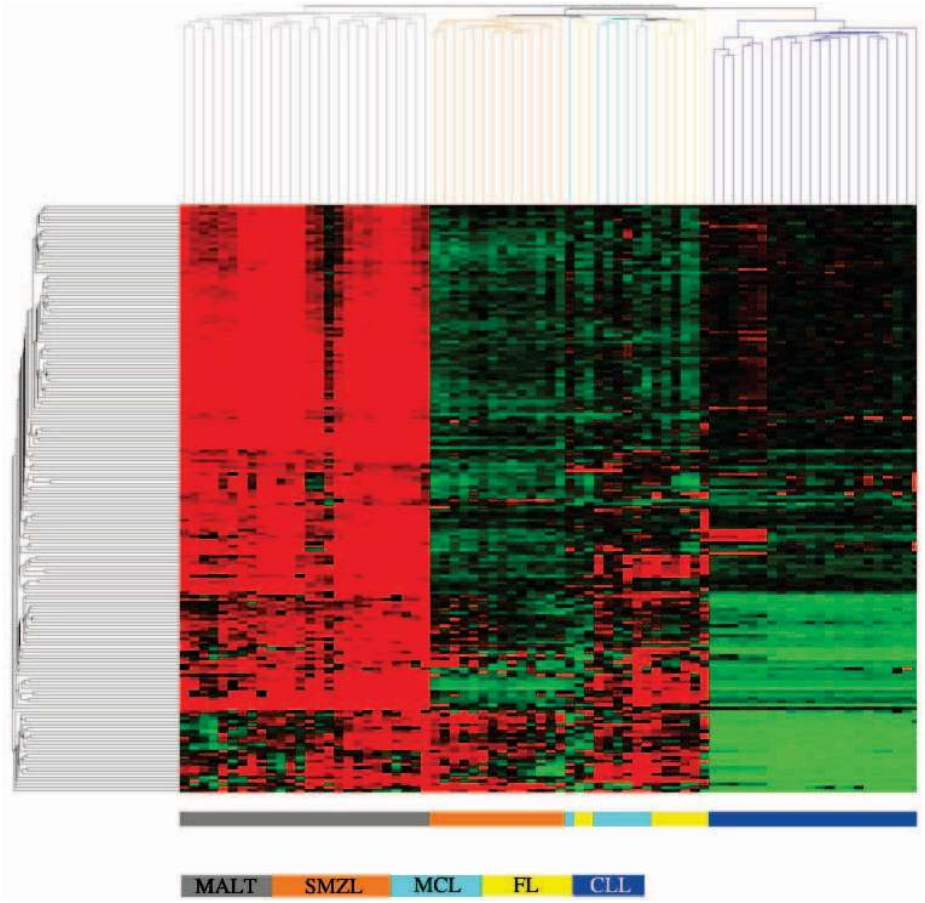


Figure 6.1 - Summary of bioinformatics strategy to combine MALT lymphoma, FL, MCL, SMZL and CLL microarray data and identify the genes highly and specifically expressed in MALT lymphoma.
Both the U133AB and U133plus2 sets were normalised using RMA and combined using median polish. The normalised set was then subjected to multivariate ANOVA and Bayesian statistics (using eBayes) analysis. Common probes from both analyses were reduced by subjecting them to self organising maps (SOM) and volcano plot analyses. The remaining probes were further reduced using biological knowledge and studies form the literature.

Multivariate ANOVA with Bonferroni's stringent multiple testing criterion yielded 4907 probes whereas Baysian statistical analysis (which tends to shrink the data and eliminate non-variant probes using model fitting) gave rise to 390 probes. The common set between the two analyses was 234 probes. SOM and Volcano plot analysis further filtered the set to 178 probes. The 178 probes were ranked according to their fold change between MALT lymphoma and the other lymphomas. The top 20 probes were selected and known genes that are expressed in non-neoplastic cells were excluded.

Using this approach, 5 probes were selected; 2 probes mapped to *Dermatopontin*, one to *Decorin*, one to *Tetraspanin 8* and one to *Lactoferrin*. Fold change, minimum and maximum raw expression signals and coefficient of variation were calculated for the 5 probes across the 26 MALT lymphoma and the 51 other lymphoma cases separately (Table 6.1). The descriptive statistics showed that *Lactoferrin* had the lowest CV (67%) and the highest average (1027.05) in the MALT lymphoma group indicating that *Lactoferrin* is expressed uniformly at higher level in the MALT lymphoma group (Table 6.1). Plot of the raw expression microarray *Lactoferrin* data confirmed that it is most highly expressed in MALT lymphoma group compared to the other 3 genes (Figure 6.1). This in addition to the fact that a literature search showed Lactoferrin to inhibit the immunostimulatory effect on human B cells (Britigan *et al.*, 2001) and might play a role in lymphocyte migration in lymphoid malignancy (de Sousa *et al.*, 1978) making it the most likely phenotypic marker and was used for downstream validation.

A



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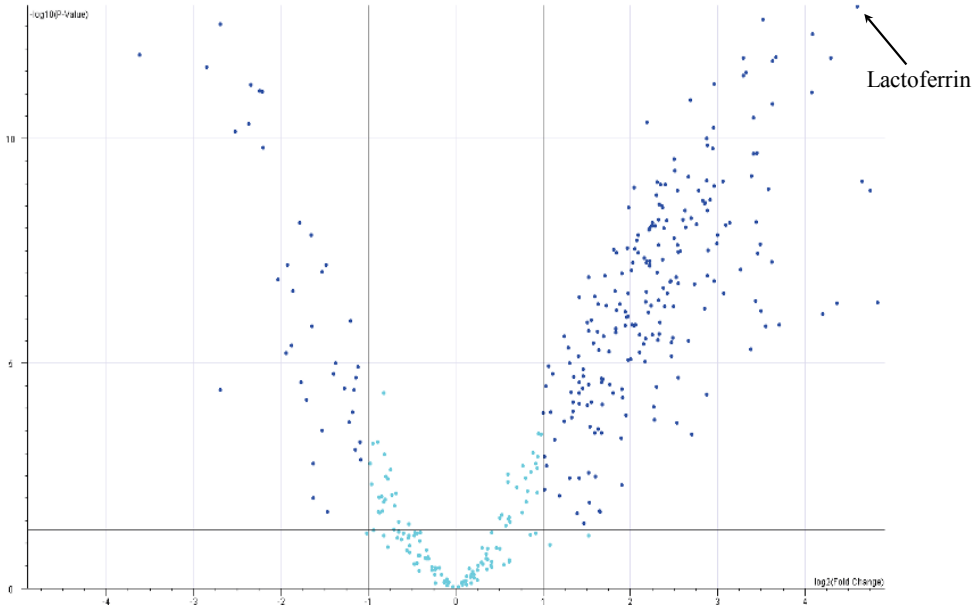


Figure 6.2 - (A) Supervised hierarchical clustering of MALT lymphoma, FL, MCL, SMZL and CLL (B) Volcano plot of MALT v other lymphomas with an arrow showing *Lactoferrin* position near the top.

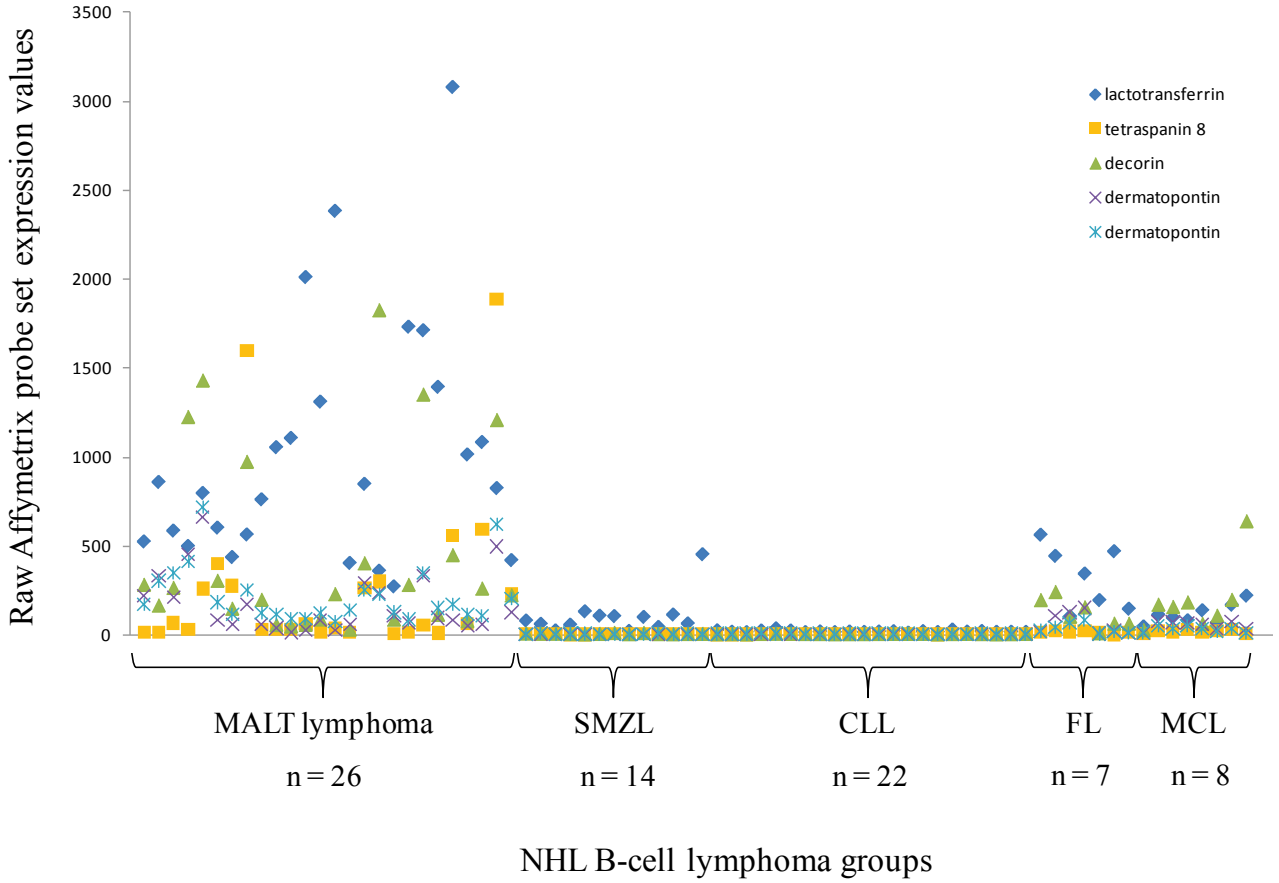


Figure 6.3 - Variation of the raw gene expression microarray data of *Lactoferrin* across the 5 lymphoma groups.
Five probes came in the final analysis two of them localised to the Dermatopontin gene. The groups used are; 26 MALT lymphomas, 14 SMZLs, 22 CLLs, 7 FLs and 8 MCLs.

Table 6.1 - Descriptive statistical properties of the 5 potential phenotypic marker probes calculated for MALT and other lymphomas separately.

Probe Name	Gene Name	Fold Change	MALT Average	MALT SD	MALT CV	MALT Max	MALT Min	mphomas Avera	Lymphomas SD	Lymphomas CV
202018_s_at	Lactoferrin	9.61	1027.05	683.09	0.67	3081.00	274.80	106.92	133.93	1.25
203824_at	Tetraspanin 8	23.59	266.72	468.33	1.76	1887.00	10.85	11.30	7.02	0.62
211896_s_at	Decorin	8.29	457.07	519.97	1.14	1829.00	34.28	55.13	105.75	1.92
213068_at	Dermatopontin	7.66	173.85	167.19	0.96	666.10	17.90	22.70	34.23	1.51
213071_at	Dermatopontin	13.00	220.28	161.25	0.73	718.10	77.23	16.94	17.33	1.02

6.4.2 Validation of *Lactoferrin* expression in MALT lymphoma

The expression of *Lactoferrin* in MALT lymphoma and other lymphoma subtypes was first compared using qRT-PCR on microdissected tumour cells from FFPE tissues. All the expression values of *Lactoferrin* in SMZL were negative, most likely due to tissue degradation and thus the SMZL data were not used in the analysis. *Lactoferrin* mRNA expression was the highest in the 44 MALT lymphomas group as compared to 11 MCL and 13 FL.

Although the range of *Lactoferrin* expression in MALT lymphoma was rather large, there was only small overlap between MALT lymphoma and FL or MCL. Not surprisingly, the Mann-Whitney U test showed a significant difference in the gene expression between MALT lymphoma and FL or MCL (Figure 6.4). Thus *Lactoferrin* could be a potential marker for MALT lymphoma.

To further validate this, immunohistochemistry was performed with Lactoferrin antibody (ab15811) on 5 gastric MALT lymphoma, 2 FL and 2 MCL. The preliminary immunohistochemical results showed high and scattered staining in MALT lymphoma compared to tonsils (Figure 6.5). The intensity and distribution of staining was similar between five cases of MALT lymphoma, FL and MCL (data not shown), probably because this anti-Lactoferrin antibody seem to detect inflammatory infiltrating tissue which can occur as a result of the lymphoma so it was a poor choice to use for this study. Thus, immunohistochemistry need to be repeated with perhaps a different anti-Lactoferrin antibody such as the polyclonal rabbit anti-human (product no. A0061, DakoCytomation, Cambridge, UK) or the monoclonal mouse anti-human (Clone 1A1, product no. H86024M, Meridian Life Science, Maine, USA) where both were successfully used on clear cell carcinomas FFPE tissue (Giuffre *et al.*, 2007). Another antibody is the polyclonal rabbit anti-human anti-

Lactoferrin (product no. 07-685, Upstate (Millipore), Massachusetts, USA) which was successfully used on nasopharyngeal carcinoma tissue microarray (Zhou *et al.*, 2008). However, it has also been shown that Lactoferrin binding in B-lymphocytes may increase during certain stages of cell maturation (Butler *et al.*, 1990) and Lactoferrin is expressed in cells from the upper gastrointenstinal tract (possibly related to mucosal defence mechanisms) but has low background staining (Mason *et al.*, 1978), thus immunostaining with either of the two antibodies mentioned above need to be interpreted with caution.

