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Biological Pathways in B-cell Non-Hodgkin's Lymphoma

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*The beginning of knowledge is the discovery of something
we do not understand.*

- Frank Herbert

To my family

ABSTRACT

Non-Hodgkin's lymphomas have become more prevalent in the past 20 years. They constitute a diverse group of lymphoid tumors that have been understood and classified according to their clinical behaviour, anatomic location, morphology, immunophenotype, cytogenetics, and gene expression profile. Microarray profiling yields information about large number of genes, which can be involved in different functional pathways of importance for the biology of lymphomas.

In this thesis, we started to look at major types of B-cell lymphomas by using the microarrays, with the aim to have a macroscopic view of the signaling pathways involved in their biology. Functional signatures such as cell-cycle, apoptosis, cytokine-cytokine receptor interaction, T-cell receptor, B-cell receptor (BCR), cell adhesion, and NF- κ B activation describe multiple dysregulated pathways in lymphomas. The BCR gene expression delineates distinct functional heterogeneity within each lymphoma. In CLL, the BCR genes were positively correlated to the expression of the pathways for CREB, MAPK/ERK5, BCR Signaling and MET. In MCL, the BCR genes were additionally coregulated to GC T-helper cell, integrins, CXCR4 and blood pan-B cell genes. In FL, BCR genes showed positive correlation to the mTOR-, GC B-cell-, Akt- and BCR Signaling-, blood pan B-cell- and ERK5-pathways while in DLBCL they were co-expressed with GC-B-cell, blood pan-B cell and c-myc targets. Interestingly, targets for BLIMP-1, an important transcription factor, were positively correlated to the expression of BCR genes in all the four mentioned lymphomas (Paper I).

During unsupervised clustering, we observed that BCR genes and TCL1 oncogene were expressed in the same cluster. Variation in the level of gene array expression of *TCL1A* was significantly associated with gene signatures recognizing B-cell lymphoma biology, such as germinal center, BCR, NF- κ B (and its target genes), death, MAP kinases, TNFR1, TOLL, and IL1R. High expression of TCL1 was related to poor prognosis in CLL and MCL in microarray and immunohistochemistry respectively. (Paper II).

Thus, we found in these studies (Paper I and II) that BCR and TCL1 are important to lymphoma biology, while they also define striking heterogeneity within known lymphoma types.

The role of somatic hypermutation of the B-cell receptor in the lymphoma biology was demonstrated (paper III); we identified here surrogate markers or genes in the cell machinery that accompany the process. Markers of SHM (such as RAD51C and CDK7) and ongoing SHM (TFDP1 and POLA) are some of the genes involved in DNA repair and replication. CDK7 and RCC1, separately and together, showed prognostic value in Mantle Cell Lymphoma on immunohistochemistry (Paper III).

In the end, we narrowed our attention to Follicular Lymphoma (FL) and the microenvironment. Using a unique approach of flow cytometry and automated microscopy scoring in FL, we identified that the clinical outcome was independently predicted by different immune cells: CD4+ T-cells and macrophages were associated with poor prognosis while cytotoxic T-lymphocytes and T-regulatory cells were associated with better prognosis (Paper IV).

The results presented in the thesis provide insight and understanding of lymphoma biology that would facilitate discovery of markers of prognostic and hopefully therapeutic importance.

LIST OF PUBLICATIONS

- Mohit Aggarwal , Margarita Sánchez-Beato, Gonzalo Gómez-López, Fatima Al-Shahrour, Nerea Martínez, Antonia Rodríguez, Elena Ruiz-Ballesteros, Francisca I. Camacho, Alberto Pérez-Rosado, Paloma de la Cueva, María J Artiga, David G. Pisano, Eva Kimby, Joaquín Dopazo, Raquel Villuendas, Miguel A. Piris

I. Functional signatures identified in B-cell Non-Hodgkin Lymphoma profiles

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- Mohit Aggarwal , Raquel Villuendas, Gonzalo Gomez, Socorro M Rodriguez-Pinilla, Margarita Sanchez-Beato, David Alvarez, Nerea Martinez, Antonia Rodriguez, Maria E Castillo , Francisca I Camacho, Santiago Montes-Moreno, Jose A Garcia-Marco, Eva Kimby, David G Pisano and Miguel A Piris

II. TCL1A expression delineates biological and clinical variability in B-cell lymphoma

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III. Somatic hypermutation signature in low grade B-cell Lymphomas

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- Björn Engelbrekt Wahlin, Mohit Aggarwal, Santiago Montes-Moreno, Luis Francisco Gonzalez, Giovanna Roncador, Lydia Sanchez-Verde, Birger Christensson, Birgitta Sander, Eva Kimby

IV. A unifying model of the microenvironment of follicular lymphoma: outcome is predicted by programmed death-1-positive, regulatory, cytotoxic and helper T cells and macrophages

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Related work (not included)

- Ruiz-Vela A, Aggarwal M, de la Cueva P, Treda C, Herreros B, Martín-Pérez D, Dominguez O, Piris MA.

V. Lentiviral (HIV)-based RNA interference screen in human B-cell receptor regulatory networks reveals MCL1-induced oncogenic pathways.

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ABBREVIATIONS

ADCC	Antibody-dependent Cell-mediated Cytotoxicity
AID	Activation-induced cytidine deaminase (AICDA)
AKT	v-akt murine thymoma viral oncogene homolog 1
APC	Antigen Presenting Cell
ATLV	adult T cell leukaemia virus
B29	CD79b molecule, immunoglobulin-associated beta
BAD	BCL2-associated agonist of cell death
BAFF	tumor necrosis factor (ligand) superfamily, member 13b
BCL2	B-cell CLL/lymphoma 2
BCL6	B-cell CLL/lymphoma 6
BCL-xL	BCL2-like 1
BCR	B-cell receptor
BL	Burkitt lymphoma
BLIMP-1	B-lymphocyte-induced maturation protein 1
BLNK	B-cell linker
BM	Bone Marrow
B-NHL	B-cell Non-Hodgkins Lymphoma
BTK	Brutons Tyrosine Kinase
CB1	Canabinoid Receptor-1
CD10	membrane metallo-endopeptidase
CD30	tumor necrosis factor receptor superfamily, member 8
CD4	CD4 molecule
CD40	CD40 molecule, TNF receptor superfamily member 5
CD40L	CD40 antigen ligand; T-B cell-activating molecule; TNF-related activation protein;
CDK7	Cyclin Dependant Kinase-7
cFOS	FBJ murine osteosarcoma viral oncogene homolog
CGH	Comparitive Genomic Hybridisation
CHK1	CHK1 checkpoint homolog
CIITA	class II, major histocompatibility complex, transactivator
c-JUN	jun oncogene/AP-1
CLL	Chronic Lymphocytic Leukemia
c-myc	avian myelocytomatosis viral oncogene homolog
COX2	cyclooxygenase 2
CR2	complement component (3d/Epstein Barr virus) receptor 2
CSR	Class Switch Recombination
CTL	Cytotoxic T Lymphocyte
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
CXCR4	chemokine (C-X-C motif) receptor 4
CXCR5	chemokine (C-X-C motif) receptor 5/BLR1
DLBCL	Diffuse Large B-Cell Lymphoma
DNMT1	DNA Methyl Transferrase-1
EBV	Epstein Barr Virus
ERK	extracellular signal-regulated kinase 2/mitogen-activated protein kinase 1
Fas	TNF receptor superfamily, member 6/CD95
FDC	Follicular Dendritic Cell
FFPE	formalin-fixed paraffin-embedded
FGF	Fibroblastic Growth Factor
FISH	Flourescent In Situ Hybridisation
FL	Follicular Lymphoma
FRC	fibroblastic reticulum cells

FLIPI	Follicular Lymphoma International Prognostic Index
FOXP3	forkhead box P3
GALT	Gut Associated Lymphoid Tissue
GC	Germinal Center
GEP	Gene expression profiling
GPCR	G-Protein Coupled Receptors
HHV8	Human Herpesvirus 8
HIV	Human Immune-deficiency Virus
HSC	Haematopoietic Stem Cell
HSF1	heat shock transcription factor 1
HTLV	Human T-lymphotropic virus Type I
ICAM1	intercellular adhesion molecule 1/CD54
ID3	inhibitor of DNA binding 3
IgVH	Immunoglobulin V Heavy Chain
IgVL	Immunoglobulin V light chain region
IHC	Immuno Histo Chemistry
IL1R	Interleukin 1 receptor
IL2	Interleukin 2
IL4	Interleukin 4
IL5	Interleukin 5
IL6	Interleukin 6
iNOS	nitric oxide synthase 2, inducible
IPI	International Prognostic Index
IRF4	interferon regulatory factor 4
ITAM	Phosphorylated immunoreceptor signaling motifs
JAK3	Janus kinase 3 [
JNK	mitogen-activated protein kinase 8
JunB	jun B proto-oncogene
Kcells	Killer Cells
LTalfa	Lymphotoxin alfa
MALT	Mucosa Associated Lymphoid Tissue
mb1	proteasome (prosome, macropain) subunit, beta type, 5/PSMB5
MCL	Mantle Cell Lymphoma
MDM2	Mdm2 p53 binding protein homolog
MEK	mitogen-activated protein kinase kinase kinase 1
MHC-I	Major Histocompatibility Complex-I
MHC-II	Major Histocompatibility Complex-II
MUM1	melanoma associated antigen (mutated)
MZBCL	Marginal Zone B Cell Lymphoma
NFkB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NHL	Non-Hodgkin Lymphoma
NKcell	Natural Killer Cell
p38	mitogen-activated protein kinase 14
p53	tumor protein p53
p53RE	p53 response element
PAX5	paired box 5/ B cell specific activator protein
PCR	Polymerase Chain Reaction
pCREB	phosphorylated cAMP responsive element binding protein 1
PD-1	Programmed Death-1
PI3K	phosphoinositide-3-kinase, catalytic, gamma polypeptide
PIM1	pim-1 oncogene
PKA	Protein Kinase-A
PKB	Protein Kinase-B
PKC	Protein Kinase-C

PLC	Phospholipase C
PLC γ 2	Phospholipase C gamma 2
POLA	Polymerase A
qPCR	quantitative Polymerase Chain Reaction
RAD51C	DNA repair protein RAD51 homolog 3
RBC	Red Blood Cells
RCC1	regulator of chromosome condensation 1
SAPK	mitogen-activated protein kinase 9/jnk2
SHM	Somatic Hyper Mutation
SMZL	Splenic Marginal Zone Lymphoma
SPIB	Spi-B transcription factor (Spi-1/PU.1 related)
Spry2	Sprouty2
STAT3	signal transducer and activator of transcription 3
Syk	spleen tyrosine kinase
TBM	Tingible body macrophages
TCL1A	T-cell Leukemia-1 A oncogene
TCM	Central Memory T-cells
TEM	Effector Memory T cells
TH	Thymocyte
TMA	Tissue Microarray
TNF	Tumor Necrosis Factor
TOLL	toll-like receptor 4
TRAF	TNF receptor-associated factor
TRAIL	tumor necrosis factor (ligand) superfamily, member 10/CD253
Treg	Regulatory T-cell
TSG	Tumor Suppressor Gene
WBC	White Blood Corpuscles
VEGF	Vascular Endothelial Growth Factor
VH	Hypervariable region on heavy chain
VLA4	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
XBP1	X-box binding protein 1
ZAP70	zeta-chain (TCR) associated protein kinase

INTRODUCTION

Lymphomas are of two major types Hodgkin and Non-Hodgkin lymphoma (NHL), the later divided in B- and T-cell lymphoma. This study is focussed towards major types of B-cell lymphoma.

Molecular biology of lymphoma is determined by an interaction between tumor cell and microenvironment¹, whereby it determines the activation of B-cell receptor, NF-kB, and other signaling pathways, eventually regulating apoptosis and cell cycle². To date, little has been done to analyze simultaneously the most frequent lymphoma types with the aim of identifying commonly deregulated pathways, i.e., non-lymphoma-type-specific alterations that could be of importance for lymphoma biology.

In lymphomas, primary and secondary cytogenetic alterations have been identified that involve oncogenes such as *c-myc translocation*, mostly t(8;14), in Burkitt Lymphoma (BL)³, *bcl-2 translocation* t(14;18) in Follicular Lymphoma (FL)⁴ and *CyclinD1 translocation* t(11;14) in Mantle Cell Lymphoma (MCL)⁵. Moreover, deregulation of *TCL1A* has been described in Chronic Lymphocytic Lymphoma (CLL)⁶, and *BCL-6* deregulation in DLBCL⁷. Still there is a need to identify more accurate molecular markers⁸, and to apply the accumulated knowledge about lymphoma to find more specific drugs.

Some molecular markers such as CyclinD1, Bcl6, Bcl2, TCL1A and CD30 as well as surface antigens on lymphoma- and different immune-cells in the microenvironment can be detected by immunohistochemistry and flow cytometry. Such markers are of diagnostic and might be of prognostic importance.

(I) THE NORMAL LYMPHATIC SYSTEM

The lymphatic system is of importance for the defence against infection and cancer and consists of bone marrow, thymus, spleen, lymph nodes and lymphatics (the small vessels that link the lymph nodes and returns excess fluid to the circulation). The lymph nodes (located all over the body with clusters in the neck, armpits and groin, as shown in Fig1) contain lymphocytes, a certain type of white blood cells. The right lymphatic duct and the thoracic duct drain lymph fluids into two veins that come together to form the inferior vena cava, which passes into the heart. The cisterna chyli is a widened portion of the thoracic duct, where fluids from several lymph-collecting vessels are received. The spleen removes and destroys worn-out red blood cells, but is also important in the fight against infections. Bone marrow in the long bones is the seat of haematopoiesis.

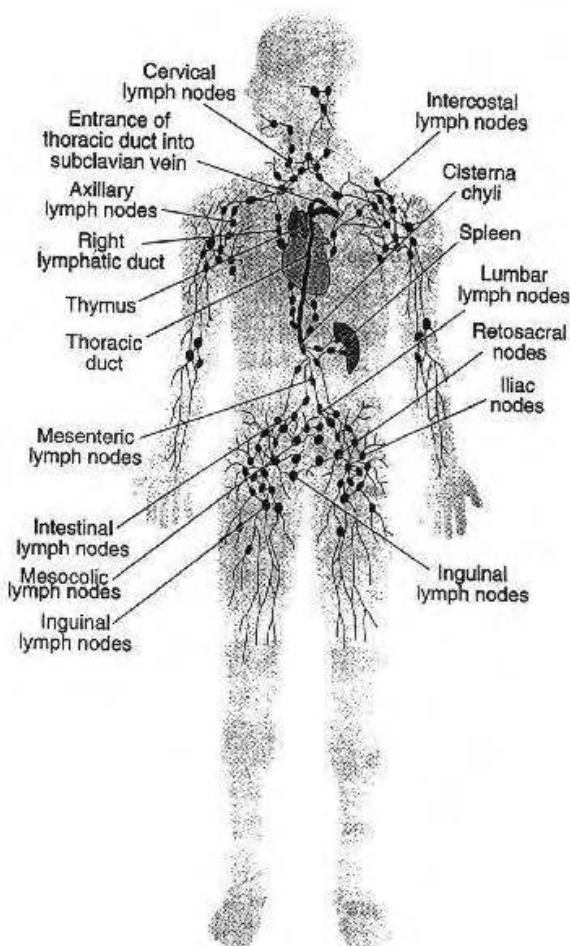


Fig.1 Anatomic location of lymph nodes in human body

Functions of the lymphatic system:

- Bone marrow, lymph nodes and other lymphoid tissues are the site of clonal production of immunocompetent lymphocytes and macrophages in the specific immune response.
- The pressure and volume of the extracellular fluid is maintained by lymphfluid returning excess water and dissolved substances from the interstitial fluid to the circulation.

1. **Bone Marrow:** It is a store house of stem cells for haematopoiesis (WBCs, RBCs and platelets) interspersed in meshwork of mesenchymal or stromal cells in the spine and the cavity of long bones. The mesenchyme and osteoblasts secrete supporting factors necessary for the survival of stem cells. All the cells of the immune system are initially derived from the bone marrow. They form through a process called haematopoiesis. During haematopoiesis, bone marrow-derived stem cells differentiate into either mature cells of the immune system or into precursors of cells that migrate out of the bone marrow to continue their maturation elsewhere. The bone marrow produces B cells, natural killer cells, granulocytes and immature thymocytes, in addition to red blood cells and platelets. Tumours may often originate from the transformation of normal stem cells, similar signaling pathways may regulate self-renewal in stem cells and cancer cells, and cancer cells may include 'cancer stem cells'. The latter are rare cells with indefinite potential for self-renewal that drive tumorigenesis. It has been recently shown that that DNA methylation also plays a crucial role for cancer stem cells⁹. When Hematopoietic Stem Cells (HSCs) formed just a little Dnmt1 (DNA methyltransferase 1), the HSCs lost their potential for self-renewal and the HSCs were restricted in their formation of B cells and T cells⁹.

B-lymphocytes (B-cells) are produced in the bone marrow and require bone marrow stromal cells and their cytokines for maturation. During its development, each B-lymphocyte becomes genetically programmed through a series of gene-splicing reactions to produce an antibody capable of binding a specific epitope of an antigen. Antibodies on the surface of B-lymphocytes (surface immunoglobulin, sIg) function as B-cell receptors.

B-lymphocytes are able to recognize soluble antigens as well as microbes such as viruses bound to the B-cell receptor. In addition, B-lymphocytes appear to survey antigen-presenting dendritic cells and acquire antigens from their surface.

The first signal for the activation of a naive B-lymphocyte occurs when B-cell receptors on the surface of the B-lymphocyte bind epitopes of antigens having a corresponding shape. A second signal is also needed for the activation of the naive B-lymphocyte. This is provided when a component of the complement system called C3b binds to the microbial surface. C3b is subsequently degraded to C3d which, in turn, binds to a complement receptor called CR2 on the surface of the B-lymphocyte.

Once bound, the antigen is engulfed, placed in a phagosome, and degraded with lysosomes. During this process, protein antigens are broken down into a series of peptide epitopes. These peptides eventually bind to grooves in MHC-II molecules that are then transported to the surface of the B-lymphocyte.

B cells occupy a unique position in immune responses because they express immunoglobulin (Ig) and class II MHC molecules on their cell surface. They therefore are capable of producing antibody with the same specificity as that expressed by their immunoglobulin receptor; in addition they can function as an antigen presenting cell to a helper T cell. Activation of the T cell results in the production of cytokines that enable the specific B cell to become activated to produce soluble antibodies. Binding of an antigen to the immunoglobulin receptor delivers one signal to the B cell, but that is insufficient. Second signals delivered by costimulatory molecules are required; the most important of these is CD40L on the T cell that binds to CD40 on the B cell to initiate delivery of a second signal.

2. Lymph nodes: Lymph nodes are small encapsulated organs located along the pathway of lymphatic vessels. They vary from about 1 mm to 1 to 2 cm in diameter and are widely distributed throughout the body, with large concentrations occurring in the areas of convergence of lymph vessels. They serve as filters through which lymphocytes percolates on its way to the blood. The microanatomy of lymph node (dark zone, light zone) and B- and T-cell interaction in germinal center during affinity maturation is beautifully shown in images and movies in Science¹⁰.

3. Diffuse Lymphatic Tissue and Lymphatic nodules: The alimentary canal, respiratory passages, and genitourinary tract are guarded by accumulations of lymphatic tissue that are not enclosed by a capsule (i.e. they are *diffuse*) and are found in connective tissue beneath the epithelial mucosa. These cells intercept foreign antigens and then travel to lymph nodes to undergo differentiation and proliferation. Local concentrations of lymphocytes in these systems and other areas are called *lymphatic nodules*. In general these are single and random but are more concentrated in the GI tract in the ileum, appendix, caecum, and tonsils. These are collectively called the Gut Associated Lymphatic Tissue (GALT). MALT (Mucosa Associated Lymphatic Tissue) includes these plus the diffuse lymph tissue in the respiratory tract.

4. The thymus: The thymus is where immature lymphocytes differentiate into T-lymphocytes. The thymus is fully formed and functional at birth. Characteristic features of thymic structure persist until about puberty, when lymphocyte processing and proliferation are dramatically reduced and eventually eliminated and the thymic tissue is largely replaced by adipose tissue. The lymphocytes released by the thymus are carried to lymph nodes, spleen, and other lymphatic tissue where they form colonies. These colonies form the basis of T-lymphocyte proliferation in the specific immune response. T-lymphocytes survive for long periods and recirculate through lymphatic tissues.

The transformation of primitive or immature lymphocytes into T-lymphocytes and their proliferation in the lymph nodes is promoted by a thymic hormone called *thymosin*. Occasionally the thymus persists

after puberty and the continued secretion of thymosin and the production of abnormal T-cells may contribute to some autoimmune disorders. Conversely, lack of thymosin may also allow inadequate immunologic surveillance and thymosin has been used experimentally to stimulate T-lymphocyte proliferation to fight lymphoma and other cancers.

5. **The spleen:** The spleen filters the blood and reacts immunologically to blood-borne antigens. This is both a morphologic (physical) and physiologic process. In addition to large numbers of lymphocytes the spleen contains specialized vascular spaces, a meshwork of reticular cells and fibers, and a rich supply of macrophages which monitor the blood. Connective tissue forms a capsule and trabeculae which contain myofibroblasts, which are contractile. The human spleen holds relatively little blood compared to other mammals, but it has the capacity for contraction to release this blood into the circulation during anoxic stress. White pulp in the spleen contains lymphocytes and is equivalent to other lymphoid tissue, while red pulp contains large numbers of red blood cells, which are filtered and degraded here.

The spleen functions in both the immune and the hematopoietic systems. Immune functions include: removal of antigens from the blood, proliferation of lymphocytes and production of antibodies. Hematopoietic functions include: formation of blood cells during fetal life, removal and destruction of aged, damaged and abnormal red cells and platelets, retrieval of iron from hemoglobin degradation and storage of red blood cells.

PHYSIOLOGY OF THE IMMUNE SYSTEM:

Adaptive (acquired) immunity refers to antigen-specific defence mechanisms that are designed to remove specific antigens. After an antigen contact, this immune response takes several days to become protective.

There are two major branches of the adaptive immune responses:

1. Humoral immunity: involves the production of antibody molecules in response to an antigen and is mediated by B-lymphocytes (*as already discussed*).
2. Cell-mediated immunity: involves different types of T- cells; cytotoxic T-lymphocytes, activated macrophages and activated NK cells. Cytokines are released from T-lymphocytes in response to an antigen.

Helper T cells (CD4+ T-cells)

- Express the CD4 protein on their surface
- Assist other leukocytes in immunological processes.

Sub-population of CD4 cells may suppress the activity of

- (i) B cells via the production of gamma interferon
 - (ii) Cell mediated immune responses via the production of IL-4 and IL-10.
- Present peptide antigens associated with MHC class II on the surface of Antigen Presenting Cells (APCs). The T-cell receptors and CD4 molecules on effector T4-lymphocytes bind to

these MHC-II molecules with the bound peptide epitope on the B-lymphocyte. Once activated, they divide rapidly and secrete cytokines. This enables the effector T4-lymphocytes to produce cytokines such as interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-6 (IL-6). Depending on cytokine signals received, these cells differentiate into TH1, TH2, TH3, TH17, T-Follicular Helper, or one of other subsets, which secrete different cytokines.

Collectively these cytokines:

- a. Enable activated B-lymphocytes to proliferate.
- b. Enable antibody producing cells to switch the class or isotype of antibodies being produced.

Cytotoxic T cells (CTLs, CD8+ T cells)

- Express the CD8 protein on their surface
- Destroy virally infected cells and tumor cells
- Implicated in transplant rejection.
- Associated with MHC class I

Memory T cells (CD4+ or CD8+)

- Persist long after an infection has resolved.
- Expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections
- Subtypes: central and effectors^{11, 12}.

The central memory T-cell subset (TCM) represents the memory stem cells due to their capacity for self-renewal. They are characterised by high cytokine-induced STAT5 phosphorylation. The gene expression profile and flow cytometry of Effector cells (TEM) is different from central memory cells. Also, TEM have been shown to develop effector functions more rapidly than TCM^{12, 13}. TCM are able to release significant amounts of helper cytokines, such as IL-2^{14, 15}. Genes associated with lymphoid homing are highly over-expressed in TCM¹⁵.

Regulatory T cells (Treg cells, Suppressor T cells)

- Two types: naturally occurring (CD4+CD25+FoxP3+) from thymus
- adaptive (Tr1/Th3 cells) during immune response.
- Are crucial for the maintenance of immunological tolerance.
- Shut down T cell-mediated immunity toward the end of an immune reaction
- Suppress auto-reactive T cells that escaped the process of negative selection in the thymus.

Natural killer cells (CD16, CD56+)

- Natural killer T cells (NKT cells) bridge the adaptive immune system with the innate immune system.

- Once activated, the expanded NK cells help to produce a cytokine known as interleukin-10, which effects immunoregulation and inflammation control.
- Recognize and eliminate tumor cells and cells infected with herpes viruses.

Killer cells (K cells) are probably not a separate cell type but rather a separate function of the NK group. K-cells contain immunoglobulin Fc receptors on their surface and are involved in a process known as Antibody-dependent Cell-mediated Cytotoxicity (ADCC). ADCC occurs as a consequence of antibody being bound to a target cell surface via specific antigenic determinants expressed by the target cell. Once bound, the Fc portion of the immunoglobulin can be recognized by the K-cell. Killing then ensues by a mechanism similar to that employed by CTLs.

(II) B-CELL NON-HODGKIN'S LYMPHOMAS

Non-Hodgkin's lymphomas (NHL) are the result of the malignant (cancerous) growth of B or T cells. Although both cell-types can develop into lymphomas, B-cell lymphomas are much more common, accounting for 85% of all cases of NHL compared to T-cell lymphomas, which account for 15% of all cases, according to the American Cancer Society. The major types of B-cell lymphomas are summarised in Table 1.