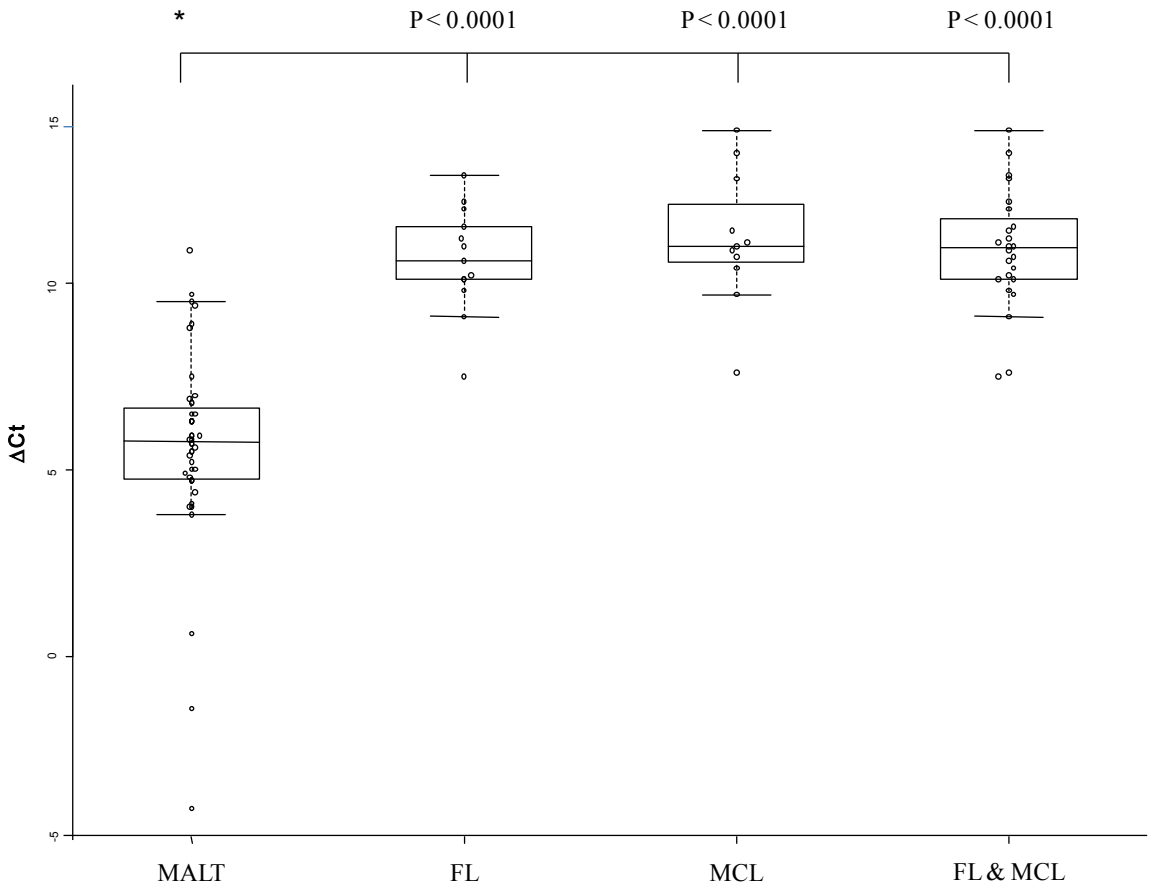
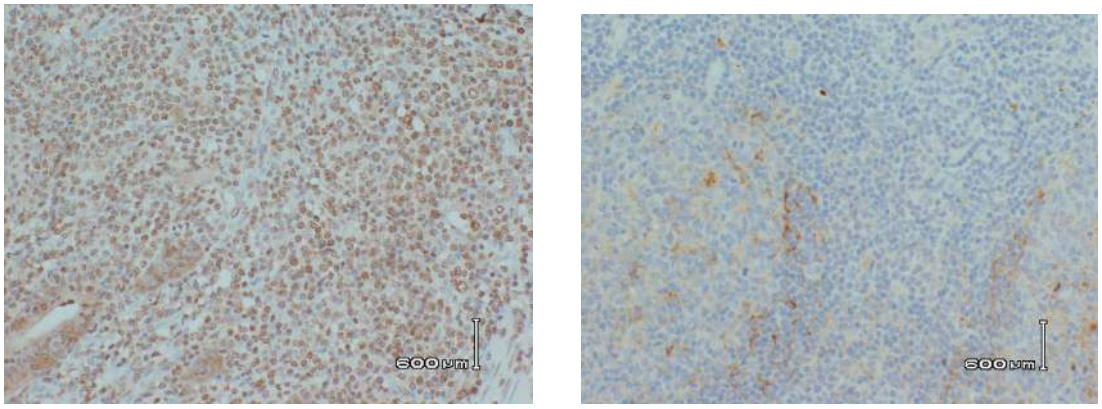


Figure 6.4 - Validation of *Lactoferrin* expression in 44 MALT, 13 FL and 11 MCL lymphomas by real-time quantitative RT-PCR.

This was performed in triplicate using RNA samples extracted from tumour cells microdissected from paraffin-embedded tissue sections. Asterisk indicates statistical significant differences between FL, MCL and combined FL with MCL and MALT lymphoma group by Mann-Whitney non-parametric statistical test. The medians are indicated by horizontal bars in the rectangular boxes. Error bars show the standard deviation of the results in each group. High values reflect low transcript expression and vice versa.



MALT lymphoma Tonsil

Figure 6.5 - Validation of Lactoferrin expression in MALT lymphomas by immunohistochemistry.

Summary of Lactoferrin immunohistochemistry showing high and scattered staining in MALT lymphoma compared to tonsils. The pattern of staining between MALT lymphoma, FL and MCL was similar.

6.5 Discussion

By analysis of gene expression profiles of MALT lymphoma, FL, MCL, SMZL and CLL, several genes including *Lactoferrin* were found to be highly expressed in MALT lymphoma whereas the other genes were not found to be highly expressed in MALT lymphoma (Figure 6.3). High expression of *Lactoferrin* in MALT lymphoma was also confirmed by qRT-PCR and awaiting further validation by immunohistochemistry.

Lactoferrin belongs to a family of iron-binding proteins that modulate iron metabolism, haematopoiesis and immunologic reactions. Lactoferrin is an iron binding glycoprotein with approximate weight of 78 kDa. It is present in secretary fluids of mammals and contained in secondary granules of neutrophils (Boxer *et al.*, 1982) that defends against microbial pathogens in innate immunity. It has been shown to be bacteriostatic and bactericidic against various infectious agents including *H. pylori* (Ellison, III, 1994). The specific receptor for Lactoferrin has been reported on several cell types including mitogen-stimulated human peripheral blood lymphocytes (Maneva *et al.*, 1983), macrophages (Maneva *et al.*, 1983), platelets and epithelial cells of human mammary gland (Bennett *et al.*, 1983). It is thought that receptor binding is the first step in cell functions related to Lactoferrin. For instance, interaction of Lactoferrin with cells of the immune system induces the release of cytokines (Van Snick *et al.*, 1976) and can protect mice against a lethal dose of *E. coli* in experimental infection (Van Snick *et al.*, 1976). Lactoferrin is also capable of promoting the proliferation of phytohaemagglutinin-stimulated human peripheral blood lymphocytes (Maneva *et al.*, 1983), as well as both human B- and T- cell lines (Dagg *et al.*, 1981).

Unmethylated CpG dinucleotide motifs in bacterial DNA, as well as oligodeoxynucleotide (ODN) containing these motifs, are potent stimuli for many host immunological responses. Lactoferrin has been shown to inhibit CpG (ODN) stimulation of CD86 expression in the

human Ramos B-cell line leading to a decrease in the cellular uptake of ODN, a process required for CpG bioactivity (Britigan *et al.*, 2001). Lactoferrin binding of CpG-containing ODN may serve to modulate and terminate host response to immunostimulatory molecules such as Heparin and LPS at mucosal surfaces and sites of bacterial infection (Britigan *et al.*, 2001).

Lactoferrin has also been shown to play a role in the control of lymphoid cell migration (de Sousa *et al.*, 1978) perhaps via the control of chemokines and cytokine release. Thus, Lactoferrin plays an important role in B-cell physiology.

Mounting evidence from the literature suggest that there is a link between increase in Lactoferrin expression and *H. pylori* infection and gastric inflammation. Microarray analysis of antral biopsies from patients with and without *H. pylori* infection showed *Lactoferrin* expression to be 4.2 folds higher in patients with *H. pylori* infection versus normal controls (Mannick *et al.*, 2004). Microarray and qRT-PCR analysis showed *Lactoferrin* expression was upregulated in gastric biopsies with *H. pylori* infection with 19.4 fold change in *H. pylori* positive compared to negative biopsies by qRT-PCR (Wen *et al.*, 2004). In a similar study, *Lactoferrin* expression had 14.6 and 16.8 fold induction in *H. pylori* positive compared to negative biopsies using microarray and qRT-PCR respectively (Wen *et al.*, 2007). Molecular analysis of *H. pylori* associated gastric inflammation in chronically infected and immune mice, showed Lactoferrin to be expressed 38 fold higher in *H. pylori* infected mice compared to naïve animals (Rahn, 2003). In addition, a study by Zou et al showed that supplementation with Lactoferrin could be effective in increasing eradication rates of anti-*H. pylori* therapy, and could be helpful for patients with *H. pylori* eradication failure (Zou *et al.*, 2009) and Hirata et al. showed that faecal Lactoferrin levels were elevated and this was

found to be useful in the detection of colorectal diseases including MALT lymphoma (Hirata *et al.*, 2007).

Analyses of B-cell malignancies comprising cells representing different maturational stages showed variable Lactoferrin expression (Butler *et al.*, 1990). Acute lymphoblastic leukaemia (ALL) derived from progenitor B cells, and hairy cell leukaemia (HCL) derived from late activated memory B cells do not express surface Lactoferrin. In addition, EBV-transformed B-cell lines, representing activated B cells, were virtually negative for Lactoferrin. The same study showed that CLL had the highest percentage of surface Latoferrin positivity (Butler *et al.*, 1990). Microarray analysis in this thesis showed that mRNA expression of Lactoferrin in CLL is minimal. However the CLL microarray data were obtained from a study by Calin *et al.* (Calin *et al.*, 2008) in public domain and no CLL tissue was available, thus it was not possible to confirm this finding by qRT-PCR as was the case with FL and MCL. If the qRT-PCR results confirm the microarray data, it can be hypothesised that perhaps the binding (but not the expression) of Lactoferrin to B lymphocytes might increase during certain stages of cell maturation. This might slightly reduce the use of Lactoferrin as a potential phenotypic marker for MALT lymphoma, however the diagnosis of MALT lymphoma is not usually confused with CLL/SLL, as the latter typically is $CD5^+$ and $CD23^+$. Furthermore, the disease usually manifests as CLL (rather than SLL) with an associated with lymphocytosis at presentation, a feature not associated with MALT lymphoma. Attempts of Lactoferrin immunohistochemistry with commercial antibody failed to yield any conclusive results and validation of Lactoferrin expression in MALT lymphoma remains to be investigated.

CHAPTER 7 – GENERAL DISCUSSION

7.1 MALT lymphoma is a distinct entity within the lymphomas but with heterogeneity between MALT lymphoma with and without chromosome translocation as indicated by gene expression profiling

Gene expression microarray results showed that MALT lymphoma is an entity distinct from other lymphoma subtypes, FL (thought to originate from germinal centre B cells) and MCL (thought to originate from the mantle zone B cells) (Kuppers, 2005). Significantly, MALT lymphomas expressed at high levels, many of the genes related to response to external biotic stimulus. On the other hand, in FL, immune response related genes were highly expressed, and in MCL, cell cycle related genes were highly expressed.

Unsupervised clustering between translocation positive and negative MALT lymphomas showed no clear separation between these cases indicating overlap in the molecular mechanism between the two groups. In all unsupervised clustering analyses, translocation negative MALT lymphomas with BCL10 nuclear staining tended to cluster with translocation positive cases suggesting that such cases at the molecular level resemble more to those with chromosome translocation.

7.2 Overview of the molecular mechanisms underlying the pathogenesis of MALT lymphoma

Although unsupervised clustering analyses showed considerable overlap in the gene expression profiles between MALT lymphomas with and without chromosome translocation, there was a significant difference in the expression of NF-κB target genes between the two subgroups. Exhaustive and systematic GSEA of various molecular pathways and biological processes, showed that gene sets related to inflammation, immune responses, chemokine and

GPR signalling, were differentially over-represented between these different subgroups. Importantly, several of these molecular pathways or biological processes also lead to NF-κB activation. These findings were also reinforced by independent analyses of differentially expressed genes between MALT lymphomas with and without translocation using hypergeometric tests. These observations provide several novel insights into the molecular mechanisms of both translocation-positive and negative MALT lymphomas which potentially explain their different clinical and histological presentations.

7.2.1 Aberrant molecular mechanisms of translocation positive MALT lymphoma

GSEA and leading edge analyses revealed a common core subset of genes that were overexpressed in translocation positive cases and a high proportion of them were NF-κB target genes involving multiple related biological processes or molecular pathways.

Potentially critical immune receptors highly expressed in translocation positive MALT lymphoma were TLR6, CD40, CD83, CD1D and CD69 as shown by GSEA of gene expression microarray study in this thesis.

TLR6 typically forms heterodimers with TLR2 on the cell surface to recognize bacterial antigens (Gomariz *et al.*, 2007). TLR2/TLR6 signalling activates not only the IKK complex that leads to activation of the NF-κB transcriptional factor, but also the MAP kinase p38 and Jun amino-terminal kinase (JNK) that lead to activation of the AP-1 transcriptional factor (Akira *et al.*, 2004). Hence, over-expression of TLR6 in translocation-positive MALT lymphoma could potentially augment the NF-κB activity mediated by MALT lymphoma associated oncogenic products and also activate the MAP kinase pathways. Data from this thesis in Baf3 B cells and Jurkat T cells showed that expression of TLR6, in the presence of

TLR2, enhanced both BCL10 and API2-MALT1 mediated NF-κB activation. This effect was particularly significant upon LPS stimulation suggesting a potential biological cooperation between MALT lymphoma associated translocations and TLR signalling in lymphomagenesis. In addition, data from this thesis demonstrated that TLR6 with TLR2 enhanced API2-MALT1, BCL10 and MALT1 mediated AP-1 activation in BJAB B cells regardless of LPS stimulation.