Following is of interest about B- and T-cell cell lymphoma:

- Clonal lymphoproliferative disorders
- Increasing incidence over past 40 years for unknown reasons
- 50,000 new cases in US per year, many HIV related. In Sweden, the incidence has been increasing¹⁶.
- Heterogenous types of neoplasms; diagnosis of "NHL" or lymphoma gives far less information than specific type.
- Putative cell of origin known for most B cell lymphomas¹⁷ but not in most T cell lymphoma. Most B-lymphomas are derived from germinal-centre (GC) B cells or from B cells that have passed through the GC. The GC is surrounded by a mantle zone of naive B cells (mostly CD5+). In the spleen the marginal zone is a B-cell-rich zone located between B-cell follicles and the T-cell area. The origin of marginal-zone B cells probably includes post-GC memory B cells and naive B cells involved in T-cell-independent immune responses.
 - (i) MALT lymphomas & nodal marginal-zone B-cell lymphomas are derived from marginal-zone B cells
 - (ii) Splenic marginal-zone B-cell lymphomas comprise both follicular and marginal-zone B cells, and often carry unmutated variable (V)-region genes. These lymphomas might therefore be derived from naive B cells prone to undergo marginal-zone B-cell differentiation.
 - (iii) MCL are believed to be derived from CD5+ (naive) B cells of the mantle zone, about 20–30% of cases carry mutated V-region genes, indicating that they have passed through the GC.
 - (iv) CLL cells have mutations in V-region genes in 50% of cases. Both mutated and unmutated subsets of B-CLL have been proposed to derive either from CD5+ B cells, memory B cells or marginal-zone B cells.
 - (v) Post-transplant lymphomas, which often develop in patients after organ transplantation, are often derived from antigen-selected, BCR-expressing GC B cells
 - (vi) Gene-expression profiling identified two main subtypes of diffuse large B-cell lymphoma (DLBCL): one with a profile resembling GC B cells (GC-type), and the other resembling in-vitro-activated B cells (ABC-type)
 - (vi) Primary mediastinal B-cell lymphomas are derived from post-GC B cells of the thymus.
- Cytogenetic translocation, that puts a proto-oncogene or apoptotic gene next to a gene that is constitutively active in lymphocytes, is common.
- 80-85% are B cell, 15 % T-cell but NK cell are rare

- Monoclonality, as determined by antigen receptor gene rearrangement (immunoglobulin or T cell receptor)
- Characteristic patterns of tissue involvement occur, such as follicular lymphomas in B cell areas, T cell lymphomas in paracortical zones
- Most tumors are widely disseminated at diagnosis, requiring systemic therapy for cure. In adults, most common subtypes are follicular lymphoma, diffuse large B cell lymphoma, CLL/SLL and multiple myeloma
- Risk factors: immunodeficiency (primary or secondary), autoimmune disorders (Sjogren's, rheumatoid arthritis, Hashimoto's thyroiditis) in which also chronic antigen stimulation is operating, radiation, chemotherapy and transplantation.
- Chronic antigen stimulation: viral disease (HIV, ATL, KSHV/HHV8, HTLV-1, hepatitis C, EBV), and Helicobacter pylori infection. Some of the infectious diseases also leads to immunodeficiency as HIV.
- Presenting symptoms: lymph node enlargement, B symptoms, extra nodal growth, and involvement of blood, bone marrow, GI-tract, skin or CNS. Sometimes mediastinal involvement (especially lymphoblastic and mediastinal large B cell lymphoma)
- **International Prognostic Index (IPI):** high IPI score is related to a poorer prognosis - age at diagnosis >60 years, presence of B-symptoms, performance status 2-4 vs. 0-1, elevated serum LDH, more than 1 nodal or extranodal sites of disease, advanced vs. localized disease.
- **Follicular Lymphoma Prognostic Index (FLIPI):** high FLIPI score is related to a poorer prognosis - age at diagnosis >60 years, anemia (hemoglobin < 120g/L), elevated serum LDH, more than 4 nodal sites of disease, advanced stage III-IV.
- **Treatment:** addition of rituximab (anti-CD20 antibody) to most regimens has lead to improved survival in B-cell lymphomas
- **Differential Diagnosis:** florid immunoblastic proliferations in infectious mononucleosis or other viral infections, particularly in children; autoimmune lymphoproliferative syndromes in patients with Fas or FasL deficiency , EBV+ post-transplant lymphoproliferative disease

Table1. The major types of B-cell NHL

B-cell NHL	Immunophenotype	Putative cell of origin	Cytogenetics	Clinical Features
Small lymphocytic lymphoma (SLL) /Chronic Lymphocytic Leukemia (CLL)	Pan-B+;CD5+;CD23+; CD10-; sIgM faint, IgM or/and IgD+	Unmutated-CLL stem cell Mutated- B-cell undergone Germinal Center reaction	Del 13q/miR 15,16a ¹⁸ Del 11q/ATM Del 17p/p53 Trisomy 12	Lymphadonopathy, splenomegaly Anemia Trc penia
Lymphoplasmacytic lymphoma (LPL)/ Waldenstroms Macroglobulinemia	Pan-B+; CD5-; CD10-; cylgM+	Peripheral B-lymphocyte transforming into plasma cell with mutated IgV genes and ongoing mutations	t(9;14)(p13;q32) PAX5/IgH (50% of cases) 6q del/ BLIMP ¹⁹ (-) ²⁰	Indolent low-grade disease; IgM monoclonal protein in the blood
Follicular lymphoma	Pan-B+; CD10+/-; CD5-; slg+	Centrocytes / centroblasts of germinal centre origin with somatic hypermutation of the IgV genes and ongoing mutations (antigen driven stimulation)	t(14;18)(q32;q21) / BCL2 + (70-80% of cases)	Indolent. Advanced stages predominate. Grade I-IIIa (indolent) IIIb is different from indolent grade FL.
Diffuse Large B- cell lymphoma	CD19+;CD22+;CD10-/+ ; Slg+	Large transformed B-cells harbouring somatic hypermutation of the Ig genes (ongoing mutations in some cases)	• t(14;18) and p53 mutations (20% of the cases) • t(3;V)(q27;V)/ BCL6 rearr (6-30% of cases) • t(8;14)(q24;q32) or variants c-MYC rearr (7-10% of cases)	▫ Usually aggressive ▫ Immunoblastic lymphoma (Kiel classification) do worse than centroblastic lymphomas ▫ ABC type of DLBCL has worse prognosis than the GC type.
Burkitt's lymphoma	Pan-B+; TdT-; CD10+; CD5-; slgM+	Peripheral B-cells that have encountered the antigen and harbours mutated Ig genes	t(8;14)(q24;q32) or variants /	Extremely aggressive disease
Burkitt-like lymphoma	Pan-B+; TdT-; CD10-/+ CD5-; slg+	Peripheral B-cells that have encountered the antigen	• t(8;14) or variants (25% of cases) • t(8;14)+ t(14;18) (30% of cases)	▫ Aggressive disease ▫ Cases with dual 8;14 and 14;18 translocations have a worse outcome
Mantle cell lymphoma	Pan-B +; CD5+; CD23-; CD10-/+; slgM+ bright	CD5+ B-cells of the follicle mantle mutated as well as unmutated IgV gene sequences	t(11;14)(q13;q32) / BCL1 rearr (50-90%)	▫ Advanced stages predominate ▫ Short survival ▫ Complex karyotype carries an unfavourable prognostic significance
Marginal zone B-cell lymphoma (MZBCL)	pan-B+; CD5-/+; CD10-; CD23-; CD11c+/-; cylg + (40% of the cells), sIgM+ bright; slgD-	Marginal zone lymphocytes harbouring hypermutated IgV genes	t(11;18)(q21;q21) / PI2 / MLT fusion (30-50% of the low-grade MALT)	Extra-nodal low-grade MALT lymphoma
			t(1;14)(p21;q32) del(7)(q22-31) (40% of the cases)	Splenic MZBCL
			+3/+3q (30-70% of the cases)	Nodal, extra-nodal and splenic MZBCL

(III) SOMATIC HYPERMUTATION

Somatic hypermutation (SHM) is a mechanism inside cells that is part of the way the immune system adapts to the antigens or microbes that confront it. SHM diversifies the receptors used by the immune system to recognize antigens and allows the immune system to adapt its response to the new threats during the lifetime of an organism. It involves a programmed process of mutation affecting the variable regions of immunoglobulin genes. Unlike germline mutations, SHM affects only individual immune cells, and the mutations are not transmitted to progeny.

Somatic hypermutation (SHM) of the Ig genes is required for affinity maturation of the humoral response to foreign antigens. It is known that an enzyme called Activation-Induced (Cytidine) Deaminase, or AID^{21, 22} is important in the process of SHM. AID is specifically expressed in germinal center centroblasts and is indispensable for this process. However, AID is also involved in Class Switch Recombination (CSR), hence cannot be treated as a pathognomic hallmark of SHM process^{23, 24}.

Experimental evidence supports the view that the mechanism of SHM involves deamination of cytosine to uracil in DNA by AID^{21, 22}. A cytosine:guanine pair is thus directly mutated to a uracil:guanine mismatch.

(a) Uracil residues are not normally found in DNA, therefore, to maintain the integrity of the genome most of these mutations must be repaired by high-fidelity DNA mismatch repair enzymes. The uracil bases are removed by the repair enzyme, uracil-DNA glycosylase²².

(b) Error-prone DNA polymerases are then recruited to fill in the gap and create mutations^{21, 25}. The synthesis of this new DNA involves error-prone DNA polymerases, which often introduce mutations either at the position of the deaminated cytosine itself or neighboring base pairs.

During the B-cell response to T-cell-dependent antigens, the B cells undergo a rapid proliferative phase in the germinal centre. This is accompanied by the introduction of mutations into the immunoglobulin (Ig) variable region (V) genes. The B cells are then selected according to the affinity of the encoded immunoglobulin for antigen, resulting in affinity maturation of the response. During B cell division the immunoglobulin variable region DNA is transcribed and translated. The introduction of mutations in the rapidly-proliferating population of B cells ultimately culminates in the production of thousands of B cells, possessing slightly different receptors and varying specificity for the antigen, from which the B cell with highest affinities for the antigen can be selected. The B cells with the greatest affinity will then be selected to differentiate into long-lived plasma cells producing antibody and memory B cells contributing to enhanced immune responses upon reinfection.

Mistargeted somatic hypermutation is a likely mechanism in the development of B-cell lymphomas²⁶. When a B cell recognizes an antigen, it is stimulated to divide (or proliferate). During proliferation, the B cell receptor locus undergoes an extremely high rate of somatic mutation that is at least 10⁵-10⁶ fold greater than the normal rate of mutation across the genome. Variation is mainly in the form of single base substitutions, with insertions and deletions being less common. These mutations occur mostly at "hotspots" in the DNA, known as hypervariable regions. These regions correspond to the complementarity determining regions; the sites involved in antigen recognition on the immunoglobulin²⁷. The exact nature of this targeting is poorly understood, although is thought to be controlled by a balance of error-prone and high fidelity repair²⁸. This directed hypermutation allows for the selection of B cells that express immunoglobulin receptors possessing an enhanced ability to recognize and bind a specific foreign antigen.

It is possible that SHM could be a cause of malignancy since it has been shown that the process may generate DNA strand breaks and is known to be able to generate insertions and deletions. Such events may mediate the translocation of genes--a process that is pivotal in the evolution of many lymphomas. Since SHM can occur outside the Ig loci, this process may be involved in distant translocation events²⁹. The importance of SHM is briefly highlighted in some of the lymphoma types:

DLBCL: SHM is now known to also mutate *non-Ig* genes. Mistargeted SHM was first identified in *Bcl6* in approximately half of GC-derived malignancies³⁰ and a minor fraction of normal GC-derived B cells²⁹. The *CD95/Fas* gene was subsequently shown to be mutated in a limited set of normal and malignant post-GC B cells³¹. *Pax5*, *Pim1*, *RhoH/TTF*, and germ-line *c-myc* oncogenes were reported to be hypermutated in DLBCL³². With the exception of *CD95/Fas*, all known hypermutated *non-Ig* genes undergo translocations into the *Ig* locus. SHM of these four oncogenes in DLBCL is predicted to reflect selection, as shown for the CD95/Fas death domain mutations associated with resistance to apoptosis³³. It is important to remember that Bcl6 and GC formation are essential for somatic hypermutation.³⁴ It was found that mutations in the genes encoding the BCR accessory proteins B29 (Igβ, CD79b) and mb1 (Igα, CD79a) occur at frequencies lower than but follow the characteristic pattern in *Ig* genes in a broad spectrum of GC- and post-GC-derived malignant B cell lines, as well as in normal peripheral B cells.³⁵ B29 and mb1 are the first *non-Ig* genes shown to be targeted by SHM that are not proto-oncogenes or tumor suppressor genes. In addition, neither gene is known to undergo translocation or to be directly involved in tumorigenesis.

CLL: It is now a well accepted fact that there are two types of CLL: mutated and unmutated type. The unmutated type of CLL is the one where the cell of origin is a primitive or progenitor stem cell originating in bone marrow and has not undergone the germinal center reaction where SHM takes place. The unmutated CLL is known to be of poor prognosis and correspond to high expression of ZAP70³⁶ and TCL1. Surface IgM that constitutes the BCR and the motif in p53RE region of BCL6 are targets of aberrant somatic hypermutation in CLL. In addition to unmutated status of IgVH region, abnormalities of the TP53 gene are associated with a particularly severe prognosis in

patients with CLL³⁷. Microarray analysis identified BCL6 as a primary target of p53. The BCL6 gene contains a p53 response element (p53RE) residing within the TMDR (translocations, mutations, and deletions region). This p53RE contains a motif known to be preferentially targeted by somatic hypermutation and frequently dysregulated in B-NHL³⁸.

MCL: includes a major subset with unmutated IgVH genes and a smaller subset displaying mutated IgVH genes. Presence of somatic hypermutation in the 'mutated' subset suggests either exposure to a germinal centre (GC) environment or that somatic hypermutation has been acquired in a non-GC context. Furthermore, a preferential IgVH gene utilization has been revealed in MCL, where IgVH3-21 and IgVH4-34 are the most predominant. MCLs with an IgVH 3-21 usage almost exclusively share the same light chain (IgVL 3-19) use and this subset is also related to a better prognosis. MCL cases utilising the IgVH 3-21 gene display less chromosomal alterations than MCLs using other IgVH genes. Thus IgVH 3-21(+) tumours may represent a distinct MCL sub entity with a possible role for antigens in MCL development³⁹.

(IV) MICROARRAYS

The morphology of the tumor and immunohistochemistry (IHC) staining have historically been used for the laboratory diagnosis of malignant lymphomas by the pathologists. Also evaluation of BM trephine biopsy sections with these techniques is well-established in the staging of malignant lymphoma. Technology advances with invention of flow cytometry made it possible to identify surface antigen markers that could accurately diagnose the lymphoma type in cases of doubtful morphology. Thus, both immunohistochemistry and immunophenotyping are now routinely used for the laboratory diagnosis of lymphomas. The schema for immunphenotyping for indolent and mantle cell lymphoma is shown in Fig2 below:

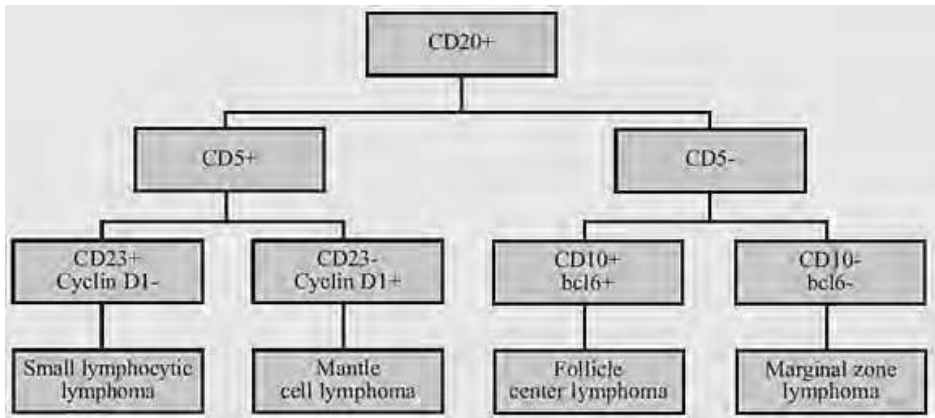


Fig2. Immunphenotyping in indolent B-cell Lymphomas and MCL

However, enough was not enough: soon it was found that even tumors with same diagnosis had variable outcomes and response to treatments. Cytogenetic assessment through use of FISH and CGH arrays was a significant step forward, and pathognomic chromosomal alterations like t(11:14) for MCL and t(8:14) for BL was used to confirm diagnosis. More recently, the advent of gene expression profiling unfolded new horizons to discovery of the genes assignable to specific cellular pathways and has forwarded our understanding about the heterogeneity that exists in lymphoma. Microarrays aided in sub-classifying lymphomas biologically and in the understanding of the functional or cellular processes that are dysregulated.