The growth of gastric MALT lymphoma cells is critically dependent on their cognate interaction with *H. pylori* specific tumour infiltrating T cells, involving co-stimulatory molecules such as CD40/CD40L (D'Elios *et al.*, 2003; Hussell *et al.*, 1996). A recent study showed that CD40 signalling could enhance both API2-MALT1 and MALT1 mediated NF-κB activation in B cells (Ho *et al.*, 2005). In addition, previous studies have also indicated a role of B-cell receptor signalling in *H. pylori* induced MALT lymphomagensis (Craig *et al.*, 2010). In this thesis, the potential cooperation between expression of MALT lymphoma associated oncogenes and BCR and CD40 signalling were investigated. As expected, functional studies in WEHI B cells showed additive effect between CD40L stimulation and BCL10 in NF-κB activation. GSEA of the expression microarray data demonstrated that the expression of co-stimulating molecules CD40 and CD83 was enriched in translocation positive MALT lymphoma, further supporting their role in lymphomagenesis. In line with this hypothesis, the GSEA also revealed up-regulation of CD1D in translocation positive MALT lymphoma, which is involved in the presentation of microbial lipid and lipopeptide antigens to T cells (Brigl *et al.*, 2004). Thus, it is tempting to speculate that the microbe mediated immune responses including T cells may also play a role in the pathogenesis of translocation positive MALT lymphoma. The potential involvement of TLR, CD40 and CD83 signalling in translocation positive MALT lymphoma may explain the finding that rare

cases of translocation positive gastric MALT lymphoma respond to *H. pylori* eradication (Liu *et al.*, 2002b; Wundisch *et al.*, 2005).

CD69 is an early cell activation antigen expressed on the surface of activated immune cells by small subset of T and B cells in peripheral lymphoid organs (Hara *et al.*, 1986). Although the precise function of CD69 in B cells is largely unknown, it is a well-described activation marker in several cell types, and its expression is up-regulated in marginal zone B cells upon TLR stimulation (Rubtsov *et al.*, 2008). CD69 is frequently expressed in low-grade B-cell lymphomas, and in FL, its expression is associated with poor treatment outcome (de Jong *et al.*, 2009; Erlanson *et al.*, 1998). Also, it has been shown that CD69 functions downstream of IFNα and IFNβ, and possibly other activating stimuli, to promote lymphocyte retention in lymphoid organs (Shiow *et al.*, 2006). The finding here of enriched expression of CD69 in translocation-positive MALT lymphoma further implicates its role in lymphomagenesis.

Among the genes up-regulated in translocation positive cases, IRF4 showed a significant increase in cases with t(1;14) chromosomal translocation. IRF4 encodes a transcriptional factor and is expressed in activated B cells and cells showing plasma cell differentiation (Pernis, 2002). It is required during an immune response for lymphocyte activation and the generation of immunoglobulin-secreting plasma cells. In line with its known expression pattern, IRF4 is highly expressed in the plasma cell component of MALT lymphoma. Furthermore, plasma cell differentiation of neoplastic B cells is most prominent in MALT lymphoma with t(1;14) translocation. IRF4 is transcriptionally activated by t(6;14)(p25;q32) in multiple myeloma where gene expression profiling and genome wide chromatin immunoprecipitation analysis uncovered an extensive network of IRF4 target genes and showed that although IRF4 is not genetically altered in most myelomas, they are nonetheless

addicted to an aberrant IRF4 regulatory network that fuses the gene expression programmes of normal plasma cells and activated B cells (Shaffer *et al.*, 2008). Strong IRF4 expression has also been found in several lymphoma subtypes including lymphoplasmacytic lymphoma, 75% of diffuse large B-cell lymphoma and primary effusion lymphoma, which are not associated with t(6;14) (Falini *et al.*, 2002). IRF4 is one of the molecules found in the ABC-DLBCL signature (Alizadeh *et al.*, 2000). The oncogenic activity of IRF4 is thought to be related to its transcriptional repression of IFN-inducible genes, and thus the suppression of the anti-proliferative effects of IFN (Hrdlickova *et al.*, 2001; Pernis, 2002). NFκB transactivates IFN, and IFNα and IFNβ were moderately up-regulated in t(11;18) or t(1;14) positive MALT lymphoma. A simultaneous up-regulation of IRF4 may block the side-effects of NF-κB on IFN activation.

In addition to immune receptors discussed above, several potentially critical chemokine receptors were highly expressed in translocation positive MALT lymphoma and they included CCR2, CXCR4, CCR5 and CCR7 as shown by GSEA of gene expression microarray study in this thesis.

Several homeostatic chemokines have been shown to play an important role in mucosal immunology including germinal centre formation, homing and trafficking of activated mucosal B cells. Germinal centre formation involves the trafficking and positioning of lymphocytes in the organized lymphoid tissue (Moser *et al.*, 2001) and homing mechanisms play a role in B-cell recruitment to the secondary lymphoid tissue. CCR7 expression is up-regulated in activated B cells allowing them to acquire the capacity to migrate into the T-cell zone and to follicles in Peyer's patches, where the CCR7 ligands, CCL19 and CCL21 are highly expressed (Okada *et al.*, 2002; Reif *et al.*, 2002). CCR7 is shown to play a central role

in regulation of normal mucosal lymphocyte re-circulation and homeostasis, particularly in the stomach (Hopken *et al.*, 2007). CXCR4 is expressed in B cells at multiple stages of their development. It is required for retention of B-cell precursors in the bone marrow. CXCR4-deficient B-cell precursors that migrated prematurely became localised in splenic follicles despite their unresponsiveness to CXCL13. CXCR4 is also critical for B-cell homing to the Peyer's patches and splenic marginal zone (Nie *et al.*, 2004). In both low grade B-cell NHL and classic Hodgkin lymphomas, CCR7 and CXCR4 over-expression were associated with a wide lymph node spread, supporting their role in lymphoma pathogenesis (Lopez-Giral *et al.*, 2004; Hopken *et al.*, 2002; Trentin *et al.*, 2004).

B cells express CCR5 on their cell surfaces, and RANTES, one of four chemokine ligands of CCR5, is mitogenic for B cells (Rabkin *et al.*, 1999). Thus, it is tempting to speculate that RANTES and hence CCR5 may play a role in lymphoma expansion by avoiding immune surveillance.

CCR2 (CC chemokine receptor 2) is a receptor for MCP-1 which attracts monocytes and T cells to sites of injury as part of the inflammatory response. Two isoforms of CCR2, namely CCR2A and CCR2B have been cloned (Charo *et al.*, 1994; Charo, 1999). It has been suggested that these two isoforms of the receptor might be splice variants of a single gene (Charo *et al.*, 1994). CCR2B is the major form of the receptor readily detected in monocytes, whereas CCR2A is less abundant (Charo, 1999). The physiological role of CCR2A is not fully understood (Charo, 1999). Data in this thesis has shown that CCR2A and CCR2B were differentially expressed between translocation positive and negative MALT lymphoma cases with greater variability in CCR2A expression. Flaishon *et al.* described a novel role for CCR2 and its ligand CCL2/JE in inhibiting the chemotactic response of immature B cells to the chemokine CXCL12/stromal cell-derived factor 1 (SDF-1), suggesting that CCR2 and its

ligand act as negative regulators of the homing of immature B cells (Flaishon *et al.*, 2004). They also showed that CCR2 is transcribed in immature B cells, while its mRNA is dramatically down regulated at the mature B cells stage. CCR2-deficient mice showed massive accumulation of immature B cells in the lymph nodes in comparison with wild type mice (Flaishon *et al.*, 2004). Beside its expression in immature B cells, CCR2 is found to be expressed in mature B-cell neoplasms such as marginal zone B-cell lymphoma (Trentin *et al.*, 2004). It has been shown that CCR2 is up-regulated in response to the formation of superoxide free radical molecules and that IL-2 induced the expression of CCR2 in T lymphocytes, which correlated with the response of these cells to MCP-1 in chemotaxis assays (Loetscher *et al.*, 1996). Also, IL-10 selectively up-regulated the expression of CCR2 in monocytes by prolonging the mRNA half-life (Sozzani *et al.*, 1998). More recently, CCR2 was shown to mediate hematopoietic stem and progenitor cell trafficking to sites of inflammation (Si *et al.*, 2010). However, it is still unclear how the up-regulation of CCR2 expression contributes to MALT lymphoma development or whether it is induced by the presence of oxygen free radicals which would occur during *H. pylori* mediated inflammatory responses. It may be that infection with *H. pylori* leads to chronic inflammation which may later cause the B cells to initiate the expression of CCR2 amongst other chemokines leading to their trafficking to inflammation sites. Once the inflammation subsides, CCR2 expression becomes reduced, however some of the cells within the inflammatory site may acquire an oncogenic even such as t(11;18)/API2-MALT1 leading to the constant expression of CCR2 via aberrant NF-κB and MAPK pathways activation. Over-expression of CCR2A and B, CCR5 and CXCR4 has been shown to activate NF-κB, Jak/STAT and MAPK pathways (Okada *et al.*, 2002). This, together with the TCR, BCR and CD40 signalling may form a

positive feedback autoregulatory loop in API2-MALT1 or BCL10 mediated NF-κB activation, thus leading to constitutive NF-κB activation.

7.2.2 Aberrant molecular mechanisms of translocation negative MALT lymphoma

In contrast to translocation-positive MALT lymphoma, translocation-negative cases were characterised by expression of a strong inflammatory gene signature. GSEA and leading edge analysis also revealed common core subset genes involving several related biological processes or molecular pathways, which were enriched in translocation-negative MALT lymphoma. The top examples included proinflammatory cytokines IL8 and IL1β, molecules involved in B- and T- cell interaction such as CD86, CD28 and ICOS, several chemokine and chemokine receptors, NR4A3 (also known as MINOR) and TLR2 (Figure 3.10).

IL8 and IL1β are the hallmark of a proinflammatory cytokine profile in response to *H. pylori* infection. IL8 is critical for neutrophil infiltration and activation, while IL1β induces gastrin release, inhibits acid secretion and promotes apoptosis of epithelial cells (McNamara *et al.*, 2008). The finding of over-expression of these proinflammatory cytokines in translocation-negative gastric MALT lymphomas, indicates the presence of active *H. pylori* infection. In keeping with this, translocation-negative gastric MALT lymphomas show a higher number of blast cells than translocation-positive cases (Okabe *et al.*, 2003). In addition, a number of chemokines and chemokine receptors was highly expressed in the translocation-negative cases. This may reflect the trafficking and retention of various immune cells in response to an active *H. pylori* infection.

Most importantly, GSEA showed up-regulated expression of the surface molecules involved in B- and T- cell interaction namely CD86, CD28 and ICOS in translocation-negative gastric

MALT lymphoma. Although residual reactive follicles may be present and contribute to the high CD86, CD28 and ICOS expression in translocation negative cases, the germinal centre markers CD10 and BCL6 were expressed in much lower levels in MALT lymphoma (Figure 3.3). More importantly, over-expression of CD86 in tumour cells was clearly demonstrated by qRT-PCR on microdissected samples and immunohistochemistry. In line with the these findings, a previous study showed significantly higher CD86 expression in gastric MALT lymphomas that responded to *H. pylori* eradication than those resistant to the therapy (66% VS 10%) (de Jong *et al.*, 2001). Although the chromosome translocation status in these cases is not available, it is most likely that the cases responded to *H. pylori* were translocation-negative (Liu *et al.*, 2002b). Taken together, these findings suggest that there is an active immune response to *H. pylori* infection in translocation-negative gastric MALT lymphoma, and this most likely underscores the tumour cell survival and expansion, and thus determines its response to *H. pylori* eradication.

NR4A3 is another molecule significantly enriched and over-expressed in translocation negative MALT lymphoma. NR4A3 is a member of the nerve growth factor-1B (NGF1B, or NR4A1 or Nur77) subfamily of nuclear orphan receptors. In T cells, NGF1B and NR4A3 are involved in TCR mediated cell death and thymocyte negative selection (He, 2002). These nuclear orphan receptors are also involved in the apoptotic process of other cell types in response to external signals (Hashida *et al.*, 2007). The function of NR4A3 in B cells is currently unclear. Nonetheless, NR4A3 is one of the top over-expressed genes in cured, as opposed to fatal/refractory, DLBCL (Shipp *et al.*, 2002). It is possible that over-expression of NR4A3 in lymphoma cells may predispose them to apoptosis following *H. pylori* eradication and elimination of the microbial mediated immune stimulation.

7.2.3 Molecular mechanisms of BCL10, MALT1 and API2-MALT1 mediated NF-κB activation

Cytospin and immunohistochemistry data from this thesis showed that over-expression of BCL10 led to its subcellular localisation in the nucleus. Co-immunoprecipitation data showed that over-expression of BCL10 led to its interaction with MALT1 in BJAB B cells. Functional data showed that only BCL10 over-expression with LPS stimulation led to IκBβ degradation. In addition, subcellular localization and co-IP data showed more BCL10 in the presence of MALT1 reflecting the possiblity that increased MALT1-BCL10 interaction might lead to increase in BCL10 expression. However, BCL10 was shown to interact with transcription factor IIB which plays an important role in the assembly of transcription activators that make up the RNA polymerase II pre-initiation complex (Liu *et al.*, 2004c) suggesting a possible role as a transcriptional activator. Taken together, it can be hypothesised that excess BCL10 interacts with MALT1 either by affecting its stabilisation or its expression. Either way, the cytoplasmic level of BCL10 and MALT1 needs to be maintained to allow the formation of the CBM signalosome upstream of the NF-κB subunits. Closer to the NF-κB subunits, BCL10 over-expression together with stimulation by LPS in B cells or CD3/CD28 in T cells stimulation led to constitutive NF-κB activation via IκBβ degradation. It has been shown that TCR/CD28 co-stimulation induces IκBα, IκBβ and IκBε degradation (Li *et al.*, 2005). IκBα and IκBβ use slightly different mechanisms of NF-κB activation. One hypothesis proposes that IκBα masks the NLS of p65 in addition to the fact that it contains nuclear export sequencing that enables newly synthesized IκBα to shuttle nuclear NF-κB/Rel dimers into the cytoplasm (Phelps *et al.*, 2000). In contrast, IκBβ does not contain nuclear export sequence and is able to mask the NLS domains of both p65 and p50. While detailed control of IκB nuclear import and export has yet to be defined, these unique

properties of IκBα and IκBβ are thought to provide the fine tuned regulation of NF-κB/Rel proteins, whereby IκBα controls transient NF-κB/Rel activation and IκBβ regulates sustained NF-κB/Rel activity. Studies using embryonic fibroblasts derived from various IκB knockout mice (Hoffmann *et al.*, 2002), showed that IκBα resulted in high oscillatory NF-κB nuclear activity, whereas IκBβ displayed a constant steady increase in nuclear NF-κB activity that plateaued without subsequent decline, allowing sustainable NF-κB activation in the case of prolonged stimulation (Hoffmann *et al.*, 2002). Also, studies have shown that when IκBβ is degraded, NF-κB activation becomes persistent; even though newly synthesized IκBα accumulates to high levels in unstimulated cells. This is partly because the newly synthesised IκBβ is an unphosphorylated protein that binds to a portion of newly made NF-κB and sequesters it from IκBα. The unphosphorylated IκBβ, however, fails to mask the NLS and DNA binding domain on NF-κB, resulting in the nuclear uptake of the unphosphorylated NF-κB/IκBβ complex (Suyang *et al.*, 1996). Thus, data from this thesis showed that over-expression of BCL10 leads to IκBβ degradation causing constitutive NF-κB activation. This partly explains the biochemical mechanisms behind the NF-κB reporter assay observations showing that BCL10 expression together with surface receptor stimulation led to increased NF-κB activation. In addition, phosphorylated BCL10 was shown to form a complex with another NF-κB inhibitor, BCL3, to enter the nucleus (Yeh *et al.*, 2006), thus it might be that BCL10 may move to the nucleus with BCL3. However, details of BCL10 nuclear import and export as well as the function of nuclear BCL10 remain to be determined. Collectively, it can be hypothesised that BCL10 may indirectly (e.g. via interaction with the IKK complex or some unknown molecule) degrades IκBβ, since BCL10 is not part of the transcription factor complex that binds the NF-κB promoter, nor does it affect the ability of NF-κB to bind the DNA. This degradation of IκBβ may cause different NF-κB to transactivate slightly different

set of NF-κB target genes thus explaining the slightly different gene expression profiles of the NF-κB target genes between MALT lymphoma with and without chromosome translocation.