Gene expression profiling has made significant difference to our understanding of haematological malignancies in general and B-cell lymphomas in particular. DNA microarray technology allows visualisation of the pattern of gene expression involving all of the characteristics of individual cell types, whether they are normal or neoplastic, including the steps of differentiation within cell lineages. In this technique, fragments of selected genes are placed on a matrix to produce a gene microarray, which is then hybridized with complementary DNA synthesized from tumor-cell messenger RNA. A fluorescent label on the complementary DNA allows the relative levels of expression of each

species of messenger RNA to be determined. Whole genome microarrays will probably be used to identify critical subgroups of genes, whose expression can be detected through the use of disease-specific microarrays or protein expression. Advanced bioinformatic tools facilitate identifying this critical subgroup of genes hidden in the large set of genes on the gene chip. Under ideal circumstances, we always aim for finding a master gene—that is causing the lymphoma, is diagnostic of the disease and can be targeted for therapy. A gene of prognostic importance is verified both at the RNA and protein level using qPCR and TMA respectively. Subsequently the marker with good specificity as well as sensitivity is chosen for multicentric studies on full tissue sections before approval into general use.

A prognostic marker should ideally be negative/very weak in one group/ normal tissue and positive in other, much like diagnostic markers. However, discovery for such ideal or perfect markers takes place with time. Mostly, arbitrary cut-offs are used when survival characteristics are significantly different between high and low expression for a marker. Arbitrary cut-offs are manual and individual perceptions vary; thus creating challenges for universal acceptance in the routine use. Hence, the search for perfect candidates goes on.

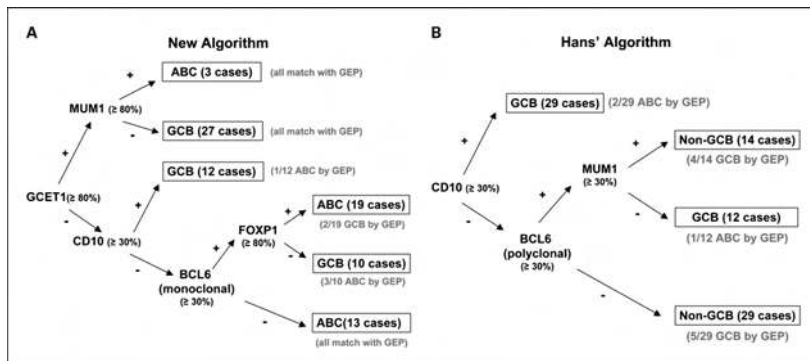
Gene expression profiling (GEP) provides a complete picture of the transcriptome, which reflects the specific activation/differentiation states of a given cell population. The advent of GEP in the field of hematology has allowed advances to be made in defining the molecular taxonomy of lymphomas. For example, Shipp et al. could define DLBCL into three types based on their gene profiles: Oxidative Phosphorylation, Cell cycle-BCR and Stromal/Microenvironment⁴⁰. Tumors that are otherwise morphologically identical can now be classified according to molecular patterns predictive of distinct clinical outcomes. Success with such applications has led to the development and implementation of diagnostic and prognostic strategies based solely on microarray data⁴¹.

Molecular-profiling data on DLBCL is the classical example in lymphomas⁴². Gene arrays helped discovery of two major subtypes of DLBCL: (a) the GC subtype- GEP resembling normal germinal center B cells, and (b) the ABC- GEP resembling activated B cells⁴³ & (c) “unclassified” group^{5, 42}. The GC subtype has better prognosis than the ABC subtype, independent of the IPI⁴⁴, when patients are treated with CHOP or R-CHOP. Their data indicate that the gene-expression patterns of GC and ABC subtypes resemble those of normal germinal-center cells and activated lymphocytes — findings consistent with Lennert and Luke's classification of large-cell lymphomas of B-cell lineage into germinal-center and immunoblastic subtypes⁴⁵.

The challenge is how GEP discoveries could be reduced to best practice, both within the context of clinical trials and the diagnosis/prognosis of cancer in clinical routine. The common approach in translational research has been to “translate” GEPs into protein-based tests such as IHC that can be done routinely on FFPE tissue biopsies. Though RNA from FFPE or frozen tissues can be used for GEP based subclassification or subtyping of tumors with limited number of classifier genes, proteins are appealing as analytes: they are generally well preserved in archival tissue blocks, and

tissue-based analyses provide additional potentially valuable information about the expression of markers within both tumor and stromal cells.

In DLBCL, Hans et al. developed an IHC algorithm⁴⁶, which relied on three markers, MUM1/IRF4, CD10, and BCL6 that are differentially expressed in GC and ABC subtypes of DLBCL. However, the “Hans” algorithm could match GEP classification with 80% concordance. Choi et al. improved the “Hans” algorithm by incorporating two additional markers, GCET1 and FOXP1, which are associated with the GC and ABC DLBCL subtypes, respectively⁴⁷. The “Choi” algorithm reported 93% concordance. Thus by using IHC Hans et al and Choi et al prognostically relevant subgroups of DLBCL can be defined (fig 3).



(Fig3 taken from Choi W W et al. Clin Cancer Res 2009;15:5494-5502)

Thus, the advances in genomics have made progress in IHC possible. However, the process of IHC requires standardization, wherein the process encounters following challenges:-

- The markers should be both specific and sensitive.
- Slide-to-slide variation is inevitable when tumors are stained on individual slides. The use of tissue microarrays (TMA) mitigates this effect in research studies, but is not yet a feasible approach on daily basis for individual patient.
- Regional variation in staining intensity exists in large biopsy sample. TMAs tend to overlook this problem from their sheer small size. The staining intensity can vary because of antibodies.
- The decisions points should ideally be positive or negative staining, but usually are based on pattern identification and cut-offs agreed on by panel of pathologists. Judgement of IHC staining intensity & percent-positive tumor cells is largely subjective, which has facilitated development of robotic computers that can perform multiparametric quantification of proteins in individual cells in tissue sections and can generate quantitative results. New tissue imaging technologies, such as ARIOL provide the choice between bright-field microscopy as well as fluorescence, preserving the ability to assess tissue architecture while allowing performing flow cytometry like analysis on solid sections. However, these platforms still are nascent technologies and used as research tools.

- (e) Presence of standard procedures for the fixation and processing of tissues as well as the performance of IHC across pathology departments. They should perform well and give similar results in multi-center studies.
- (f) The success of markers or algorithms is tied to specific therapies and their predictive power is altered in untreated patients or with new treatments. It is hoped that the discovery of targeted therapies in future will overcome this limitation. One such example is that of FL, where subsets of immune cells determine the prognosis in the patient. However, the use of Immunotherapy with Rituximab is known to alter the stromal milieu wherein the prognostic role of specific marker gets altered⁴⁸.

(V) FUNCTIONAL PATHWAYS IN B-CELL LYMPHOMA

Below are the general features of some of the signaling pathways found relevant in our studies (Fig5):

B-cell Receptor (BCR) Signaling

BCR signaling is known to play a very important role in the pathogenesis through an upstream survival signal in B-NHLs. Normal pre-B lymphocytes have down-regulated expression of Bcl-2, favouring apoptosis. However, the expression of a functional BCR leads to signaling that up-regulates Bcl-2 expression and rescues these cells such that they relocate in the peripheral blood and become mature B cells^{49, 50}. Therefore, during development, B cells are continuously under selective pressure to express functional BCRs. This implies the existence of a basic BCR-mediated signal that provides maintenance of the B cell homeostasis⁵¹. The nature of this constitutive signal is distinct from an antigen-driven signal that leads to proliferation and clonal expansion of the mature B cells, and therefore it is better defined as a basal, or “tonic”, signal^{52, 53}. Perhaps B cells require constitutive low-level receptor engagement with low-affinity autoantigens for survival⁵⁴. Conversely, the tonic signal could be the result of a steady-state level of signaling in unstimulated cells, generated by an equilibrium between positive and negative regulators downstream of the BCR⁵³. It has been demonstrated in vivo experiments that the BCR can cooperate with the MYC oncogene to accelerate lymphomagenesis, and that this acceleration is increased when the BCR is stimulated by cognate antigen⁵⁵. The presence alone of a specific BCR (BCR^{HEL}) seems to intensify the effect of the tonic signal and, when the specific BCR^{HEL} and its cognate sHEL antigen are present, the tonic signal becomes a full strength signal. Another experiment supporting the requirement for BCR signaling in lymphomagenesis involved the silencing of signaling components of the BCR—Igα/Igβ. Tumours isolated from EμMYC/BCR^{HEL} mice were transduced with lentivirus encoding shRNA directed against either Igα or Igβ. The transduced tumour cells were then transplanted into Rag^{-/-} mice that were incapable of any T cell responses to the virus (and thus also incapable of any T-dependent B cell responses). In the absence of Igα/Igβ signaling, the transplanted tumours failed to expand in the immunodeficient recipients.

This result not only confirms the role of BCR signaling in lymphomagenesis but also implies that continuous signaling by the BCR is required for the tumor to thrive.

Ligand-induced BCR signaling induces receptor oligomerization, Igα/β immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation, and activation of the spleen tyrosine kinase (SYK), which initiates downstream events and amplifies the initial BCR signal. BCRs also transmit low-level tonic survival signals in the absence of receptor engagement. Of great interest is the role of proximal BCR signaling molecule, Syk. Once activated, Syk propagates the BCR signal by associating with adaptor proteins and phosphorylating important signaling intermediates, including B-cell linker protein (BLNK), PI3K (phosphatidylinositol 3-kinase) and PLCγ2 (phospholipase Cγ2)⁵⁶. The signaling cascade then proceeds with the activation of downstream signaling molecules that regulate the cellular

response, such as the Akt, extracellular signal-regulated kinase (ERK), c-JUN NH₂-terminal kinase and the nuclear factor (NF)- κ B transcription factor.

Syk has been found to be constitutively phosphorylated through antigen-independent mechanism and contributes to the growth of malignancy FL, DLBCL, MCL^{57, 58, 59, 60}, and CLL⁶¹.

Targeted Syk inhibition prevents increase in leukemic cell viability induced by sustained BCR engagement and inhibits BCR-induced Akt activation and Mcl-1 upregulation. Thus, Syk inhibition could provide a double therapeutic benefit by disrupting both antigen-dependent and antigen-independent signaling pathways that regulate leukemic cell survival in CLL. Aberrant expression and activation of the Src-family kinase Lyn, which can activate Syk by phosphorylating Y352, has also been observed in CLL⁶².

DLBCL that has transcriptome signature of BCR type can be effectively targeted by Syk specific inhibitors. Therefore, SYK-dependent tonic BCR signaling is an important and potentially targetable survival pathway in some, but not all, DLBCLs⁵².

BCR-mediated signaling via phosphorylation of Btk, Syk, Erk1/2, and p38 occurs more rapidly in tumor B cells from FL samples than in infiltrating nontumor B cells, achieved greater levels of per-cell signaling, and sustains this level of signaling longer than in nontumor B cells⁶³.

Germinal Center

The germinal center (GC) reaction is crucial for T cell-dependent immune responses and is targeted by B cell lymphomagenesis. The changes that occur in B cells during GC transit are as follows: naïve B cells → centroblasts → centrocytes → memory B cells.

Naïve B cells are characterized by the expression of cell cycle-inhibitory and antiapoptotic genes, as seen on GEP. The transition of a naïve to a GC B cell is associated with a dramatic change in the expression of genes associated with cell proliferation and DNA mismatch repair genes. *c-Myc* protooncogene is not expressed in the CB. The naïve B cell → CB transition is associated with significant and specific changes in the expression of genes controlling apoptosis. The CB → CC transition involves changes in the expression of genes known to be expressed in B cells undergoing Ig gene rearrangements (immature or “transitional” B cells). The CC → memory B cell transition involves most genes associated with activation, proliferation, and DNA metabolism regains expression levels comparable to those found in the naïve B cells, implying that most post-GC memory cells return to quiescence⁶⁴.

GC of lymphoid organs is the main structure where antigen-activated B cells diversify their immunoglobulin genes by SHM to generate high-affinity antibodies. Most of the cells also undergo CSR to generate antibodies with specialized effector functions⁶⁵. SHM and CSR are associated with DNA-strand breaks. GC B cells use specialized mechanisms that allow for the activity of those DNA-modifying processes without inducing a DNA-damage response, foremost by inhibiting the p53-dependent and p53-independent responses. Centroblasts, which are GC B cells that undergo active SHM, are programmed to proliferate extremely rapidly and thereby generate a large number of immunoglobulin mutations in a short time from which high-affinity antibodies can be selected. Moreover, these cells have a pro-apoptotic programme that ensures the rapid elimination of B cells expressing newly generated antibodies with suboptimal binding characteristics. The transcription factor BCL-6 (B-cell lymphoma 6) is the master regulator of GC B-cell differentiation, as it mediates the repression of genes involved in negative cell cycle regulation as well as the inhibition of genes involved in B-cell activation, plasma-cell and memory B-cell differentiation, and in the response to genotoxic stress. GC B cells that produce high-affinity antibodies are selected to differentiate into plasma cells and memory B cells through specific gene expression changes that coordinately regulate proliferation, apoptosis and differentiation. Essential for these processes are several major transcriptional regulators that, besides BCL-6, include PAX5 (paired box protein 5), NF-κB (nuclear factor-κB), IRF4 (interferon-regulatory factor 4), BLIMP1 (B-lymphocyte-induced maturation protein 1), and XBP1 (X-box binding protein 1)^{66, 65, 41}. Genes critically involved in the regulation of proliferation, apoptosis and differentiation during the GC response are occasionally dysregulated by genomic alterations — such as chromosomal translocations or aberrant SHM in regulatory regions — resulting from errors during antibody gene modifications. Therefore, the production of high-affinity antibodies comes at the risk of oncogenic transformation.

FL, DLBCL and BL originate by transformation of germinal center (GC) B cells. FL and BL are characterized by recurring reciprocal chromosome translocations that fuse *Ig* genes either with the *BCL2* cell survival gene or with the *MYC* proto-oncogene, respectively. Also, reciprocal Chr translocations involving the *BCL6* transcriptional repressor gene and more than 20 partner loci, including the *Ig* genes, typify DLBCL⁶⁷. These and additional tumor-promoting genes are subsequently expressed under the control of *Ig* regulatory sequences rather than their native regulatory elements, resulting in dysregulated expression. Interestingly, *MYC*, *BCL6*, and other genes, such as *TCL1*, are also expressed at high levels in many NHLs in the absence of activating translocations. In these cases, inappropriately high expression is a consequence of other genetic or possibly epigenetic alterations and is likely contributory to the transformation process. B-cell lymphomas in *pE μ -B29-TCL1* transgenic (TCL1-tg) mice develop after long latencies. This indicates that high expression of TCL1 is insufficient to drive transformation and that other genetic or epigenetic changes, presumably occurring in the GC, are required. Aberrant DNA break repair associated with the normal GC processes of *Ig* SHM and CSR serve as possible sources for complementing mutations to facilitate complete transformation. *pE μ -B29-TCL1* lymphomas require the GC to develop and strongly suggest that transformation arises as a result of tumorigenic changes to key GC-related functions and pathways.

TCL1 affects BCR signaling and CD40L-dependent FAS-induced death pathways that set the stage for GC-associated increases in AID expression in p53-suppressed B cells, leading to tolerated genetic errors including aneuploidy and aberrant MYC activation⁶⁸.

CD40 Signaling

In addition to BCR and GC, another important pathway that plays a central role in tandem is the CD40 signaling pathway in B-NHLs.

CD40 is a TNFR family member. Binding of CD40 with its counter receptor, CD154 (CD40L, CD40 ligand), acts on APCs and T-Cells in a bi-directional fashion, mediating both humoral and cellular immune responses. B-Cells depend on CD40 for survival, for expression of costimulatory molecules like B7 (to interact with T-Cells), GC formation, memory generation, Ig CSR and production of numerous cytokines (IL-1, IL-6, IL-8, IL-10, IL-12, TNF-Alpha, MIP1Alpha and cytotoxic radicals)⁶⁹. CD40-mediated signal transduction activates multiple pathways such as NF- κ B, MAPK and STAT3⁷⁰ that regulate gene expression through activation of activating proteins, c-Jun, ATF2 (Activating Transcription Factor-2) and Rel transcription factors. Several TRAF proteins, including TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6, have been shown to interact with CD40 and to play critical roles in CD40-mediated pathways⁷¹. TRAF3 can initiate signaling pathways that lead to the activation of p38 and JNK (c-Jun NH2-terminal kinase) but inhibits Act1-dependent CD40-mediated NF-KappaB activation and initiates CD40L-induced apoptosis. TRAF2 is required for activation of SAPK (Stress-Activated Protein Kinase) pathways and also plays a role in CD40-mediated surface molecule upregulation, IgM secretion in B-Cells and up-regulation of ICAM1 gene⁷¹. Activation of SAPK/JNK and p38 is mediated via TRAF6 whereas ERK1/2 activity is potentially mediated via other TRAF members. Other pathways activated by CD40 stimulation include the JAK3-STAT3 and PI3K-Akt, which may contribute to the antiapoptotic properties conferred by CD40L in B-Cells⁷². CD40 directly binds to JAK3 and mediates STAT3 activation followed by up-regulation of ICAM1, CD23, and LT-Alpha.

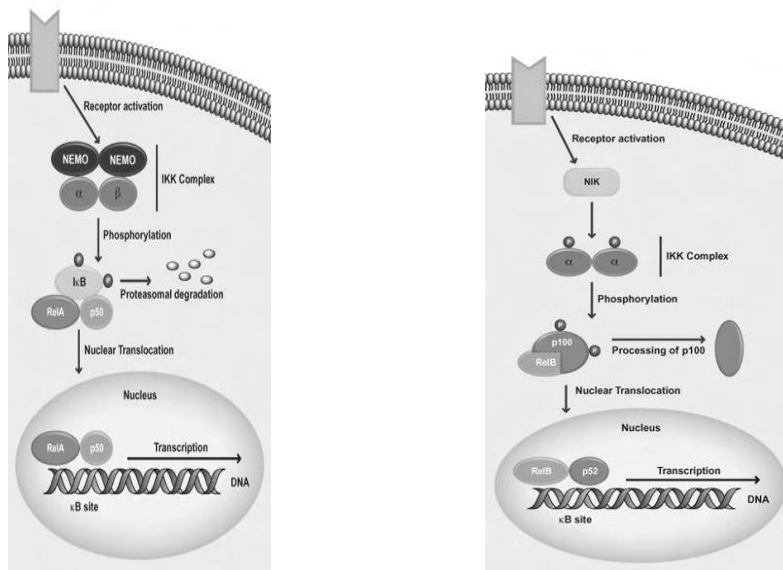
CD40 is widely expressed in B cell lymphomas. Aberrant CD40L expression, which may lead to constitutive CD40 engagement, has been observed in several malignancies, including CLL, MCL, BL and FL^{73, 74, 75, 76}. Constitutive CD40 signaling in B cells induced selective and constitutive activation of the noncanonical NF- κ B pathway and the mitogen-activated protein kinases Jnk and extracellular signal-regulated kinase. LMP1/CD40-expressing mice older than 12 mo developed B cell lymphomas of mono- or oligoclonal origin at high incidence, thus showing that the interplay of the signaling pathways induced by constitutive CD40 signaling is sufficient to initiate a tumorigenic process, ultimately leading to the development of B cell lymphomas⁷⁷.

CD40 signaling translocates NF κ B factors such as c-Rel in naïve and memory B-cells but only in a small subset of GC cells. This shows that GC expansion occurs in the absence of CD40 signaling, which may act only in the initial and final stages of the GC reaction⁷⁸. BCL6 cooperates with constitutive CD40 signaling to rapidly transform p53-deficient primary mouse B cells in vitro towards lymphoma formation. Constitutive CD40 signaling alone does not transform p53-deficient B cells, indicating that BCL6 acts specifically as an immortalizing oncogene in this system⁷⁹.

The antiapoptotic signaling of CD40, which interferes with TRAIL-induced apoptosis in FL B cells, involves NF- κ B-mediated induction of c-FLIP and Bcl-x_L which can respectively interfere with caspase 8 activation or mitochondrial-mediated apoptosis. These findings suggest that a cotreatment with TRAIL and an inhibitor of NF- κ B signaling or a blocking anti-CD40 Ab could be of great interest in FL therapy⁸⁰.

NFκB Pathway

The transcription factor NF-κB is a tightly regulated positive mediator of T- and B-cell development, proliferation, and survival. The controlled activity of NF-κB is required for the coordination of physiologic immune responses. However, constitutive NF-κB activation can promote continuous lymphocyte proliferation and survival and has recently been recognized as a critical pathogenic factor in lymphoma. Various molecular events lead to deregulation of NF-κB signaling in B-NHLs either upstream or downstream of the central IκB kinase⁸¹. These alterations are prerequisites for lymphoma cell cycling and blockage of apoptosis⁸². NFκB (nuclear factor kappa beta) is a transcription factor that plays important roles in the immune system^{83, 84, 85-89, 90}. NFκB regulates the expression of cytokines, inducible nitric oxide synthase (iNOS), cyclo-oxygenase 2 (COX-2), growth factors, inhibitors of apoptosis and effector enzymes in response to ligation of many receptors involved in immunity including T-cell receptors (TCRs), BCRs and members of the Toll-like receptor/IL-1 receptor super family. Pathological dysregulation of NFκB is linked to inflammatory^{91, 92} and autoimmune diseases as well as cancer⁹³. The two pathways of NFκB mechanism are shown in Fig4:-



CANONICAL PATHWAY⁹⁴

NON-CANONICAL PATHWAY

Fig4. Two types of NFκB Pathways

The non-canonical pathway is responsible for the activation of p100/RelB complexes and occurs during the development of lymphoid organs responsible for the generation of B and T lymphocytes. Only a small number of stimuli are known to activate NFκB via this pathway and these factors include lymphotoxin B, B cell activating factor (BAFF)⁹⁵ and casein kinase¹⁹⁶.

The coiled-coil domain normally keeps CARD11 in a latent state in unstimulated B lymphocytes, but when these cells encounter foreign antigen, the coiled-coil region switches from its latent state to one that causes the CARD11 protein to aggregate and stimulate the NFκB pathway. This process is mimicked pathologically by the activating mutations in ABC type of DLBCL⁹⁷.

MAPK Signaling

The MAPKs are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. There are four major groups of MAPKs in mammalian cells—the ERKs (Extracellular signal-Regulated Kinases), the p38MAPKs, the JNKs (c-Jun NH₂-terminal Kinases) and the ERK5 (Extracellular signal-Regulated Kinase-5). The ERK signaling cascade is activated by a wide variety of receptors involved in growth and differentiation including GPCRs (G-Protein Coupled Receptors), RTKs (Receptor Tyrosine Kinases), integrins, and ion channels. Important target of ERK is NF-KappaB: IKK-Alpha (I-KappaB Kinase-Alpha). IKK-Alpha phosphorylates I-KappaB-Alpha, which leads to ubiquitination and then leads to the degradation of I-KappaB-Alpha by the Proteasome, resulting in the translocation of NF-KappaB to the nucleus. In the nucleus it binds to its consensus sequence (5'-GGGACTTTC-3') and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control, and apoptosis^{98, 99}. Other nuclear targets of ERK include the MSKs (Mitogen- and Stress-activated protein Kinases), CREB, c-Myc, HSF1 (Heat-Shock Factor-1), Paxillin and many more transcription factors¹⁰⁰. Activation of the JNK signaling cascade generally results in apoptosis, although it has also been shown to promote cell survival under certain conditions⁹⁹. p38 MAPK is a crucial mediator in the NF-kappaB-dependent gene activation induced by TNF. MAPK pathways are involved in many pathological conditions, including cancer and other diseases. Therefore, a better understanding of the relationship between MAP kinase signal transduction system and the regulation of cell proliferation is essential for the rational design of novel pharmacotherapeutic approaches¹⁰¹.

TCL1 transgenic mice (TCL1-tg), in which TCL1 is ectopically expressed in mature lymphocytes, develop multiple B- and T-cell leukemia and lymphoma subtypes, supporting an oncogenic role for TCL1 that probably involves AKT and MAPK-ERK signaling pathway augmentation. Additional, largely unknown genetic and epigenetic alterations cooperate with TCL1 during lymphoma progression. Sprouty proteins are context-dependent negative or positive regulators of MAPK-ERK pathway signaling. Repression of Spry2 expression in TCL1-tg mouse and human B-cell lymphomas and cell lines is associated with dense DNA hypermethylation. Spry2 expression was induced in normal splenic B cells by CD40/B-cell receptor costimulation and regulated a negative feedback loop that repressed MAPK-ERK signaling and decreased B-cell viability. Conversely, loss of Spry2 function hyperactivated MAPK-ERK signaling and caused increased B-cell proliferation. Combined, these results implicate epigenetic silencing of Spry2 expression in B lymphoma progression and suggest it as a companion lesion to ectopic TCL1 expression in enhancing MAPK-ERK pathway signaling¹⁰².

MAPK signal transduction is very important for cell proliferation and cell cycle progression and has become an important target in many kinds of cancers. Dysregulation of several growth factor receptors connected to p38MAPK occurs in FL transformation. Dysregulation of p38MAPK target genes may induce growth factors and cytokine genes and thus exert an autocrine-paracrine effect that further promotes lymphoma progression¹⁰³.