A hypothesis on the molecular mechanisms underlying MALT lymphoma with and without chromosomal translocation can be generated from the data described above. In translocation positive MALT lymphoma, over-expression of API2-MALT1, BCL10 or MALT1 activates the canonical NF-κB pathway. Canonical NF-κB activation is augmented by B-cell receptor signalling, TLR signalling and potentially CCR2 signalling. The non-canonical NF-κB pathway may be activated by CD40 and LTβ receptor signalling. Activation of the canonical and non-canonical NF-κB pathways leads to enhanced expression of the NF-κB target genes, particularly TLR6, CCR2A, CCR2B CD69, IRF4 and BCL2. Over-expression of these immune receptors may provide a further positive feedback to the activation of the NF-κB pathways. In addition, expression of TLR6 and CCR2 may trigger activation of the MAPK pathway. Over-expression of BCL2 is expected to promote tumour cell survival. In essence, the above chromosome translocations cause constitutive NF-κB activation with expression of their target genes forming a potential positive feedback loop, and the relentless NF-κB activation, which in the case of gastric MALT lymphoma confers its resistance to *H. pylori* eradication (Figure 7.1)

In translocation negative MALT lymphoma, the ongoing inflammatory and immune responses maintain active cognate B- and T- cell interaction via co-stimulating molecules CD86/CD28, B7RP1/ICOS, which are the major determinants of tumour cell survival and thus explain, in the cases of gastric MALT lymphoma, their responses to *H. pylori* eradication (Figure 7.1).

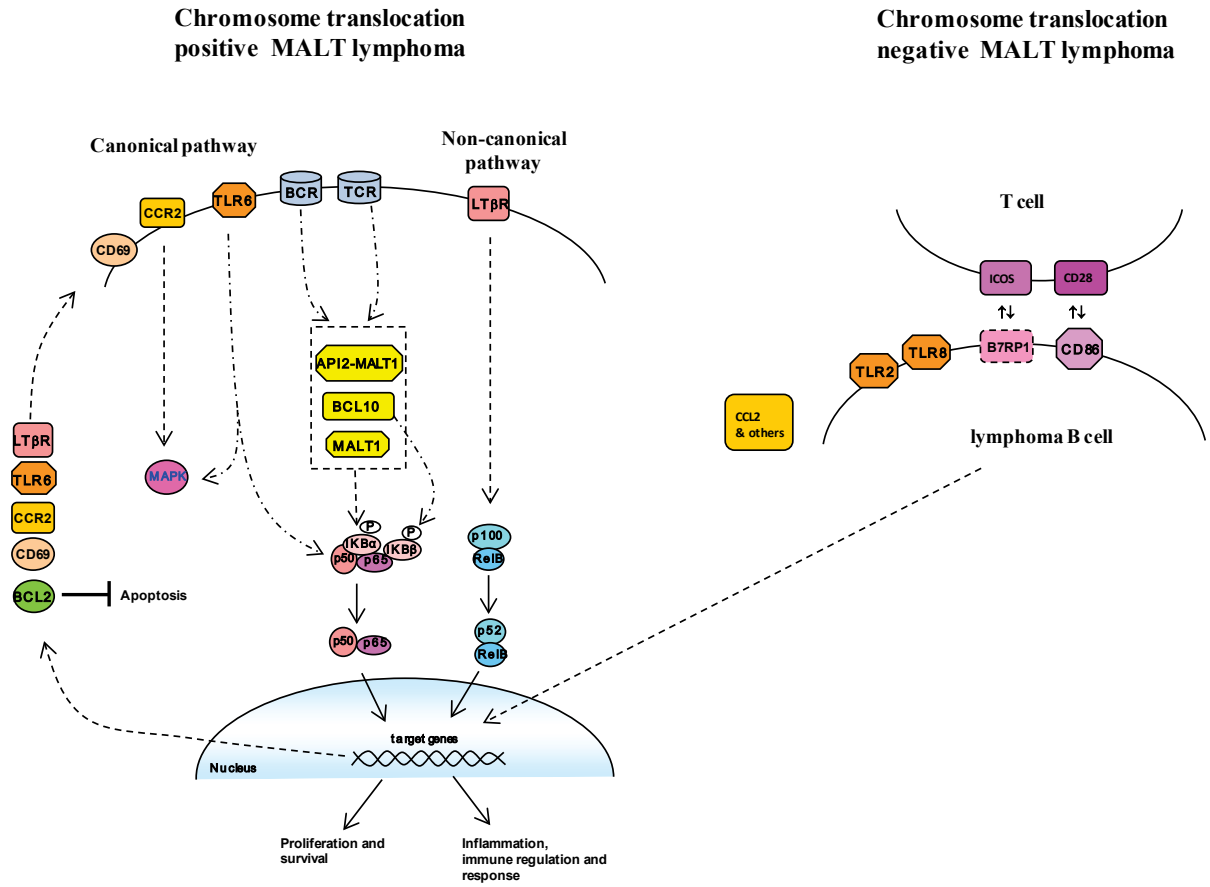


Figure 7.1 - Summary and hypothesis on molecular mechanisms of MALT lymphoma with and without chromosomal translocation.

In translocation positive MALT lymphoma, over-expression of *API2-MALT1*, *BCL10* and *MALT1* activates the canonical NF-κB pathway (e.g. in the case of *BCL10* via degradation of IκBβ), leading to enhanced expression of the NF-κB target genes, particularly *TLR6*, *CCR2*, *CD69* and *BCL2*. Over-expression of *TLR6* may provide a further positive feedback to the activation of the NF-κB pathway. Similar positive feedback may also be expected from the CCR2 signalling, and in addition both TLR6 and CCR2 may trigger activation of the MAPK pathway. The pathogenic implication of enhanced *CD69* expression is currently unknown. Over-expression of *BCL2* is expected to promote the tumor cell survival. In essence, the above chromosome translocations cause constitutive NF-κB activation with the expression of their target genes forming a potential positive feedback loop, and the relentless NF-κB activation, which in the case of gastric MALT lymphoma, confers its resistance to *H. pylori* eradication.

In translocation negative MALT lymphoma, the ongoing inflammatory and immune responses maintain active cognate B- and T- cell interaction via the co-stimulating molecules; CD86/CD28, B7RP1/ICOS, which are the major determinants of tumor cell survival and thus explain, in the cases of gastric MALT lymphoma, their responses to *H. pylori* eradication.

7.3 MALT lymphoma specific phenotypic marker identification

Comparison of MALT expression microarray data with other lymphomas demonstrated Lactoferrin to be highly expressed in MALT lymphoma but not in other lymphoma subtypes. This was confirmed by qRT-PCR showing *Lactoferrin* to be significantly over expressed in all MALT lymphoma cases compared to FL or MCL. Currently, the only reported marker for marginal zone lymphomas is myeloid cell nuclear differentiation antigen (MNDA), a nuclear protein expressed by myeloid cells and a subset of B cells (Kanellis *et al.*, 2009). It was shown to be expressed in normal tissue by a subset of the marginal zone B cells as well as subgroups of CLL, MCL and DLBCL, but it is highly expressed by MALT, SMZL and nodal marginal zone lymphoma (NMZL) and rarely expressed in FL making it potentially a useful marker for distinguishing between NMZL and FL (Kanellis *et al.*, 2009). Both microarray and qRT-PCR results from this thesis showed that *Lactoferrin* mRNA is more highly expressed in MALT compared to other lymphomas. It can be hypothesised that if the Lactoferrin protein expression follows a similar pattern as its mRNA expression, then Lactoferrin may prove to be a better phenotypic marker for MALT lymphoma than MNDA.

Lactoferrin belongs to a family of iron-binding proteins that modulate iron metabolism, haematopoiesis and immunologic reactions. It has been shown that Lactoferrin which is present at mucosal surfaces and neutrophil specific granules (Baggiolini *et al.*, 1970) (Raphael *et al.*, 1989), readily binds CpG-containing DNA which binds to B cells via interaction with DNA on the cell surface (Bennett *et al.*, 1983), mediated through the highly charged N-terminal sequence of Lactoferrin (Kawasaki *et al.*, 2000). Lactoferrin inhibited CpG ODN stimulation of CD86 expression in the human Ramos B cell line and decreased cellular uptake of ODN, a process required for CpG bioactivity. Lactoferrin binding of CpG-containing ODN may serve to modulate and terminate host response to immunostimulatory

molecules such as CD86 and CD80 at mucosal surfaces and sites of bacterial infection. (Britigan *et al.*, 2001). In addition, various studies show that there is an increase in Lactoferrin expression and *H. pylori* infection and gastric inflammation (Mannick et al) (Wen and Wen). Also, clinical studies showed that supplementation with Lactoferrin could be effective in increasing eradication rates of anti-*H. pylori* therapy, which could be helpful for patients with *H. pylori* eradication failure (Zou et al.) and that fecal Lactoferrin levels were elevated and this was found to be useful in the detection of colorectal diseases including MALT lymphoma (Hirata et al.).

Since the starting point of MALT lymphoma is thought to be infection with a pathogen and most of the samples analysed were *H. pylori* positive gastric MALT lymphoma, this may explain why Lactoferrin seem to be more highly expressed in MALT compared to other lymphomas. Besides its inhibitory effect on CpG-containing ODN in human B cells, Lactoferrin has been shown to affect phenotypic changes in immature B-cell populations and has an effect on the antigen presenting function of these cells (Zimecki *et al.*, 1995). Lactoferrin also plays a role in the maturation of cells of the immune system and, together with the demonstration of the involvement of Lactoferrin in the maturation of T cells (Zimecki *et al.*, 1991), this provides evidence that Lactoferrin can enhance the induction phase of the immune response.

In conclusion, Lactoferrin is a likely phenotypic marker for MALT lymphoma but further confirmation by immunohistochemistry is needed.

7.4 Conclusions

The results detailed in this study allow the following conclusions to be drawn:

1) Unsupervised clustering of MALT lymphoma with FL and MCL shows that MALT lymphoma is a distinct entity. Nonetheless, there is an overlap in the gene expression profiles between translocation positive and negative MALT lymphomas as both activate NF-κB pathway but leading to the expression of different sets of NF-κB target genes.
2) Translocation positive MALT lymphoma was characterised by an enhanced expression of NF-κB target genes, particularly CCR2, TLR6, CD69, IRF4 and BCL2.
3) Translocation negative MALT lymphoma was featured by active inflammatory and immune responses to *H. pylori* infection. Tumour cell interaction with infiltrating T cells through co-stimulatory molecules (especially CD86/CD28) may have an important role in their survival and clonal expansion.
4) *In vitro* assays show cooperation between the expression of MALT lymphoma associated oncogenes and signalling via surface receptors including BCR, TLR and TCR. Such cooperation may be operational *in vivo*.
5) BCL10 expression with surface receptor stimulation leads to IκBβ degradation. Over-expression of BCL10 together with LPS stimulation in BJAB B cells may also activate NF-κB inactivated by IκBβ.
6) Comparison of MALT lymphoma expression microarrays with other lymphomas showed Lactoferrin to be a putative MALT lymphoma specific marker.

7.5 Future perspectives

This thesis has identified novel mechanisms involved in MALT lymphoma pathogenesis, however many questions remain to be addressed.

7.5.1 CCR2 involvement in the molecular mechanism of MALT lymphomagenesis

Genes highly expressed in translocation positive MALT lymphoma include CCR2A and B isoforms. It would be useful to determine the role of each of these in MALT lymphomagenesis. This can be done in a similar way to the functional experiments carried out on TLR6 by identifying which pathways (e.g. NF-κB and MAPK) they affect and whether there is a synergy between them and MALT lymphoma associated oncogenes in the activation of those pathways. Once this is established, it would be helpful to construct inducible stable cellular models with each of the above genes together with MALT lymphoma associated oncogenes to confirm any synergy between each of the above genes and MALT lymphoma associated oncogenes. Migration assays can be carried out on the cellular model to study the effect of CCR2 expression on B-cell migration. Expression microarray experiments can be carried out on the cellular models by expression of CCR2 alone and together with MALT lymphoma associated oncogenes. GSEA and GO analysis will then lead to the identification of the specific pathways affected by the above genes that may be involved in MALT lymphomagenesis. Validation of genes involved in those pathways can be carried out on MALT lymphoma patient samples.

7.5.2 Nuclear BCL10 function

Identification of BCL10 binding partners would help to determine the functions of nuclear BCL10 which may help to explain its role in the regulation of the NF-κB pathway and its interaction with MALT1. Co-immunoprecipitation of nuclear BCL10 can be carried out by isolating the nuclear BCL10 fraction from BCL10 BJAB cells. BCL10 has been shown to form a complex with BCL3 which is a transcriptional co-activator of NF-κB (Yeh *et al.*, 2006), thus co-IP products of nuclear BCL10 can be investigated for the potential presence of NF-κB subunits as potential binding partners by Western blotting. Co-IP extracts can also be investigated for the presence of unknown binding partners. Extracts can be separated by PAGE and the proteins visualised by silver staining. The proteins within bands unique to extracts from cells expressing nuclear BCL10 protein can be identified by mass spectrometry. Confirmation of the identity of any putative nuclear BCL10 binding partners can then be achieved by Western blotting of co-IP products for the presence of these targets. Site-specific mutagenesis of BCL10 expression constructs could be carried out to determine the exact region of the BCL10 protein required for binding to these partners.

BCL10 has been shown to bind to transcriptional activator TBII (Liu *et al.*, 2004b). The role of BCL10 as a transcriptional activator could be investigated by Chromatin immunoprecipitation (ChIP) assay on cell lines with over-expressed BCL10 and compared to those with normal BCL10 expression. This will answer the question whether BCL10 may play a role as a transcriptional activator and if so whether it affects MALT1 expression amongst other genes which might partly explain the mechanism by which over-expression of BCL10 leads to MALT1 interaction.