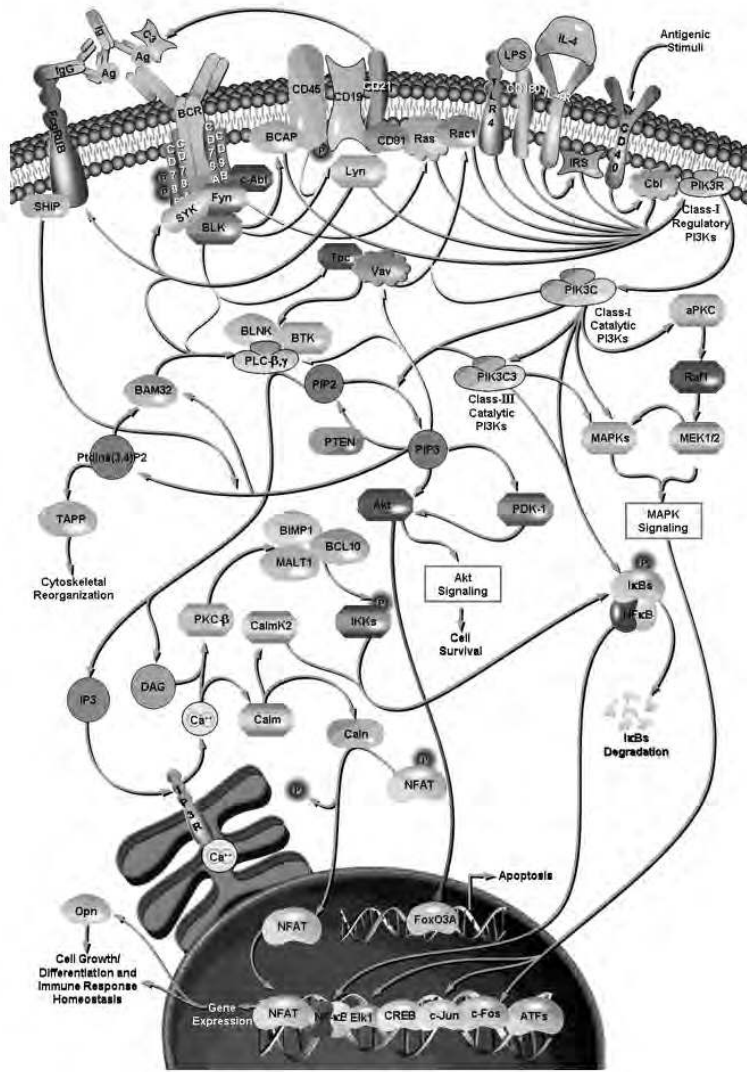


Fig5: Signaling pathways

PI3K-AKT Signaling

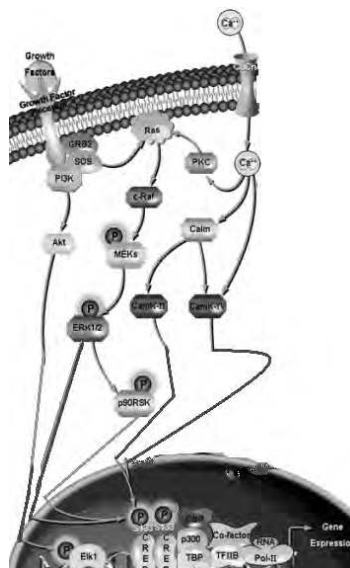
PI3Ks (Phosphoinositide-3-Kinases) regulate numerous biological processes, including cell growth, differentiation, survival, proliferation, migration and metabolism. In the immune system, impaired PI3K signaling leads to immunodeficiency, whereas unrestrained PI3K signaling contributes to autoimmunity and leukemia. The Class I and III PI3Ks basically facilitate B-cell development through defined stages, resulting in at least three distinct lineages of mature B-lymphocytes. In B-cells, PI3K is activated within seconds of antigen-receptor triggering. The BCR plays a critical role in recognition of antigens and activation of B-cells. The BCR Ig is associated with Ig-Alpha/CD79A and Ig-Beta/CD79B heterodimer. The BCR membrane Ig subunits bind antigen and cause receptor aggregation, while the Ig-Alpha/Ig-Beta subunits transduce signals to the cell interior^{104, 105, 106}. Engagement of BCR-antigen complex activates intracellular protein tyrosine kinases like SYK, BTK (Bruton's Tyrosine Kinase) and Fyn (Fyn Oncogene Related to Src). These tyrosine kinases phosphorylate the co-receptors CD19 and BCAP (B-Cell Adaptor Protein) at the YXXM motifs, which provide binding sites for PI3Ks. CD19 is one of the main regulators of PI3K activity in B-cells. CD19 has an important, but not indispensable, role in PI3K activation rather it is required for sustained PI3K activation after BCR stimulation. The co-receptor complex is also composed of CD21 and CD81. CD21 binds opsonised antigenic particles and activate complement component-C3, a reaction central to complement function in the immune response and sustained BCR signaling. Many other transmembrane receptors are known to modulate specific elements of BCR signaling. A few of these are CD45 and Fc-GammaRIIB (Low Affinity Immunoglobulin Gamma Fc Region Receptor-IIIB). CD45 occurs as a component of a complex of proteins associated with the antigen receptor, and may regulate signal transduction by modulating the phosphorylation state of the co-receptors like CD19^{105, 106, 107}. Moreover for B-cell development, the capacity of CD19 to promote thymus-dependent immune responses is linked to its capacity to recruit and activate PI3K. Understanding the contributions of PI3Ks to lymphocyte biology may contribute to the development of treatments for immune-related diseases, including autoimmunity, leukaemia and graft rejection¹⁰⁸.

Activation of Akt can begin with several events, mainly the binding of a Ligand to a Receptor in the cell membrane. Most common Ligands activating Akt include growth factors, cytokines, mitogens and hormones. PI3K is recruited to the phosphotyrosine residues (consensus sequence pYXXM) via SH2 domains in the regulatory domain (p85), and is therefore targeted to the inner cell membrane. The actions of Akt in the cell are numerous and diverse, but all result in anti-apoptosis, or pro-cell proliferation effects. These physiological roles of Akt include involvement in metabolism, protein synthesis, apoptosis pathways, transcription factor regulation and the cell cycle. Akt inhibits apoptosis by phosphorylating the BAD component of the BAD/BclXL (Bcl2 Related Protein Long Isoform) complex. Phosphorylated BAD leads to dissociation of the BAD/BclXL complex, thus allowing cell survival. Akt activates IKK, which ultimately leads to NF- κ B activation and cell survival. AKT impair Chk1 through phosphorylation, ubiquitination, and reduced nuclear localization to promote

genomic instability in tumor cells. Akt and its upstream regulators are deregulated in a wide range of solid tumors and hematologic malignancies, hence the Akt pathway is considered a key determinant of biologic aggressiveness of these tumors, and a major potential target for novel anti-cancer therapies^{109, 110}. Akt also phosphorylates MDM2. MDM2 is phosphorylated at many sites, only two of which have been identified. Ser166 is phosphorylated by Akt. Akt phosphorylation of MDM2 allows its entry into the nucleus where it targets p53 for degradation¹¹¹. Akt also activates TERT (Telomere Reverse Transcriptase), which is responsible for telomere maintenance and DNA stability.

CREB Pathway

The richness of CREB signaling is greatly increased by its responsiveness to multiple intracellular signal transduction cascades and the potential for this family of transcription factors to induce and suppress gene expression renders them ideally suited for regulating gene expression. The cAMP/CREB signaling pathway has been strongly implicated in the regulation of a wide range of biological functions such as growth factor-dependent cell proliferation and survival and glucose homeostasis. Upon stimulation of cellular GPCR (G-Protein-Coupled Receptors) and growth factor receptors, AC (Adenylate Cyclase) is activated, by G-proteins: GN-Alpha, GN-Beta and GN-Gamma leading to increases in cAMP. This in turn activates PKA by dissociating the regulatory (PKAR) from the catalytic (PKAC) subunits. In the basal state, PKA resides in the cytoplasm as an inactive heterotetramer of paired regulatory and catalytic subunits. Induction of cAMP liberates the catalytic subunits. This activated PKAC then recruits the Ca²⁺/CalmK-IV (Calmodulin (Calm)-dependent Kinases), MEK (MAPK/ERK Kinases)/ ERK1/2 (Extracellular Signal-Regulated Kinases) and together they translocate to the nucleus. The accumulation of cAMP in response to activation of GPCR also induces PLC-γ (Phospholipase-C-Gamma) that catalyzes the formation of DAG (Diacylglycerol), a PKC activator through PI (Phosphatidylinositols). PI3K is responsible for activation of Akt/PKB (Protein Kinase-B) which directly or indirectly affects CREB. In lymphoma, GC signaling through CD40 or the BCR activates pCREB-dependent genes leading to TORC2 phosphorylation, cytosolic emigration, and TCL1 repression. Signaling via cAMP-inducible pathways inhibits TCL1 repression and reduces apoptosis which is consistent with the prosurvival role of TCL1 before GC selection. The CREB/TORC2 regulatory mode controls the normal program of GC gene activation and repression that promotes B cell development and circumvents oncogenic progression¹¹². This is represented in Fig6.



BLIMP-1 Targets

This zinc finger protein is specifically expressed in a subset of germinal center B cells and plasma cells. The differentiation of mature B cells into antibody-secreting plasma cells is controlled by the presence or absence of transcription factors, including B-lymphocyte-induced maturation protein 1 (BLIMP1) and B-cell lymphoma 6 (BCL6), respectively. Recent work has enabled the genes that are targeted by these transcriptional repressors to be identified and has provided an insight into how they interact to control terminal B-cell differentiation.

Forced expression of BLIMP1 drives mature B cells to differentiate into plasma cells. Targets of BLIMP1-dependent repression, such as c-MYC, CIITA and PAX5, have been identified previously, but the control of these genes is not sufficient to explain how BLIMP1 regulates terminal B-cell differentiation. To investigate this further, Shaffer et al. carried out a DNA-microarray analysis of the changes in gene expression that are induced by BLIMP1¹¹³. Totally 228 genes are repressed and 32 genes are induced in B-cell lines do overexpress BLIMP1 compared with control cells. In many cases, microarray targets were confirmed by independent messenger-RNA and protein-expression assays. BLIMP1 was shown to promote B-cell terminal differentiation by turning off the expression of genes that are associated with cell-cycle progression, and DNA synthesis and repair, as well as those that are involved in mature B-cell function. These include several transcription factors, such as BCL6 and two newly identified targets, SPI-B and ID3. BLIMP1 also down regulates the expression of genes that are required for immunoglobulin class switching (such as AID, KU70, KU86, DNA-PKCs and STAT6), thereby inhibiting this process. Of the genes that are turned on, of particular interest is the transcriptional activator XBP1, which is a crucial regulator of plasma-cell differentiation. However, the modest increase in XBP1 mRNA in the presence of BLIMP1 indicates that other pathways are also likely to activate XBP1 transcription during plasmacytic differentiation. Therefore, BLIMP1 promotes plasmacytic differentiation by switching off genes that are required for mature B-cell function and switching on plasma-cell genes.

The down regulation of expression of BCL6 by BLIMP1 is consistent with earlier work showing that BCL6 is highly expressed in germinal-centre B cells, but that it is downregulated as B cells differentiate into plasma cells. BCL6 has been shown previously to repress the transcription of BLIMP1, so Shaffer et al. conclude that a reciprocal regulatory loop might exist whereby BCL6 and BLIMP1 antagonize the expression of each other.

But, how does BCL6 repress the expression of BLIMP1 and other target genes? Work about the interactions between BCL6 and the BLIMP1 promoter has shown that BCL6 represses BLIMP1 expression by blocking the transcriptional activity of AP1 factors¹¹⁴.

(VI) MICROENVIRONMENT IN B-CELL NON-HODGKIN'S LYMPHOMAS

The germinal centers (GCs) of lymphoid follicles contain two different micro environmental zones: the "dark" zone and the "light" zone. The "dark" zone at the base of the follicular center contains tightly packed centroblasts, whereas the "light" zone, at the apex, contains smaller non dividing centrocytes. The follicular dendritic cell (FDC) network extends throughout the GC, but is much sparser in the dark than in the light zone. Some CD4 T cells are found in the light zones. There is a sharp demarcation around the whole follicle center, which is highlighted by fibroblastic reticulum cells (FRCs). Tingible body macrophages (TBMs), containing nuclear debris in their cytoplasm, are located throughout the GCs, but their number is highest in the basal light zone⁶⁵. Studies using in vivo imaging show that zones within GCs are plastic and that both B and T cells traffic between compartments in a dynamic fashion¹¹⁵. Noticeably, tumor cells of these malignancies reside and proliferate in close association with cellular environment that retains key features of normal GC cellular microenvironment.

FOLLICULAR LYMPHOMA

Role of microenvironment in pathogenesis:

In FL, previous original data from gene expression profiling analyses have been confirmed by immunohistochemical and flow cytometry studies, showing the *in loco* presence of the main components of the anti-tumor immune response, such as specific CD4⁺ T-helper lymphocytes^{116, 117, 118} cytotoxic cells including CD8⁺ T lymphocytes^{119, 120} and CD68⁺ macrophages^{121, 122}. Other studies have also highlighted the presence of specific CD57⁺ T-helper cells¹¹⁹, signal transducer and activator of transcription 1 (STAT-1)-positive tumor-associated macrophages¹²² and FOXP3⁺ regulatory T cells. Dysfunctional immune profiles in the tumor microenvironment of FL can also lead to reprogrammed immune cells such as tumor-associated macrophages. The dual nature of the polarized M1 and M2 macrophages implies differences that may be exhibited as either antitumor cytotoxic activity or facilitated tumor growth and progression¹²³. Finally, follicular dendritic cells are able to activate caspases, thus preventing the spontaneous apoptosis of FL tumoral B cells¹²⁴.

Role of microenvironment in diagnosis:

In FL, the tumor cells reside and proliferate in follicular structures in close association with helper T cells and FDCs^{125, 126}. Neoplastic follicles in FL contain, in addition to FDC (CD21+, CD23+) other non-neoplastic cells normally found in GC including macrophages (CD68+) and GC T cells (CD31, CD41, CD57+, PD1+, and CXCL131). So the lymphoma cells seem to retain key features of normal GC B cells including the interaction with T cells and FDCs in the follicular microenvironment¹²⁷. The differential diagnosis of the GC-derived lymphomas from their reactive counterparts such as hyperplasia is possible through their micro environmental findings. In approximately 85% of grade I and grade II FL, GC cells express BCL2 protein. The remaining 15% of grade I and grade II FL and about the 50% of grade III FL are BCL2 negative. Thus, BCL2 protein

expression can be useful in identifying neoplastic follicles, but absence of BCL2 protein does not exclude FL diagnosis. In the BCL2 negative FL cases confusion with floridly reactive lymph node is usually avoided on the basis of the following micro environmental findings:

- First, in contrast to reactive GC where CB and CC occupy different zones, in FL the two types of cells are randomly distributed.
- Furthermore, although the meshworks of FDC are present in reactive and neoplastic follicular areas, these may be sparser in neoplastic than in normal follicles and may variably express CD21 and CD23.
- Finally, tingible body macrophages (TBM) that are characteristic of reactive GCs are usually absent in FL¹²⁸

Role of microenvironment in prognosis:

The genes that best defined the prognostic signatures in follicular lymphoma are expressed primarily by T cells, macrophages, or dendritic cells, but not by the tumor cells themselves. This finding indicates that the aggressiveness of the disease is mainly determined by the cellular microenvironment of the lymphoma, and not by obvious differences in the gene-expression profiles of the malignant cells themselves, although the cellular microenvironment may well be influenced in important ways by the lymphoma cells. Thus, from the molecular standpoint, follicular lymphoma is clearly different from diffuse large-B-cell lymphomas and mantle-cell lymphomas, in which the prognostic signatures are based largely on genes expressed by the lymphoma cells. The dominant influence of the cellular microenvironment on the prognosis of follicular lymphoma probably reflects the participation of immune cells in the biology and pathogenesis of this type of tumor. Indeed, other features of follicular lymphoma also indicate the importance of the cellular microenvironment. In follicular lymphoma, the histologic features resemble the structure of normal germinal centers and show a characteristic association of the lymphoma cells with helper T cells, follicular dendritic cells, and macrophages in the follicles. The tumor cells, like normal germinal-center B cells, seem to need a close association and interaction with T cells and dendritic cells. Moreover, the macrophages that normally remove apoptotic germinal-center B cells are also likely to play a role in follicular lymphoma. The dependency of follicular lymphoma cells on their microenvironment is supported by the fact that these cells are very difficult to grow *in vitro* in the absence of stromal cells and without stimulation of the CD40 receptor, which is the main signaling pathway for interactions between B cells and T cells.