7.5.3 Lactoferrin expression in MALT lymphoma by immunohistochemistry

A practically useful specific marker of MALT lymphoma needs to be developed. Lactoferrin seems to be the most promising candidate as confirmed by qRT-PCR. However, the initial Lactoferrin antibody used to validate the microarray and qRT-PCR produced inconclusive immunohistochemical data and the immunohistochemistry need to be repeated with a different Lactoferrin antibody. Two antibodies; the polyclonal rabbit anti-human (product no. A0061, DakoCytomation, Cambridge, UK) and the monoclonal mouse anti-human (Clone 1A1, product no. H86024M, Meridian Life Science, Maine, USA) were successfully used on FFPE tissue of clear cell carcinoma (Giuffre *et al.*, 2007). Another antibody is the polyclonal rabbit anti-human anti-Lactoferrin (product no. 07-685, Upstate (Millipore), Massachusetts, USA) which was successfully used on nasopharyngeal carcinoma tissue microarray (Zhou *et al.*, 2008). MNDA was shown to be a useful marker for distinguishing between NMZL and FL. Thus comparison between MNDA and the above two Lactoferrin marker on the same series of NHL B-cell lymphomas including CLL/SLL is warranted.

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Appendix I – Details of bioinformatics analysis

I.I Preprocessing algorithm for Affymetrix expression arrays

Preprocessing Affymetrix expression arrays usually involves three steps: background adjustment, normalisation and summarisation. The bioconductor software (http://www.bioconductor.org) which use the R programming platform (http://www.r-project.org/), implements a wide range of methods for each of these steps. Self-contained routines for background correction and normalisation usually take an AffyBatch as input and return a process AffyBatch. Routines for summarisation produce exprSet objects containing expression summary values.

There are currently 4 main algorithms that can be used for preprocessing the samples; MAS5, RMA, gcRMA and dChip. Literature studies comparing the above algorithms showed that dChip does not perform in a consistant manner, however none of the comparison studies showed the best algorithm to use to preprocess data from Affymetrix expression arrays. Some studies preferred MAS5 while others suggested that either RMA or gcRMA is best. Therefore, for this thesis a novel strategy was used to preprocess the raw CEL files using both gcRMA and MAS5. This was followed by non-specific filtering of the probes to eliminate non-variant probes.

The algorithm for MAS5 non-specific filtering was as follows:

```
FOR (each probe)
{
      IF ((probe raw value > 50) in > x MALT lymphoma samples)) THEN
      {
            Label that probe as variant and keep
      }

      ELSE

      {
            Discard that probe and move to the next probe
      }
}

x = is the group containing the least samples
```

So x in this thesis = 2 because the group containing the least samples is the t(14;18) and this has 2 cases

The algorithm for gcRMA non-specific filtering was as follows:

```
FOR (each probe)
{

      Calculate the CV for the probe across all samples

      IF (probe CV > 10% across all MALT lymphoma samples) THEN
      {
            Label that probe as variant and keep
      }

      ELSE

      {
            Discard that probe and move to the next probe
      }
}
```

Once this is done, a new set of probes is constructed from common probes that passed both gcRMA and MAS5 preprocessing.

The advantage of using this strategy is that MAS5 is used to filter the probes on their absolute values, thus it will keep the low copy variant probes as well as the obvious highly variant probes. gcRMA is used to eliminate any non-variant probes across all samples, which is good where some probes might be high due to the tissue specificity and not because their contribution to the tumour part of the sample.

This strategy is totally unsupervised and proved to be the best for normalising and filtering probes from difficult studies such as in this thesis where; firstly, there are no controls as such, thus the comparison is made between groups of the same entity i.e. MALT lymphoma. Secondly, the number of cases in some of the groups is very small to be statistically feasible e.g. only two cases of t(14;18) and 4 cases of t(1;14) (3 t(1;14) and 1 t(1;2)) were available. Systematic testing of individual preprocessing algorithms and combination of (RMA and MAS5) and (RMA and gcRMA) followed by false discovery rate (FDR) multiple testing corrections showed the consistant loss of probes mapping to BCL10, TLR6 and CD69.

However gcRMA and MAS5 gave the best trade-off with MAS5 giving high number of probes with some false positive but gcRMA giving lower number of probes with few false positive. This strategy was used as the initial step for analysis of all the array data in this thesis.

I.II Analysis of differentially expressed genes

Many microarray studies are designed to detect genes associated with different groups (phenotypes), for example in this thesis, the comparison of MALT lymphoma with and without chromosome translocation cases and comparison of MALT lymphoma against other lymphomas. The distribution of gene expression data is generally parametric thus the array data was log transformed in order to make the distribution of the replicated measurements per gene roughly symmetric and close to normal. A variance stabilizing transformation derived from an *error model* for microarray measurements was employed to make the variance of the measured intensities independent of their expected value. This can be advantageous for gene-wise statistical tests that rely on variance homogeneity, because it will diminish differences in variance between experimental conditions that are due to differences in the intensity level, but differences in variance between conditions may also have gene-specific biological reasons, and these will remain untouched.

Generally, for the comparison of MALT lymphoma with and without chromosomal translocation in chapter 3, t-test (ANOVA on two groups) was applied with the *error model* to obtain set of genes that are differentially expressed between the two groups. However, for the phenotypic marker study in chapter 6, multiple group ANOVA and eBayes which is part of the limma package in bioconductor, were applied separately to the same set of probes and the common probes from each analysis were combined to create a new set.

eBayes fits the probe data to a linear model and works best when the variability of the log-ratios is as homogenous as possible across the probes, whereas multiple group ANOVA can cope with slight heterogeneity in the data. eBayes is more stringent because it shrinks the data and results in few genes across the groups whereas multiple group ANOVA is less stringent and give larger set of genes with more false positives even after the stringent Bonferroni multiple testing correction. Thus a combination of both for the phenotypic marker study proved the best strategy. Finally in order to manage the vast amount of testing between the groups, a relational database management software was constructed as described in Appendix II.

I.III Construction and annotation of gene sets for GSEA

Creation and annotation of human immune gene sets

The Gene Set Enrichment Analysis (GSEA) is a powerful technique for elucidating various groups of genes that may be important from gene expression data. However, one drawback of the current implementation of GSEA is that the gene sets are only as good as the annotation and the immunology gene set annotations from GO are poor and do not follow a certain pathway. Thus in addition to running the 4395 pathways as mentioned in section 2.2.5.4, the challenge was to see which of the immune system pathways are significantly enriched in MALT lymphomas with and without chromosome translocations. For this thesis, the genes and proteins of the essential human immunome were identified and collected by literature search, reviewing the existing databases such as Immunome at: http://bioinf.uta.fi/Immunome/, ImmTree at: http://bioinf.uta.fi/ImmTree/, immune pathways in GeneGo (http://www.genego.com/) and immune pathways in Ingenuity (http://www.ingenuity.com/). It is difficult to strictly define immunome genes. In this thesis, a pragmatic approach was taken, where the gene products have to be essential for immunity, but not be widely expressed in many cells and tissues. Using this strategy and in house software tools such as relational database management systems, human immune gene sets were created that broadly fall into the following categories:

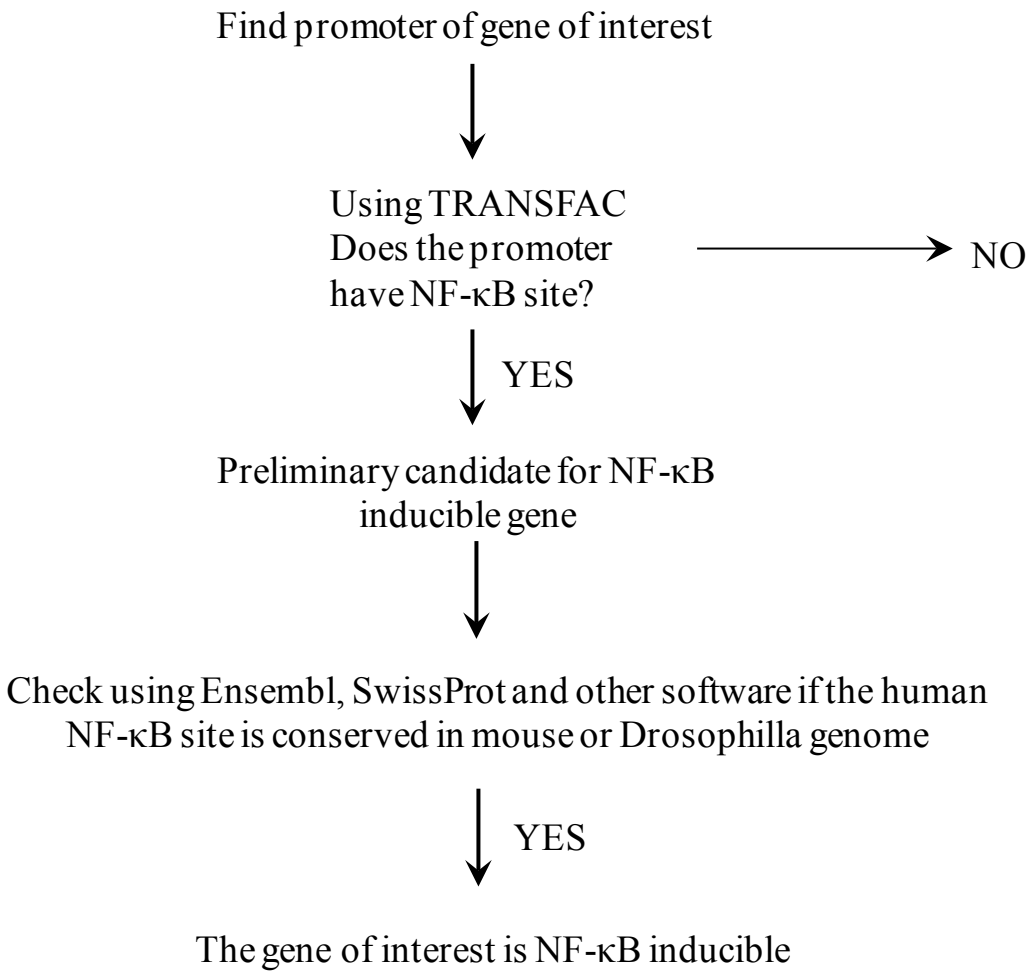
1) Antigen Presentation
2) CD genes
3) Cellular Immunity
4) Chemokine
5) Complement System
6) Humoral Immunity
7) Inflammation
8) Innate Immunity
9) Phagocytosis
10) Transcription Factor
11) B-cell receptor signalling
12) T-cell receptor signalling
13) TLR signalling pathway
13) Chemotaxis of leukocytes
14) Immune response to bacteria

Creation and annotation of NF-κB target genes set

NF-κB target gene is defined in broad terms as a gene that has a κB site in its promoter. To date there is no comprehensive list of NF-κB target genes, thus a comprehensive list of NF-κB target genes was collated by bioinformatics, literature search and Internet search of NF-κB target genes list at: http://people.bu.edu/gilmore/nf-kb/target/index.html and http://bioinfo.lifl.fr/NF-KB/

Bioinformatics strategy

Bioinformatics search algorithm is summarised as follows:



This strategy identified the following genes:

Gene symbol	Gene full name
TRIP10	thyroid hormone receptor interactor 10
IL32	interleukin 32
RCP9	calcitonin gene-related peptide-receptor component pro
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol de
LDHB	lactate dehydrogenase B
TEAD1	TEA domain family member 1 (SV40 transcriptional enh
PRDM2	PR domain containing 2, with ZNF domain
BACE2	beta-site APP-cleaving enzyme 2
SUV39H1	suppressor of variegation 3-9 homolog 1 (Drosophila)
IL1F9	interleukin 1 family, member 9
ALOX12B	arachidonate 12-lipoxygenase, 12R type
CARD15	caspase recruitment domain family, member 15
CD74	CD74 antigen (invariant polypeptide of major histocom
CXCL2	chemokine (C-X-C motif) ligand 2
DEFB4	defensin, beta 4
IL15RA	interleukin 15 receptor, alpha
TPMT	thiopurine S-methyltransferase
TLR6	toll-like receptor 6
TLR4	toll-like receptor 4
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3
PLA2G2E	phospholipase A2, group IIE
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 m
CSF2RA	colony stimulating factor 2 receptor, alpha, low-affinity
MMP8	matrix metallopeptidase 8 (neutrophil collagenase)
CCL7	chemokine (C-C motif) ligand 7
TNFRSF21	tumor necrosis factor receptor superfamily, member 21
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-depend
LAMC2	laminin, gamma 2
BCL2L10	BCL2-like 10 (apoptosis facilitator)
TNFSF6	tumor necrosis factor superfamily, member 6
CD105	homodimeric transmembrane protein which is a major gl
TNFRSF6	tumor necrosis factor receptor superfamily, member 6, d
TNFSF5	tumor necrosis factor superfamily, member 5
BM2	influenza B virus BM2
HC3	proteasome subunit HC3
SIAT8A	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransfer
TBR	tuberin
TNFRSF5	tumor necrosis factor receptor superfamily, member 5, d
RBCK1	RanBP-type and C3HC4-type zinc finger containing 1
CCR2A	chemokine (C-C motif) receptor 2 isoform A
CCR2B	chemokine (C-C motif) receptor 2 isoform B

Those genes are not found on the websites mentioned above or literature search.

Literature search

Literature search identified the following genes:

BIRC2, ICAM1, CX3CL1, NR4A3 and BCL10

Internet search

Internet search of NF-κB target genes list was carried out at:

http://people.bu.edu/gilmore/nf-kb/target/index.html and http://bioinfo.lifl.fr/NF-KB/

Summary of the categories and genes found in each is presented in the following table:

Category	Number of Genes
Cytokines/Chemokines and their modulators	29
Immunoreceptors	20
Acute phase proteins	1
Stress response genes	5
Growth factors, ligands and their modulators	15
Early response genes	3
Proteins involved in antigen	1
Cell adhesion molecules	6
Cell surface receptors	10
Regulators of apoptosis	9
Transcription factors	20
Viruses	4
Enzymes	33
Miscellaneous	41
Total no.	200

Using all 3 strategies but removing duplication a total of 271 NF-κB target genes were identified. A full list is provided in the complementary DVD attached to the back cover of this thesis.

Overall, a total of 4395 gene sets were identified including 56 custom sets which was constructed as mentioned in this appendix.

Gene set details are summarised in the following table:

Name of Gene set	No. of gene sets	No of genes	Source	Description
NF-kB target genes	1	271	http://www.nf-kb.org; http://bioinfo.lifl.fr/NF-KB; http://people	Genes contain kB binding sites in their promoters and are transactivated by NF-kB
Gene Sets from Immunome Database				
Innate immunity	1	44	http://bioinf.uta.fi/Immunome	
Antigen processing and presentation	1	45	http://bioinf.uta.fi/Immunome	
Inflammation	1	131	http://bioinf.uta.fi/Immunome	
Phagocytosis	1	18	http://bioinf.uta.fi/Immunome	
Cellular Immunity	1	100	http://bioinf.uta.fi/Immunome	
Humoural Immunity	1	103	http://bioinf.uta.fi/Immunome	
Transcription factor	1	38	http://bioinf.uta.fi/Immunome	
Complement System	1	56	http://bioinf.uta.fi/Immunome	
Chemokine receptors	1	240	http://bioinf.uta.fi/Immunome	
Cluster of differentiation (CD)	1	301	http://bioinf.uta.fi/Immunome	
Gene Sets In biological processes annotated from Gene Ontology				
Immunologially imporant genes	1	1327	http://wiki.geneontology.org/index.php/Immunologically_Imp	Genes related to immunological process and listed according to their priority score
B and T cell receptor signalling pathway	1	113	http://www.geneontology.org/	Contains 69 and 44 genes for B and T cell receptor signalling respectively
Immune response (GO:0006955)	1	596	http://www.geneontology.org/	immune response genes derived from gene ontology root category of the term GO:0006955
Adaptive immune response (GO:0002250)	1	43	http://www.geneontology.org/	adaptive immune response genes derived from gene ontology root category of the term GO:0002250
Activation of immune response (GO:0002253)	1	41	http://www.geneontology.org/	activation of immune response genes derived from gene ontology root category of the term GO:0002253
Regulation of adaptive immune response (GO:0002819)	1	11	http://www.geneontology.org/	genes from regulation of adaptive immune response derived from gene ontology root category of the term GO:0002819
Immunoglobulin mediated immune response (GO:0016064)	1	14	http://www.geneontology.org/	genes involved in immunoglobulin mediated immune response derived from gene ontology root category of the term GO:0016064
Innate immune response (GO:0045087)	1	62	http://www.geneontology.org/	genes involved with innate immune response derived from gene ontology root category of the term GO:0045087
Regulation of innate immune response (GO:0045088)	1	14	http://www.geneontology.org/	genes involved with regulation of innate immune response derived from gene ontology root category of the term GO:0002250
Regulation of immune response (GO:0050776)	1	77	http://www.geneontology.org/	genes involved in regulation of immune response derived from gene ontology root category of the term GO:0050776
Negative regulation of immune response (GO:0050777)	1	9	http://www.geneontology.org/	genes involved with negative regulation of immune response derived from gene ontology root category of the term GO:0050777
Positive regulation of immune response (GO:0050778)	1	60	http://www.geneontology.org/	genes involved with positive regulation of immune response derived from gene ontology root category of the term GO:0050778
Lymphocyte mediated immunity (GO:0002449)	1	38	http://www.geneontology.org/	genes involved in lymphocyte mediated immunity derived from gene ontology root category of the term GO:0002449
T cell mediated immunity (GO:0002456)	1	11	http://www.geneontology.org/	genes involved in t cell mediated immunity derived from gene ontology root category of the term GO:0002456
Humoral immune response (GO:0006959)	1	45	http://www.geneontology.org/	genes involved in humoral immune response derived from gene ontology root category of the term GO:0006959
Adaptive immune response (GO:0002250)	1	97	http://www.geneontology.org/	genes involved in adaptive immune response derived from gene ontology root category of the term GO:0002250
Anti apoptosis (GO:0006916)	1	165	http://www.geneontology.org/	genes involved in anti-apoptosis derived from gene ontology root category of the term GO:0006916
Caspase activation (GO:0006919)	1	40	http://www.geneontology.org/	genes involved in caspase activation derived from gene ontology root category of the term GO:0006919
Inflammatory response (GO:0006954)	1	294	http://www.geneontology.org/	genes involved in inflammatory response derived from gene ontology root category of the term GO:0006954
I-kappaB kinase NF kappaB cascade (GO:0007249)	1	41	http://www.geneontology.org/	genes involved in I-kappaB kinase NF kappaB cascade derived from gene ontology root category of the term GO:0007249
Activation of NF kappaB inducing kinase (GO:0007250)	1	13	http://www.geneontology.org/	genes involved in the activation of NF kappaB inducing kinase derived from gene ontology root category of the term GO:0007250
T cell activation (GO:0042110)	1	59	http://www.geneontology.org/	genes involved in T cell activation derived from gene ontology root category of the term GO:0042110
B cell activation (GO:0042113)	1	43	http://www.geneontology.org/	genes involved in B cell activation derived from gene ontology root category of the term GO:0042113
Chemokine (GO:0042379)	1	50	http://www.geneontology.org/	genes relating to chemokines derived from gene ontology root category of the term GO:0042379
Positive regulation of I kappaB kinase NF kappaB cascade (GO:0043123)	1	101	http://www.geneontology.org/	genes involved in the positive regulation of I kappaB kinase NF kappaB cascade derived from gene ontology root category of the term GO:0
Innate immune response (GO:0045087)	1	105	http://www.geneontology.org/	genes involved in the innate immune response derived from gene ontology root category of the term GO:0045087
Lymphocyte activation (GO:0046649)	1	137	http://www.geneontology.org/	genes involved in lymphocyte activation derived from gene ontology root category of the term GO:0046649
Regulation of T cell activation (GO:0050863)	1	70	http://www.geneontology.org/	genes involved in the regulation of T cell activation derived from gene ontology root category of the term GO:0050863
Regulation of lymphocyte activation (GO:0051249)	1	120	http://www.geneontology.org/	genes involved in the regulation of lymphocyte activation derived from gene ontology root category of the term GO:0051249
Gene sets derived from pathways annotated by GeneGo				
B Cell Lymphoma	1	50	http://www.genego.com/	Genes involved in B cell lymphoma derived from the GeneGo Metacore software
T Cell Receptor Signalling	1	46	http://www.genego.com/	Genes involved in T cell receptor signalling derived from the GeneGo Metacore software
TLR Signalling Pathway	1	82	http://www.genego.com/	Genes involved in TLR signalling pathway derived from the GeneGo Metacore software
CD28	1	71	http://www.genego.com/	CD28 pathway related genes derived from the GeneGo Metacore software
ICOS	1	93	http://www.genego.com/	Genes that are linked to ICOS pathway, derived from the GeneGo Metacore software
Chemotaxis Leukocyte	1	110	http://www.genego.com/	genes that are involved chemotaxis leukocyte, derived from the GeneGo Metacore software
Immune Response Bacteria	1	47	http://www.genego.com/	genes that are involved in bacterial immune response pathway derived from the GeneGo Metacore software
BCR Pathway	1	75	http://www.genego.com/	genes related to B cell receptor signalling pathway, derived from the GeneGo Metacore software
TCR CD28 costimulation leading to NFkB	1	70	http://www.genego.com/	genes involved in T-cell receptor signalling and CD28 costimulation leading to NFkB activation derived from the GeneGo Metacore software
Gene set derived from Pathways annotated by Ingenuity Systems				
Antigen Receptor GO	1	17	http://www.ingenuity.com	genes to do with antigen receptor signalling derived from the gene ontology part of the Ingenuity pathway analysis software
B cell activation	1	95	http://www.ingenuity.com	genes to do with b cell activation derived from the gene ontology part of the Ingenuity pathway analysis software
B cell receptor signalling	1	60	http://www.ingenuity.com	genes to do with b cell receptor signalling derived from the gene ontology part of the Ingenuity pathway analysis software
Chemokine signalling	1	36	http://www.ingenuity.com	genes to do with chemokine signalling derived from the gene ontology part of the Ingenuity pathway analysis software
T cell activation GO	1	95	http://www.ingenuity.com	genes to do with t cell activation derived from the gene ontology part of the Ingenuity pathway analysis software
T cell receptor signalling	1	57	http://www.ingenuity.com	genes to do with t cell receptor signalling derived from the gene ontology part of the Ingenuity pathway analysis software
TLR signalling	1	33	http://www.ingenuity.com	genes to do with TLR signalling derived from the gene ontology part of the Ingenuity pathway analysis software
Gene Sets from Molecular Signature Database				This is derived from the Broad Institute website at : http://www.broad.mit.edu/gsea/msigdb/downloads.jsp and enriched using IPA and GeneGo Metacore software. The molecular signature pathways that are derived are from version 2.5.
C2: Curated gene sets derived from online pathway database and publicatio	1892		http://www.broad.mit.edu/gsea/msigdb/index.jsp	C2 curated gene sets derived from online pathway database and publications
C2: Canonical pathway gene sets from pathway databases	639		http://www.broad.mit.edu/gsea/msigdb/index.jsp	Canonical pathway gene sets from pathway databases including the followings
C2: BioCarta gene sets	249		http://www.biocarta.com/	BioCarta gene sets
C2: GenMAPP gene sets	138		http://www.genmapp.org/	GenMAPP gene sets
C2: KEGG gene sets	200		http://www.genome.jp/kegg/	KEGG gene sets
C5: GO biological process gene sets	825		http://www.broad.mit.edu/gsea/msigdb/index.jsp	GO biological process gene sets
C5: GO molecular function gene sets	396		http://www.broad.mit.edu/gsea/msigdb/index.jsp	GO molecular function gene sets
Total number of gene sets	4395			
Total number of custom sets	56			

Finally, in order to manage and query the vast amount of data gathered from microarrays and gene set enrichment analysis, a relational database management software was constructed and included in the complementary DVD attached to the back cover of this thesis.

Appendix II – In house software for gene expression microarray analysis

Two main programming languages were used to write custom made software for analysis of microarray data generated from this thesis; R programming platform with use of some of the bioconductor libraries and Visual Basic programming platform embedded within Microsoft Access in order to write the relational database management software.

II.I R software

R custom software were used to preprocess and analyse the 24 CEL files from MALT lymphoma and 15 CEL files from FL and MCL microarray data, as well as to help post process some of the GSEA data and carry out unsupervised clustering and statistical analysis e.g. for qRT-PCR and identification of differentially expressed genes using Bayesian analysis such as eBayes.

A list of all the custom software is included in the complementary DVD attached to the back cover of this thesis.