CHRONIC LYMPHOCYTIC LEUKEMIA

The host microenvironment and the resulting interplay between the genetic background and environmental influences play a crucial role in disease progression, as well as in resistance to treatment. The results presented by Stamatopoulos and colleagues further support the view that a favorable microenvironment can provide the optimal combination of antigen and co-signals for expansion of the CLL-initiating cells, leading to disease progression¹²⁹. Instead, under environmental conditions which do not favor growth, proliferation of the same CLL cells is impaired, and the

molecular mechanisms preventing apoptosis prevail (Fig8). The clinical conditions of the patient reflect the overall equilibrium between these two opposing processes.

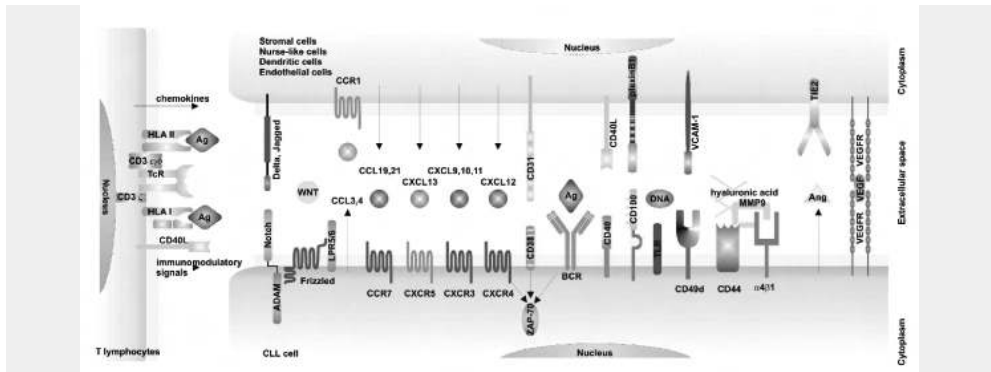


Fig 7: Main signaling pathways regulating the interactions between chronic lymphocytic leukemia cells and the microenvironment.

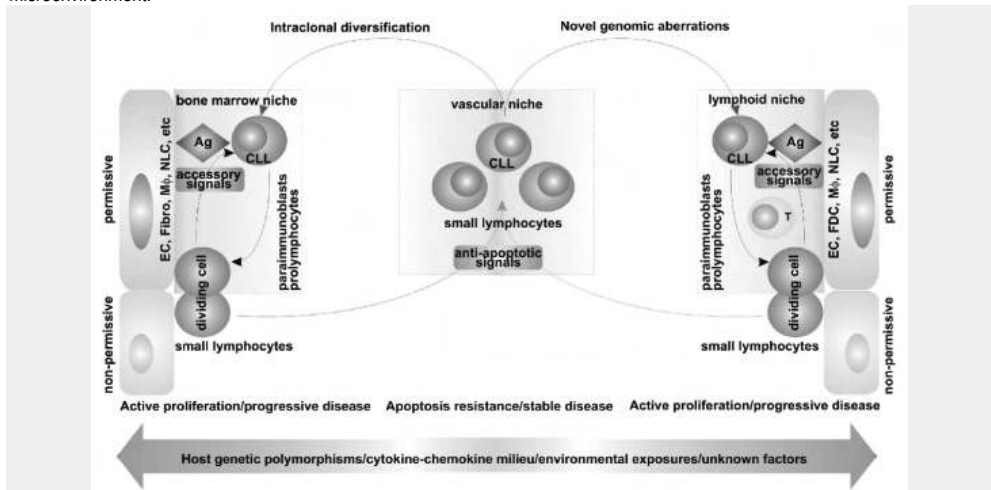


Fig8: Hypothetical model explaining the key role played by the microenvironment in maintenance and progression of chronic lymphocytic leukemia. EC: endothelial cells; Fibro: fibroblasts; Mφ: macrophages; NLC: nurse-like cells; Ag: antigen.

(Figures 7 and 8 are taken from Deaglio S, Malavasi F. *Haematologica*. 2009 Jun;94(6):752-6)

CXCR4 is expressed by most circulating CLL cells at high levels, independently from molecular markers, clinical stages or patterns of BM infiltration. BM stromal or nurse-like (NLC) cells constitutively secrete CXCL12, the ligand of CXCR4. Consequently, the CXCL12/CXCR4 axis plays a crucial role in the recruitment of neoplastic cells to growth-favorable environments¹³⁰ (Fig7).

CLL cells can also actively secrete chemokines to modify the environment by recruiting accessory cells and creating growth-permissive conditions. Although preliminary, recent results, concur that

BCR- and CD38-mediated signals effectively increase secretion of IL3 and IL4, recruiting supportive CD68⁺ macrophages and nurse like cells (NLC)^{131, 132}.

Increased angiogenesis has been consistently associated with more advanced disease phases and it is known that CLL cells themselves can secrete pro-angiogenic factors [including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and angiopoietin (Ang)] and drive the construction of new blood vessels. These newly formed vessels are characterized by increased permeability, and thus contribute to disease dissemination. CLL cells experience prolongation of survival signal due to expression of receptors for some of these pro-angiogenic factors [including VEGF receptors (VEGFR) 1 and 2 and Tie-2, the Ang receptor]¹³³.

MANTLE CELL LYMPHOMA

- (a) Role in pathogenesis and prognosis: Microenvironment is believed to play a minor role in the pathogenesis of MCL since (i) T-cell and accessory cell components are mostly sparse and (ii) prognostic gene expression signatures are dominated by proliferative tumor cell proliferation¹³⁴.
- (b) Role in drug resistance: MCL is characterized by an early, widespread dissemination and residual disease after conventional treatment. It has been shown that MSCs confer drug resistance to MCL cells that migrate beneath them. MCL cells (i) display high levels of functional CXCR4 and CXCR5 chemokine receptors and VLA-4 adhesion molecules. (ii) adhere and spontaneously migrate beneath marrow stromal cells (MSCs) in a CXCR4- and VLA-4-dependent fashion¹³⁵.

DIFFUSE LARGE B-CELL LYMPHOMA

(a) Role of microenvironment in pathogenesis

GEP studies have identified biological subtypes, defined by “BCR/proliferation”, “OxPhos” and “host response (HR)” independent of “cell of origin”. HR is made up of activated T/NK cells, macrophages, (S100+) dendritic cells, stromal cells. Their function may be either growth supportive or cytotoxic (or both)⁴⁰ (Fig9)

Distinction between ABC/GCB subtypes based on functional gene clusters, including “lymph node signature”. Lymph node signature contains macrophage and matrix information and is correlated with favourable prognosis, suggesting a cytotoxic response⁴².

immune microenvironment in DLBCL

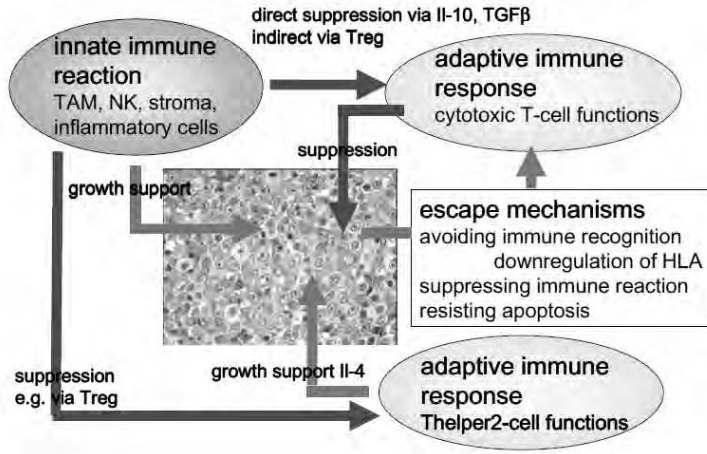


Figure 9. Immune microenvironment in DLBCL

(b) Role of microenvironment in prognosis

Immunohistochemical studies have shown two types of DLBCL^{136, 137, 138, 139, 140, 141}:

(i) Good prognosis type with effective cytotoxic response. It is characterised by:

- Presence of S100/CD1a+ IDC and CD21+ FDC
- Dense infiltrate of CTL, FOXP3+ Tregs¹³⁸ and mast cells

(ii) Poor prognostic type is characterised by dense CTL infiltrates.

Besides, the role of microenvironment has been highlighted in gene expression profiling studies as well. It was found that two stromal signatures exist in DLBCL: one that controls the mesenchymal genes and other that controls the angiogenic switch genes. DLBCL tumors with high vascularity due to angiogenic switch are known to be poor prognostic type¹⁴².

AIMS OF THE THESIS

The overall aim of the thesis was to analyze the biological or functional molecular pathways in major types of B-cell lymphoma

More specifically the aims were:

- To better understand the biology of different B-cell lymphoma subtypes in terms of functional pathways
- To find the mechanisms and role of TCL1A oncogene in MCL and CLL
- To understand the process of somatic hypermutation in B-cell lymphoma
- To identify the prognostic role of immune cell subsets in tumor microenvironment in Follicular Lymphoma

METHODS

The overall methodology is shown in Figure 10.

A) RNA Expression by DNA Microarrays (Paper I,II and II):

1)Gene expression profiling¹⁴³ was done using cDNA microarray Oncochip and data was normalised against normal lymph nodes. The lymphoma molecular subtypes were obtained on unsupervised hierarchical clustering

2)The molecular profile data was analysed using Gene Set Enrichment Analysis tool (M.I.T v2)¹⁴⁴.

3)By exploring the differential expression between lymphoma subtypes so obtained alongwith clinical risk groups, we could test for candidate genes from representative pathways as potential prognostic markers (www.gepas.org/T-rex)

B) Protein expression analysis on Tissue Microarrays (TMA) using Automated System (ARIOL) (Paper II, III and IV):

The protein expression for any gene was found on Tissue Microarrays and confirmed as validation step against full size IHC sections. The TMA for MCL used in Paper II and III are same while TMA used for FL in Paper IV is independent one.

Protein expression for TCL1 was assessed using automated microscopy which is a state-of-art technology. We also used ARIOL¹⁴⁵ to compare the expression of tumor and microenvironment markers in follicular lymphoma in solid tissue specimens from that of fluid tissues using flow cytometry (Paper IV). The cell populations were quantified using an automated scanning microscope and computerized image analysis system (Ariol SL-50; Genetix Ltd, Queensway, New Milton, Hampshire, United Kingdom) under the supervision of an expert hematologic-pathologic team. The follicular and interfollicular areas within each core were defined, since the system allows for precise demarcation by drawing boundaries on the virtual slides ARIOL provides the advantage for studying the actual distribution of lymphocyte types in the lymphoid organs, since the architecture is preserved in solid specimens.

C) **Statistics:** The Kaplan Meir survival curves for assessing the prognostic significance of a marker was done using SPSS (Paper II and III).

D) Semi-nested PCR was used to amplify the rearranged IgVH regions and compared to original sequence to assess the load of mutation. **Molecular cloning** techniques were used for assessment of ongoing mutation analysis: the PCR amplified Vh regions was cloned to TOPO vector and DNA obtained from bacterial colonies for sequencing (Paper III).

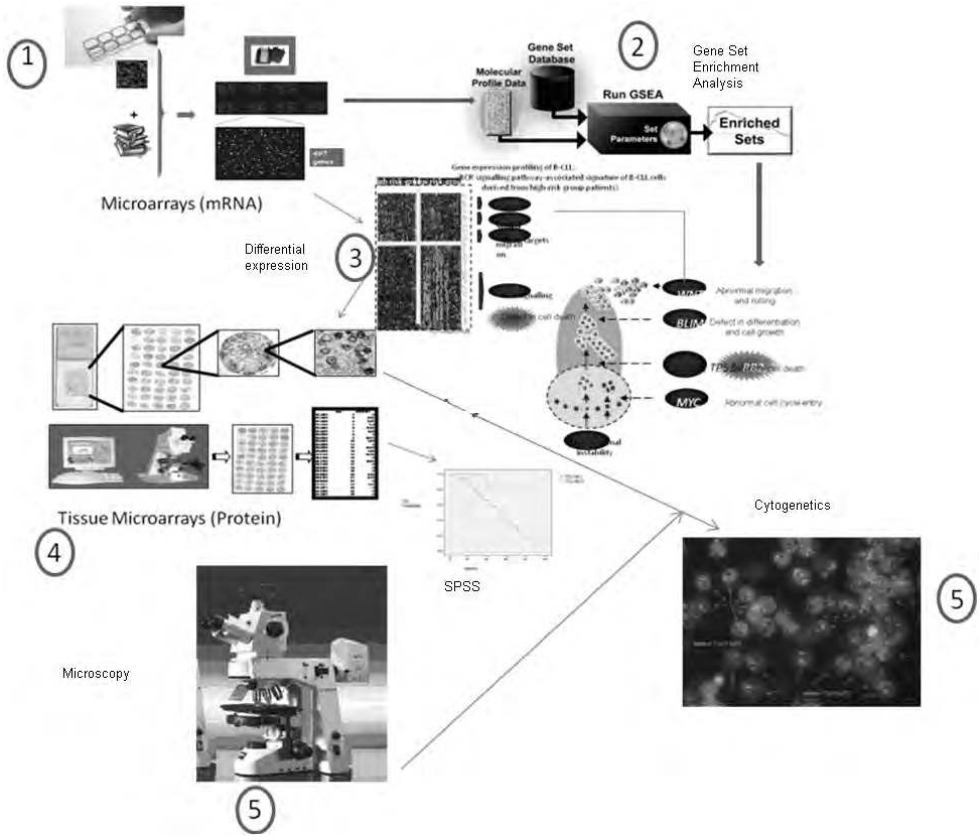


Figure 10: Methodology

RESULTS AND DISCUSSION

PAPER I

Functional signatures identified in B-cell Non-Hodgkin Lymphoma profiles

The role of functional classification is well established in DLBCL and yielded immunostain-algorithms that will pave way for future prognostic markers also in routine practise. Functional pathways have been studied in other types of lymphomas as well, although individually. Molecular biology studies of oncogenes, tumor suppressor genes, facilitatory genes or interaction of genes in signal transduction pathways continue to unfold the mystery and enhance our understanding of the pathogenic mechanisms in lymphomas.