The main software R code for pre-processing and analysing microarray data from both the U133A&B and U133plus2 platforms is as follows:

```
# Analysis of U133A&B MALT lymphoma and U133plus2 FL and MCL
# Software written by Rifat Hamoudi, 2010

library(affy)
library(gcrma)
library(genefilter)
library(gplots)
library(annotate)
library(hgu133a.db)
library(hgu133b.db)
library(hgu133aprobe)
library(hgu133bprobe)
library(gsubfn)


############################################################
#
#  Read the U133plus chips of FL & MCL
#
############################################################


setwd("/media/disk/RawArrayData/Cel Files/U133/FL_MCL")

MCL_FL<-ReadAffy()     # read affy files after changedir. Read into
AffyBatch object

sampleNames(MCL_FL)


sampleNames(MCL_FL)[1] <- "FL14"
sampleNames(MCL_FL)[2] <- "FL16"
sampleNames(MCL_FL)[3] <- "FL17"
sampleNames(MCL_FL)[4] <- "FL18"
sampleNames(MCL_FL)[5] <- "FL19"
sampleNames(MCL_FL)[6] <- "FL20"
sampleNames(MCL_FL)[7] <- "FL21"
sampleNames(MCL_FL)[8] <- "MCL22"
sampleNames(MCL_FL)[9] <- "MCL23"
sampleNames(MCL_FL)[10] <- "MCL24"
sampleNames(MCL_FL)[11] <- "MCL25"
sampleNames(MCL_FL)[12] <- "MCL26"
sampleNames(MCL_FL)[13] <- "MCL27"
sampleNames(MCL_FL)[14] <- "MCL28"
sampleNames(MCL_FL)[15] <- "MCL29"

gcrma_mcl_fl <-gcrma(MCL_FL) # normalization via gcrma
mas_mcl_fl <- mas5(MCL_FL, sc=100) # normalization via MAS5



# MCL FL groups definition

flnum <- seq(1:7)
mclnum <- c(8,9,10,11,12,13,14,15)
```

```
allnum <- c(flnum, mclnum)

# GCRMA groups implementation

flgc <- gcrma_mcl_fl[,flnum]
mclgc <- gcrma_mcl_fl[,mclnum]
allflmclgc <- gcrma_mcl_fl[,allnum]


# MAS5 groups implementation

flmas <- mas_mcl_fl[,flnum]
mclmas <- mas_mcl_fl[,mclnum]
allflmclmas <- mas_mcl_fl[,allnum]


# filter stuff on MAS5 abs values

f1<-kOverA(7, 50)# if a gene is 50 or more raw value in more than 7
samples then pass it
ff <-filterfun(f1)
masselect_flmcl <-genefilter(mas_mcl_fl, ff)
sum(masselect_flmcl)
esetmasflmcl <- mas_mcl_fl[masselect_flmcl,]

# filtering gcRMA on CV

cvfun <- cv(0.1, 1.0)
ffun <- filterfun(cvfun)
gcselect_flmcl <- genefilter(gcrma_mcl_fl, ffun)
sum(gcselect_flmcl)
esetgcrmaflmcl <- gcrma_mcl_fl[gcselect_flmcl,]


# Extract the correct set on gcRMA and MAS5

selectgenes_flmcl <- intersect(featureNames(esetmasflmcl),
featureNames(esetgcrmaflmcl))
selectgenes_flmcl
length(selectgenes_flmcl)

esetgcgoodflmcl <- gcrma_mcl_fl[selectgenes_flmcl,] # gcRMA
esetgcgoodflmcl

esetmasgoodflmcl <- mas_mcl_fl[selectgenes_flmcl,] # MAS5
esetmasgoodflmcl



gn <- featureNames(MCL_FL)
ps <- probeset(MCL_FL, gn[1:2])
probeNames(MCL_FL)[1:5]

gcrmaflmclexp <- exprs(gcrma_mcl_fl)
```

```
# eliminate AFFX and _x_
idsflmcl <- featureNames(esetmasgoodflmcl)
ids.affx <- grep("^AFFX", idsflmcl)
#noX <- grep("_x_", ids)
ids.noaffx_flmcl <- setdiff(c(1:length(idsflmcl)), ids.affx)
#ids.noaffx <- setdiff(c(1:length(idsflmcl)), noX)

esetgcfinalflmcl <- esetgcgoodflmcl[ids.noaffx_flmcl,]
esetgcfinalflmcl

esetmasfinalflmcl <- esetmasgoodflmcl[ids.noaffx_flmcl,]
esetmasfinalflmcl



############################################################
#
#  Read U133A MALT lymphomas
#
############################################################

setwd("/media/disk/RawArrayData/Cel Files/U133/MALT/HG133_A")

MALT_A<-ReadAffy()     # read affy files after changedir. Read into
AffyBatch object

sampleNames(MALT_A)

sampleNames(MALT_A)[1] <- "11_18_G0015_A"
sampleNames(MALT_A)[2] <- "11_18_G5125_A"
sampleNames(MALT_A)[3] <- "11_18_G5661_A"
sampleNames(MALT_A)[4] <- "11_18_G6071_A"
sampleNames(MALT_A)[5] <- "11_18_86_14635_Samp11V_A"
sampleNames(MALT_A)[6] <- "11_18_95_10509_Samp1F_A"
sampleNames(MALT_A)[7] <- "11_18_92_10232_Samp2F_A"
sampleNames(MALT_A)[8] <- "11_18_96_8361_Samp3F_A"
sampleNames(MALT_A)[9] <- "11_18_97_107717_Samp7V_A"
sampleNames(MALT_A)[10] <- "1_14_Bel_A"
sampleNames(MALT_A)[11] <- "1_14_G0186_A"
sampleNames(MALT_A)[12] <- "1_14_G0262_A"
sampleNames(MALT_A)[13] <- "1_2_G6389_A"
sampleNames(MALT_A)[14] <- "14_18_02_101211_Samp8V_A"
sampleNames(MALT_A)[15] <- "14_18_97_21350_Samp16V_A"
sampleNames(MALT_A)[16] <- "3_14_G0046_A"
sampleNames(MALT_A)[17] <- "NEG_G0019_A"
sampleNames(MALT_A)[18] <- "NEG_G0055_Nuc_A"
sampleNames(MALT_A)[19] <- "NEG_G0078_Nuc_A"
sampleNames(MALT_A)[20] <- "NEG_G5018_A"
sampleNames(MALT_A)[21] <- "NEG_G6352_Nuc_A"
sampleNames(MALT_A)[22] <- "NEG_88_20237_Samp12V_A"
sampleNames(MALT_A)[23] <- "NEG_92_8149_Samp4F_A"
sampleNames(MALT_A)[24] <- "NEG_91_6360_Samp5F_A"
sampleNames(MALT_A)[25] <- "NEG_96_9991_Samp6F_A"
sampleNames(MALT_A)[26] <- "NEG_89_01810_Samp13V_A"
```

```
gcrmamalt_a<-gcrma(MALT_A)   # normalization via gcrma
masmalt_a <- mas5(MALT_A, sc=100) # normalization via MAS5


# groups definition

neg_A <- c(17,18,19,20,21,22,23,24,25,26)
tr11_18_A <- c(1,2,3,4,5,6,7,8,9)
tr1_14_A <- c(10,11,12,13)
tr14_18_A <- c(14,15)

t11_18set_A <- c(tr11_18_A,neg_A)
t1_14set_A <- c(tr1_14_A, neg_A)
t14_18set_A <- c(tr14_18_A, neg_A)


# GCRMA groups implementation

posneggcrmav_A <- c(tr1_14_A, tr11_18_A, tr14_18_A, neg_A)
posneggcrma_A <- gcrmamalt_a[,posneggcrmav_A]
pData(posneggcrma_A)$sample <- c(1:25)

t11_18gc_A <- gcrmamalt_a[,t11_18set_A]
t1_14gc_A <- gcrmamalt_a[,t1_14set_A]
t14_18gc_A <- gcrmamalt_a[,t14_18set_A]


# MAS5 groups implementation

posnegv_A <- c(tr1_14_A, tr11_18_A, tr14_18_A, neg_A)
posnegmas_A <- masmalt_a[,posnegv_A]
pData(posnegmas_A)$sample <- c(1:25)

t11_18mas_A <- masmalt_a[,t11_18set_A]
t1_14mas_A <- masmalt_a[,t1_14set_A]
t14_18mas_A <- masmalt_a[,t14_18set_A]



############################################################
#
#  Read U133B MALT lymphomas
#
############################################################


setwd("/media/disk/RawArrayData/Cel Files/U133/MALT/HG133_B")

MALT_B<-ReadAffy()     # read affy files after changedir. Read into
AffyBatch object

sampleNames(MALT_B)

sampleNames(MALT_B)[1] <- "11_18_G0015_B"
sampleNames(MALT_B)[2] <- "11_18_G5125_B"
```

```
sampleNames(MALT_B)[3] <- "11_18_G5661_B"
sampleNames(MALT_B)[4] <- "11_18_G6071_B"
sampleNames(MALT_B)[5] <- "11_18_86_14635_Samp11V_B"
sampleNames(MALT_B)[6] <- "11_18_95_10509_Samp1F_B"
sampleNames(MALT_B)[7] <- "11_18_92_10232_Samp2F_B"
sampleNames(MALT_B)[8] <- "11_18_96_8361_Samp3F_B"
sampleNames(MALT_B)[9] <- "11_18_97_107717_Samp7V_B"
sampleNames(MALT_B)[10] <- "1_14_Bel_B"
sampleNames(MALT_B)[11] <- "1_14_G0186_B"
sampleNames(MALT_B)[12] <- "1_14_G0262_B"
sampleNames(MALT_B)[13] <- "1_2_G6389_B"
sampleNames(MALT_B)[14] <- "14_18_02_101211_Samp16V_B"
sampleNames(MALT_B)[15] <- "14_18_97_21350_Samp8V_B"
sampleNames(MALT_B)[16] <- "3_14_G0046_B"
sampleNames(MALT_B)[17] <- "NEG_G0019_B"
sampleNames(MALT_B)[18] <- "NEG_G0055_Nuc_B"
sampleNames(MALT_B)[19] <- "NEG_G0078_Nuc_B"
sampleNames(MALT_B)[20] <- "NEG_G5018_B"
sampleNames(MALT_B)[21] <- "NEG_G6352_Nuc_B"
sampleNames(MALT_B)[22] <- "NEG_88_20237_Samp12V_B"
sampleNames(MALT_B)[23] <- "NEG_92_8149_Samp4F_B"
sampleNames(MALT_B)[24] <- "NEG_91_6360_Samp5F_B"
sampleNames(MALT_B)[25] <- "NEG_96_9991_Samp6F_B"
sampleNames(MALT_B)[26] <- "NEG_89_01810_Samp13V_B"

gcrmamalt_b <-gcrma(MALT_B)  # normalization via gcrma
masmalt_b <- mas5(MALT_B, sc=100) # normalization via MAS5


# groups definition Bchips

neg_B <- c(17,18,19,20,21,22,23,24,25,26)
tr11_18_B <- c(1,2,3,4,5,6,7,8,9)
tr1_14_B <- c(10,11,12,13)
tr14_18_B <- c(14,15)

t11_18set_B <- c(tr11_18_B,neg_B)
t1_14set_B <- c(tr1_14_B, neg_B)
t14_18set_B <- c(tr14_18_B, neg_B)


# GCRMA groups implementation

posneggcrmav_B <- c(tr1_14_B, tr11_18_B, tr14_18_B, neg_B)
posneggcrma_B <- gcrmamalt_b[,posneggcrmav_B]
pData(posneggcrma_B)$sample <- c(1:25)

t11_18gc_B <- gcrmamalt_b[,t11_18set_B]
t1_14gc_B <- gcrmamalt_b[,t1_14set_B]
t14_18gc_B <- gcrmamalt_b[,t14_18set_B]


# MAS5 groups implementation

posnegv_B <- c(tr1_14_B, tr11_18_B, tr14_18_B, neg_B)
```

```
posnegmas_B <- masmalt_b[,posnegv_B]
pData(posnegmas_B)$sample <- c(1:25)

t11_18mas_B <- masmalt_b[,t11_18set_B]
t1_14mas_B <- masmalt_b[,t1_14set_B]
t14_18mas_B <- masmalt_b[,t14_18set_B]



############################################################
#
#  MALT_A nonspecific filtering
#
############################################################

# filter stuff on MAS5 abs values A chip

f1<-kOverA(2, 50)# if a gene is 50 or more raw value in more than 2
samples then pass it
ff <-filterfun(f1)
masselect_malt_a <- genefilter(masmalt_a, ff)
sum(masselect_malt_a)
esetmasposneg_a <- posnegmas_A[masselect_malt_a,]

# filtering gcRMA on CV

cvfun <- cv(0.1, 1.0)
ffun <- filterfun(cvfun)
gcselect_malt_a <- genefilter(gcrmamalt_a, ffun)
sum(gcselect_malt_a)
esetgcrmaposneg_a <- posneggcrma_A[gcselect_malt_a,]

# Extract the correct set on gcRMA and MAS5

selectgenes_malt_a <- intersect(featureNames(esetmasposneg_a),
featureNames(esetgcrmaposneg_a))
selectgenes_malt_a
length(selectgenes_malt_a)

esetgcgoodposneg_a <- posneggcrma_A[selectgenes_malt_a,] # gcRMA
esetgcgoodposneg_a

esetmasgoodposneg_a <- posnegmas_A[selectgenes_malt_a,] # MAS5
esetmasgoodposneg_a

gn_a <- featureNames(MALT_A)
ps_a <- probeset(MALT_A, gn_a[1:2])
probeNames(MALT_A)[1:5]

gcrmamaltexp_a <- exprs(gcrmamalt_a)

# eliminate AFFX and _x_
ids_a <- featureNames(esetgcgoodposneg_a)
ids.affx_a <- grep("^AFFX", ids_a)
```

```
ids.noaffx_malt_a <- setdiff(c(1:length(ids_a)), ids.affx_a)

#noX <- grep("_x_", ids)
#ids.noaffx <- setdiff(c(1:length(ids)), noX)


esetgcfinalposneg_a <- esetgcgoodposneg_a[ids.noaffx_malt_a,]
esetgcfinalposneg_a

esetmasfinalposneg_a <- esetmasgoodposneg_a[ids.noaffx_malt_a,]
esetmasfinalposneg_a


############################################################
#
#  MALT_B nonspecific filtering
#
############################################################


# filter stuff on MAS5 abs values B chip

f1<-kOverA(2, 50)# if a gene is 50 or more raw value in more than 2
samples then pass it
ff <-filterfun(f1)
masselect_malt_b <- genefilter(masmalt_b, ff)
sum(masselect_malt_b)
esetmasposneg_b <- posnegmas_B[masselect_malt_b,]

# filtering gcRMA on CV

cvfun <- cv(0.1, 1.0)
ffun <- filterfun(cvfun)
gcselect_malt_b <- genefilter(gcrmamalt_b, ffun)
sum(gcselect_malt_b)
esetgcrmaposneg_b <- posneggcrma_B[gcselect_malt_b,]


# Extract the correct set on gcRMA and MAS5

selectgenes_malt_b <- intersect(featureNames(esetmasposneg_b),
featureNames(esetgcrmaposneg_b))
selectgenes_malt_b
length(selectgenes_malt_b)

esetgcgoodposneg_b <- posneggcrma_B[selectgenes_malt_b,] # gcRMA
esetgcgoodposneg_b

esetmasgoodposneg_b <- posnegmas_B[selectgenes_malt_b,] # MAS5
esetmasgoodposneg_b



gn_b <- featureNames(MALT_B)
ps_b <- probeset(MALT_B, gn_b[1:2])
```

```
probeNames(MALT_B)[1:5]

gcrmamaltexp_b <- exprs(gcrmamalt_b)

# eliminate AFFX and _x_
ids_b <- featureNames(esetgcgoodposneg_b)
ids.affx_b <- grep("^AFFX", ids_b)
ids.noaffx_malt_b <- setdiff(c(1:length(ids_b)), ids.affx_b)

#noX <- grep("_x_", ids)
#ids.noaffx <- setdiff(c(1:length(ids)), noX)


esetgcfinalposneg_b <- esetgcgoodposneg_b[ids.noaffx_malt_b,]
esetgcfinalposneg_b

esetmasfinalposneg_b <- esetmasgoodposneg_b[ids.noaffx_malt_b,]
esetmasfinalposneg_b


####################################################
#
# Map all the MALT_A probes on the FLMCL
#
####################################################

maltprobes_a <- featureNames(esetmasfinalposneg_a)

esetfinalflmclgc_a <- gcrma_mcl_fl[maltprobes_a,]
esetfinalflmclmas_a <- mas_mcl_fl[maltprobes_a,]


####################################################
#
# Map all the MALT_B probes on the FLMCL
#
####################################################

maltprobes_b <- featureNames(esetmasfinalposneg_b)

esetfinalflmclgc_b <- gcrma_mcl_fl[maltprobes_b,]
esetfinalflmclmas_b <- mas_mcl_fl[maltprobes_b,]



####################################################
#
# Find common probes between MALT_A and MALT_B and FLMCL
#
####################################################

# MALT_A

flmclmaltprobes_a <- intersect(featureNames(esetmasfinalflmcl),
featureNames(esetmasfinalposneg_a))
```

```
instersectflmclmaltgc_a <- esetgcgoodflmcl[flmclmaltprobes_a,]
instersectflmclmaltmas_a <- esetmasgoodflmcl[flmclmaltprobes_a,]

instersectmaltgc_a <- esetgcgoodposneg_a[flmclmaltprobes_a,]
instersectmaltmas_a <- esetmasgoodposneg_a[flmclmaltprobes_a,]


# MALT_B

flmclmaltprobes_b <- intersect(featureNames(esetmasfinalflmcl),
featureNames(esetmasfinalposneg_b))
instersectflmclmaltgc_b <- esetgcgoodflmcl[flmclmaltprobes_b,]
instersectflmclmaltmas_b <- esetmasgoodflmcl[flmclmaltprobes_b,]

instersectmaltgc_b <- esetgcgoodposneg_b[flmclmaltprobes_b,]
instersectmaltmas_b <- esetmasgoodposneg_b[flmclmaltprobes_b,]


############################ write out ###################################

setwd("/media/Linux/malt_flmcl_res")

# MALT_A writeout

write.table(exprs(posnegmas_A), file="MALT MAS5 Achip 22283.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(posneggcrma_A), file="MALT GCRMA Achip 22283.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(esetgcfinalposneg_a), file="MALT GCRMA Filtered Achip
norm 8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(esetmasfinalposneg_a), file="MALT MAS5 Filtered Achip
raw 8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))


# MALT_B writeout

write.table(exprs(posnegmas_B), file="MALT MAS5 Bchip 22645.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(posneggcrma_B), file="MALT GCRMA Bchip 22645.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(esetgcfinalposneg_b), file="MALT GCRMA Filtered Bchip
norm 8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))
```

```
write.table(exprs(esetmasfinalposneg_b), file="MALT MAS5 Filtered Bchip
raw 8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))



# FLMCL writeout

write.table(exprs(mas_mcl_fl), file="FLMCL MAS5 U133plus2 54675.xls",
append = FALSE, sep="\t", eol="\n", row.names=TRUE, col.names=TRUE,
qmethod=c("escape", "double"))

write.table(exprs(gcrma_mcl_fl), file="FLMCL GCRMA U133plus2
54675.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(esetgcfinalflmcl), file="FLMCL GCRMA Filtered
U133plus2 norm 10652.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(esetmasfinalflmcl), file="FLMCL MAS5 Filtered
U133plus2 raw 10652.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))



# FLMCL on MALT writeout

write.table(exprs(esetfinalflmclgc_a), file="FLMCL_MALT_GC Achip
8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(esetfinalflmclmas_a), file="FLMCL_MALT_MAS Achip
8181.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(esetfinalflmclgc_b), file="FLMCL_MALT_GC Bchip
8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(esetfinalflmclmas_b), file="FLMCL_MALT_MAS Bchip
8622.xls", append = FALSE, sep="\t", eol="\n", row.names=TRUE,
col.names=TRUE, qmethod=c("escape", "double"))



# FLMCL on MALT intersect writeout

write.table(exprs(instersectflmclmaltgc_a), file="FLMCL_MALT_A_GC FLMCL
intersect 3871.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(instersectflmclmaltmas_a), file="FLMCL_MALT_A_MAS
FLMCL intersect 3871.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
```

```
write.table(exprs(instersectmaltgc_a), file="FLMCL_MALT_GC MALT_A
intersect 3871.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(instersectmaltmas_a), file="FLMCL_MALT_MAS MALT_A
intersect 3871.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(instersectflmclmaltgc_b), file="FLMCL_MALT_B_GC FLMCL
intersect 3034.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(instersectflmclmaltmas_b), file="FLMCL_MALT_B_MAS
FLMCL intersect 3034.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(instersectmaltgc_b), file="FLMCL_MALT_GC MALT_B
intersect 3034.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))

write.table(exprs(instersectmaltmas_b), file="FLMCL_MALT_MAS MALT_B
intersect 3034.xls", append = FALSE, sep="\t", eol="\n",
row.names=TRUE, col.names=TRUE, qmethod=c("escape", "double"))
```

II.II Visual Basic software for relational database management software

Two relational database management software (RDBMS) were written for this thesis. Firstly, an RDBMS entitled “ArrayExplorer RDBMS” which allowed the query and data management generated from the microarray experiments. Secondly an RDBMS entitled “GSEA RDBMS” which contained query and data management functionalities that helped in collating the information generated from GSEA.

Both of these software are included in the complementary DVD attached to the back cover of this thesis.

Appendix III – GSEA results

III.I NF-κB target gene set

A list of 271 NF-κB target genes was compiled as described in appendix I.III. 223 genes were obtained from the website:

However the remaining 48 genes were obtained through bioinformatics and literature search as described in appendix I.III. The 48 genes are listed below:

Gene symbol	Gene full name	Source
TRIP10	thyroid hormone receptor interactor 10	bioinformatics
IL32	interleukin 32	bioinformatics
RCP9	calcitonin gene-related peptide-receptor component pro	bioinformatics
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	bioinformatics
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	bioinformatics
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol de	bioinformatics
LDHB	lactate dehydrogenase B	bioinformatics
TEAD1	TEA domain family member 1 (SV40 transcriptional enl	bioinformatics
PRDM2	PR domain containing 2, with ZNF domain	bioinformatics
BACE2	beta-site APP-cleaving enzyme 2	bioinformatics
SUV39H1	suppressor of variegation 3-9 homolog 1 (Drosophila)	bioinformatics
IL1F9	interleukin 1 family, member 9	bioinformatics
ALOX12B	arachidonate 12-lipoxygenase, 12R type	bioinformatics
CARD15	caspase recruitment domain family, member 15	bioinformatics
CD74	CD74 antigen (invariant polypeptide of major histocom	bioinformatics
CXCL2	chemokine (C-X-C motif) ligand 2	bioinformatics
DEFB4	defensin, beta 4	bioinformatics
IL15RA	interleukin 15 receptor, alpha	bioinformatics
TPMT	thiopurine S-methyltransferase	bioinformatics
TLR6	toll-like receptor 6	bioinformatics
TLR4	toll-like receptor 4	bioinformatics
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	bioinformatics
PLA2G2E	phospholipase A2, group IIE	bioinformatics
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 m	bioinformatics
CSF2RA	colony stimulating factor 2 receptor, alpha, low-affinity	bioinformatics
MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	bioinformatics
CCL7	chemokine (C-C motif) ligand 7	bioinformatics
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	bioinformatics
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-depen	bioinformatics
LAMC2	laminin, gamma 2	bioinformatics
BCL2L10	BCL2-like 10 (apoptosis facilitator)	bioinformatics
TNFSF6	tumor necrosis factor superfamily, member 6	bioinformatics
CD105	homodimeric transmembrane protein which is a major gl	bioinformatics
TNFRSF6	tumor necrosis factor receptor superfamily, member 6, d	bioinformatics
TNFSF5	tumor necrosis factor superfamily, member 5	bioinformatics
BM2	influenza B virus BM2	bioinformatics
HC3	proteasome subunit HC3	bioinformatics
SIAT8A	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransfer	bioinformatics
TBR	tuberin	bioinformatics
TNFRSF5	tumor necrosis factor receptor superfamily, member 5, d	bioinformatics
RBCK1	RanBP-type and C3HC4-type zinc finger containing 1	bioinformatics
CCR2A	chemokine (C-C motif) receptor 2 isoform A	bioinformatics
CCR2B	chemokine (C-C motif) receptor 2 isoform B	bioinformatics
BIRC2	baculoviral IAP repeat-containing 2	Blood, 15th of August, 2005 ; 106(4) : 1392 - 1399
ICAM1	intercellular adhesion molecule 1 (CD54), human rhinov	Blood, 15th of August, 2005 ; 106(4) : 1392 - 1399
CX3CL1	chemokine (C-X3-C motif) ligand 1	Blood, 15th of August, 2005 ; 106(4) : 1392 - 1399
NR4A3	nuclear receptor subfamily 4, group A, member 3	Journal of Biological Chemistry, 12th of August, 2005 ; 280(32) : 29256-29262
BCL10	B-cell CLL/lymphoma 10	Journal of Biological Chemistry, 6th of January, 2006 ; 281(1) : 167 - 175

III.II Leading edge core set of NF-kB target genes enriched in MALT lymphoma with and without chromosome translocation

Rank	Gene	Description	Chromosome Band	Entrez ID	Signal to noise	Enrichment Score
Expression of genes enriched in translocation negative MALT lymphoma						
1	CXCL5	chemokine (C-X-C motif) ligand 5	4q12-q13	6374	0.530	0.020
2	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxy	1q25.2-q25.3	5743	0.519	0.039
3	NR4A3	nuclear receptor subfamily 4, group A, member 3	9q22	8013	0.485	0.057
4	CCL11	chemokine (C-C motif) ligand 11	17q21.1-q21.2	6356	0.455	0.073
5	PTGIS	prostaglandin I2 (prostacyclin) synthase	20q13.13	5740	0.416	0.087
6	IL8	interleukin 8	4q13-q21	3576	0.395	0.100
7	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	11q22.3	4314	0.388	0.114
8	CXCL2	chemokine (C-X-C motif) ligand 2	4q21	2920	0.372	0.126
9	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	4q21	2919	0.370	0.140
10	CD86	CD86 molecule	3q21	942	0.359	0.152
11	CCL2	chemokine (C-C motif) ligand 2	17q11.2-q12	6347	0.346	0.161
12	IGFBP2	insulin-like growth factor binding protein 2, 36kDa	2q33-q34	3485	0.323	0.169
13	GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	14q11.2	3002	0.322	0.181
14	VEGF	vascular endothelial growth factor	6p12	7422	0.313	0.190
15	SOD2	superoxide dismutase 2, mitochondrial	6q25.3	6648	0.313	0.202
16	IL11	interleukin 11	19q13.3-q13.4	3589	0.313	0.214
17	PLAU	plasminogen activator, urokinase	10q24	5328	0.311	0.225
18	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	1p22.2	2633	0.310	0.237
19	SDC4	syndecan 4	20q12	6385	0.292	0.242
20	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid	10p15-p14	1646	0.288	0.251
21	IER3	immediate early response 3	6p21.3	8870	0.277	0.256
22	EGR1	early growth response 1	5q31.1	1958	0.268	0.262
23	TNC	tenascin C	9q33	3371	0.268	0.272
24	PENK	proenkephalin	8q23-q24	5179	0.258	0.278
25	KITLG	KIT ligand	12q22	4254	0.252	0.283
26	PRF1	perforin 1 (pore forming protein)	10q22	5551	0.245	0.289
27	GADD45B	growth arrest and DNA-damage-inducible, beta	19p13.3	4616	0.237	0.292
28	FN1	fibronectin 1	2q34	2335	0.236	0.301
29	THBS2	thrombospondin 2	6q27	7058	0.232	0.308
30	NQO1	NAD(P)H dehydrogenase, quinone 1	16q22.1	1728	0.232	0.317
31	TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)	11p15.2	7003	0.229	0.323
32	AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1q42-q43	183	0.219	0.323
33	IL1B	interleukin 1, beta	2q14	3553	0.196	0.311
34	GIF	gastric intrinsic factor (vitamin B synthesis)	11q13	2694	0.195	0.317
35	RBCK1	RanBP-type and C3HC4-type zinc finger containing 1	20p13	10616	0.195	0.324
36	CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1	8q21.3	9420	0.188	0.324
37	IL10	interleukin 10	1q31-q32	3586	0.186	0.329
38	ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	1q32.2	1999	0.183	0.332
39	TLR2	toll-like receptor 2	4q32	7097	0.181	0.337
40	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta	3q25	5806	0.180	0.343
41	BMP2	bone morphogenetic protein 2	20p12	650	0.177	0.346
42	IL1RN	interleukin 1 receptor antagonist	2q14.2	3557	0.173	0.348
43	FCER2	Fc fragment of IgE, low affinity II, receptor for (CD23)	19p13.3	2208	0.167	0.346
44	UGCG	UDP-glucose ceramide glucosyltransferase	9q31	7357	0.167	0.352
45	BDKRB1	bradykinin receptor B1	14q32.1-q32.2	623	0.165	0.354
46	HPSE	heparanase	4q21.3	10855	0.162	0.356
47	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene	7p12	1956	0.158	0.357
48	BAX	BCL2-associated X protein	19q13.3-q13.4	581	0.150	0.353
49	CCL4	chemokine (C-C motif) ligand 4	17q12	6351	0.150	0.358
50	DIO2	deiodinase, iodothyronine, type II	14q24.2-q24.3	1734	0.146	0.357
51	ORM1	orosomucoid 1	9q31-q32	5004	0.146	0.363
52	XDH	xanthine dehydrogenase	2p23.1	7498	0.146	0.368
53	CXCL9	chemokine (C-X-C motif) ligand 9	4q21	4283	0.145	0.372
54	TFF3	trefoil factor 3 (intestinal)	21q22.3	7033	0.144	0.377
55	PTAFR	platelet-activating factor receptor	1p35-p34.3	5724	0.144	0.382
56	TNFRSF21	tumor necrosis factor receptor superfamily, member 21	6p21.1-p12.2	27242	0.141	0.383
57	KCNK5	potassium channel, subfamily K, member 5	6p21	8645	0.139	0.383
58	JUNB	jun B proto-oncogene	19p13.2	3726	0.136	0.384
59	ICOS	inducible T-cell co-stimulator	2q33	29851	0.130	0.378
60	TLR4	toll-like receptor 4	9q32-q33	7099	0.127	0.378
61	DEFB4	defensin, beta 4	8p23.1-p22	1673	0.125	0.378
62	UBE2M	ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)	19q13.43	9040	0.124	0.382
63	ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide	4q21-q23	124	0.123	0.384
64	ICAM1	intercellular adhesion molecule 1	19p13.3-p13.2	3383	0.118	0.378
65	BCL2L1	BCL2-like 1	20q11.21	598	0.117	0.381
66	FAS	Fas (TNF receptor superfamily, member 6)	10q24.1	355	0.114	0.378
67	BGN	biglycan	Xq28	633	0.113	0.382
68	PAX8	paired box 8	2q12-q14	7849	0.113	0.386
69	CTSB	cathepsin B	8p22	1508	0.112	0.387
70	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	9q31.1	19	0.111	0.390

Expression of genes enriched in translocation positive MALT lymphoma						
146	KLK3	kallikrein-related peptidase 3	19q13.41	354	-0.165	-0.161
147	UCP2	uncoupling protein 2 (mitochondrial, proton carrier)	11q13	7351	-0.171	-0.160
148	MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1	19q13.1-q13.4	11184	-0.183	-0.161
149	IFI44L	interferon-induced protein 44-like	1p31.1	10964	-0.188	-0.157
150	CCR5	chemokine (C-C motif) receptor 5	3p21	1234	-0.199	-0.157
151	IRF7	interferon regulatory factor 7	11p15.5	3665	-0.202	-0.150
152	PRDM2	PR domain containing 2, with ZNF domain	1p36.21	7799	-0.208	-0.145
153	BCL2L10	BCL2-like 10 (apoptosis facilitator)	15q21	10017	-0.218	-0.142
154	CCR7	chemokine (C-C motif) receptor 7	17q12-q21.2	1236	-0.231	-0.137
155	IRF4	interferon regulatory factor 4	6p25-p23	3662	-0.262	-0.134
156	LTB	lymphotoxin beta (TNF superfamily, member 3)	6p21.3	4050	-0.263	-0.124
157	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	2p13-p12	5966	-0.272	-0.116
158	C4BPA	complement component 4 binding protein, alpha	1q32	722	-0.273	-0.105
159	TLR6	toll-like receptor 6	4p14	10333	-0.287	-0.097
160	BCL10	B-cell CLL/lymphoma 10	1p22	8915	-0.290	-0.086
161	CD69	CD69 molecule	12p13-p12	969	-0.352	-0.078
162	TFEC	transcription factor EC	7q31.2	22797	-0.364	-0.065
163	BCL2	B-cell CLL/lymphoma 2	18q21.33	596	-0.396	-0.051
164	CCR2B	chemokine (C-C motif) receptor 2 isoform B	3p21.31	7E+05	-0.500	-0.034
165	CCR2A	chemokine (C-C motif) receptor 2 isoform A	3p21.31	7E+05	-0.927	0.000