Aims:

The objective of this project was to investigate the role of biological signatures or functional pathways involved in major types of B-cell NHL. The unique feature of the study was to analyse all the lymphoma types at the same time. The advantage of this approach gives us a macroscopic view, contrary to the microscopic view when we analyse lymphoma types separately when looking at the genetic level. Macroscopic analysis allowed us to see what dysfunctions (up regulation or down regulation) are affecting important biological pathways implicated in the biology of lymphomas. This approach allowed us:

- to understand the similarities as well as differences in the expression of pathways between different lymphoma types.
- to appreciate that such similarities and differences exist within each lymphoma type itself.

The idea was, however, not to establish a new system of sub-classification within lymphoma types or replace existing pathological or molecular classification, but to appreciate heterogeneity as defined by functional pathways within lymphoma types diagnosed under WHO diagnostic criteria. Of course it would be very interesting to identify robust molecular classification for all lymphoma types as we have for DLBCL, but demands large collection of patient samples for each lymphoma type and external validation with same or at least similar microarray platforms.

Methods:

Unsupervised clustering of cases yielded groups that could be analysed for biological understanding using the correlation clustering to enrichment score values obtained from Gene Set Enrichment analysis. The pathways were found to cluster into biologically understandable clusters, called functional signatures. These signatures are already known to play important roles in the pathogenesis of B-cell NHL and included B-cell Receptor pathway, Germinal Center, CD40 Signaling, NF-kB pathway, Apoptosis, Cytokines, MAP kinases and the stroma/Microenvironment. This validates our already known understanding of cancer development- that although oncogene/TSG are master lesions, it is the interplay and support role of all these other factors that lead to lymphoma.

We also included the SHM genes that are already known and those we had identified in Paper III into functional pathway. Therapeutic clusters were also included; this shall continue to facilitate development of targeted therapy in NHL. To explore the role of microarrays and therapeutic targets, we started a project using Connectivity Maps already used in ALL¹⁴⁶. However, large consortium studies and collaboration are needed to develop such tools solely for lymphoma therapeutics and both pre-clinical & clinical trials will be needed.

Results:

Of notable importance is the role of BCR that we explored further to understand the intra-tumoral heterogeneity. Equally interesting was the role of TCL1 oncogene in lymphoma patients, which we explored separately in paper II. The reason we chose to look through BCR defined heterogeneity is that BCR is known to have an important biological role in the pathogenesis of CLL, MCL and FL. The pathways that correlated with expression of BCR proximal genes were mainly ascribable to BCR signal pathway, B-cells, BLIMP-1 targets and GC- T helper cells and represented a spectrum of heterogeneity across patient samples from external datasets in CLL, MCL, FL and DLBCL.

Discussion:

The functional pathways clustered to biologically understandable signatures facilitating broad view of the deregulation of cell signaling in major types of B-cell Non-Hodgkin's Lymphoma. Cell cycle, BCR and GC signatures held striking variability within diagnostic lymphoma types. The presence of NFkB, apoptosis and associated pathways signature in the microenvironment suggests their role in lymphoma cells as well as the supporting cells in the microenvironment. Of interest are those genes that are targets of some of the important transcription factors such as BLIMP-1 and XBP1.

PAPER II

TCL1A expression delineates biological and clinical variability in B-cell lymphoma

Background:

Transgenic mice with TCL1 over-expression develop B and T cell malignancies of host type¹⁴⁷. μ -TCL1 transgenic mice are now widely accepted models for CLL¹⁴⁸, in which TCL1 over-expression is associated with down-regulation of miR-29 and miR-181¹⁴⁹ and correlate with the proliferative, unmutated¹⁵⁰ or ZAP70+ CLL type⁶. It is important, however, to remember that TCL1 is not the “master lesion” in CLL but an important oncogene implicated in the pathogenesis of CLL. It has been shown that TCL1 transgenic B cells primed for transformation must experience the GC environment to develop genome instability and become fully malignant⁶⁸.

The genes interacting with TCL1 have been a matter of speculation. Recently it was shown that components of the NF κ B pathway and not AKT are the central pathway for TCL1 to exert its effects. We have shown through microarray studies that TCL1 expression correlates with expression of genes from NF κ B, CREB and BCR¹⁵¹ pathways. Later, through co-immunoprecipitation technique, it was proven in CLL that Tc1 physically interacts with c-Jun, JunB, and c-Fos inhibits AP-1 transcriptional activity and activates NF-kappaB by physically interacting with the p300/CREB binding protein⁶. GC signaling through CD40 or the BCR activates pCREB-dependent genes, causes TORC2 (CREB regulated transcription co activator 2) phosphorylation, cytosolic emigration, and TCL1 repression. It was shown that CREB coactivator TORC2 directly regulates TCL1 expression¹¹².

Methods: Gene Expression Profiling, Gene Set Enrichment Analysis and Oncomine.

Results: In this study, we have demonstrated an association between TCL1A expression and essential pathways for B-cell survival, including BCR, CD40 signaling, NF κ B, TOLL and Calcium signaling pathways. The prognostic importance of TCL1 in MCL and CLL was also demonstrated in patient samples.

Discussion: The role of TCL1 is not the sole possession of CLL, but is expressed variably in MCL, FL, and DLBCL, with virtual absence from post-GC tumors¹⁵². We showed expression of TCL1 to be quite variable in DLBCL, from low to high, while it was variable but high in FL and CLL. It would be interesting to study the role of TCL1 in FL, its expression being so heterogeneous. While TCL1 expression in mature T-cell tumors is indicative of TCL1 gene translocation, TCL1 expression in B-cell tumors parallels its regulation in non-neoplastic B cells.

I explored the role of TCL1 defined heterogeneity in DLBCL and found its expression to corroborate with the molecular classification defined by Shipp et al., as shown in Table 2 (*unpublished data*).

PATHWAYS FOR DLBCL	BCR+		TCL1+	
	p	fdr	p	fdr
BCR SIGNAL PATHWAY	NS	NS	0.040619	0.160246
BLIMP-1 TARGETS	0.000	0.000	0.011236	0.05241
BLOOD PAN-BCELL	0.000	0.002	0.00726	0.039421
C-MYC TARGETS	0.049	0.123	0.005976	0.028935
GC B-CELL	0.019	0.078	NS	NS
	BCR-		TCL1-	
BM PLASMA CELL	NS	NS	0.004016	0.07148
BLOOD MONOCYTE	0.029	0.125	0.003906	0.012282
DCPATHWAY	NS	NS	0.014799	0.151217
BLOOD NKCELL	0.000	0.036	0.006085	0.077008
KRAS2 SIGNATURE	NS	NS	0.010373	0.095244
IRF3 TARGETS	NS	NS	0.008969	0.074298
REGULATORY T-CELL	NS	NS	0.019068	0.143895

How the expression of TCL1 is spread between ABC v/s GC types of DLBCL remains to be explored. We tried to learn how TCL1 plays an important role and which pathways it affects in different lymphoma types by looking into the functional pathways whose expression correlated with the expression of TCL1. Two striking results were:

- TCL1 was found to be central to most of the functional pathways that we had found in Paper I to be involved in the pathogenesis of NHL types, including the BCR signaling pathway.
- MCL and CLL showed a striking heterogeneity in microarray expression, which is why we investigated further experiments for these two lymphomas to explore if the biological exploration had a possible clinical or prognostic relevance.

Indeed, it was found in CLL that very high TCL1 mRNA expression in microarrays was associated with worse prognosis. However, it still needs to be validated at protein level in tissue microarray studies.

In MCL, protein expression in TMAs showed very high expression of TCL1 as indicator of poor prognosis. What are the molecular interactions of TCL1 in MCL can be an interesting study for future.

PAPER III

Somatic hypermutation signature in low grade B-cell Lymphomas

Background:

The study focuses on small B-cell lymphomas in which the presence of Ig somatic hypermutation has been reported to be a biological variable of clinical significance, such as CLL^{153, 154}, MCL^{155, 156} and SMZL¹⁵⁷. It was thus of interest to look for genes in the cell machinery that are switched on during the SHM process. The advantage of these findings could enable

- i) discovery of surrogate markers for SHM¹⁵⁸
- ii) finding a SHM gene signature.

Methods:

Gene expression analysis was followed by Immunohistochemistry. Ongoing mutation was measured using DNA cloning and sequencing studies. The samples were obtained from patients of CLL, MCL or SMZL. No in vitro somatic hypermutation model was used.

Results

Patients with >2% or higher SHM were found to have over-expression of genes involved in cell cycle machinery and regulation, DNA repair and replication and important transcription factors. These genes were tested for protein expression in normal as well as lymphoma affected lymph nodes and seven of these genes were found to be strongly expressed in germinal center B-cells, the seat of SHM process. Of these seven, CDK7 and RAD51C qualified as potential SHM markers with high specificity, while TFDP1 and POLA were significantly associated with ongoing SHM. Besides, CDK7 and RCC1 were both significantly associated with overall survival in MCL.

Discussion

The process of SHM involves DNA unwinding as happens during replication or transcription and DNA repair by DNA polymerases. SHM is a normal process designed in mammalian evolution for the body to respond to huge variety of antigens with precision. However, when the SHM rate becomes aberrantly high as seen in rapidly dividing cells in germinal centers, it plays role in lymphoma development. Mutated IgVH in B-cells or lymphoma cells is a hallmark of GC reaction. The involvement of deregulated machinery with genes involved in DNA repair, replication, transcription and cell cycling is thus understandable. The discovery of SHM markers in MCL should facilitate identification of poor prognostic patients. Since these markers have high specificity, they should be used in conjunction with markers that have high sensitivity to avoid false negative results. Thus CDK7 and RCC1 qualify as surrogate prognostic marker in MCL.

Gene expression profiling leads us to test a number of marker antibodies on tissue sections and verify them on whole tissue sections. In our studies, we found surrogate diagnostic markers of somatic hyper mutation in low grade lymphomas such as RAD51C, CDK7, TFDP1 and POLA. POLA is a replication

polymerase expressed in hypermutating cells but not in resting B-cells. Both CDK7 and RAD51C can activate p53, which leads to cell cycle arrest, so that the DNA repair can progress.

In Mantle Cell Lymphoma, we found that markers of somatic hyper mutation such as RCC1 and CDK7 were associated with longer overall survival and thus qualify for prognostic markers. RCC1 is produced by the gene expression of CHC1. It is involved in regulating the onset of chromosome condensation in the S-phase. CDK7 is a mediator of cell cycle progression through activation by binding to cyclins

PAPER IV

A unifying model of the microenvironment of follicular lymphoma: outcome is predicted by programmed death-1-positive, regulatory, cytotoxic and helper T cells and macrophages.

Background: The role of immune signatures in predicting the prognosis in FL was first shown by Dave et al¹⁵⁹. Subsequent studies have been performed with IHC and flow cytometry with varying results from different cohorts. During our own study on Functional Pathways (Paper I) two observations were notable in the results:

- Even though micro-environment was expressed in both the molecular subtypes of FL, FL-b had over-expression for pathways ascribable to BCR, GC and Cytokines when compared to FL-a. It is also interesting to point out that FL-b had over-expression for pathways such as CTL, NKT, Blood NK-cells CD4 T-cell differentiation, MAPK, AKT, B-cell and BCR pathway.
- BCR low or negative expression was co-related to positive or high expression for pathways ascribable to cells in the FL tumor microenvironment and cytokines. These pathways also included NFkB target genes and B-cell & T-cell inter-regulatory calcium signaling pathways.

Due to the above findings and our interest in FL we decided to further study the role of different cells in the microenvironment with focus on T-cells. We hypothesized that several immune cell subsets were important for disease outcome and their individual prognostic importance should be demonstrable in the same analysis and in competition with clinical factors.

Studies at KI had identified, verified and characterized all patients diagnosed with follicular lymphoma (FL) in Southern Stockholm County between January 1994 and January 2004 (a total of 197 verified patients after biopsy examination). The flow cytometry studies that were performed on lymph node at diagnosis were re-analyzed and a strong correlation was found between the number of CD8-positive (CD8+, cytotoxic) T cells and better survival. Multivariate analysis showed that patients with high number of CD8+ cells ran five times less risk to die and patients with medium-high number two times lower risk compared to patients with low numbers of CD8+ cells. Similar differences were also seen within each clinical FLIPI risk group. It was found following examination of immunohistochemical staining that CD8+ T cells were stationed perifollicular without much contact with the malignant B-cells, while the other (CD4+) T cells more frequently were inside the follicles. We suspected that the CD8+ T cells mainly exert its effects during treatment, after which the antigen-presenting cells can stimulate T-cell response with material from dead tumor cells¹²⁰.

Material & Methods:

To verify and further explore the above finding, we produced a tissue microarray (TMA) at diagnostic lymph nodes in the patients who had extreme clinical outcomes in the cohort: those

who died rapidly of lymphoma and those with long follow-up time and still alive without any treatment or having had very little treatment, a total of 70 patients (37 with good and 33 with poor prognosis).

For TMA we arranged millimeter-sized pistons from different biopsies side by side in the paraffin blocks for simultaneous immunohistochemical staining and histological analysis. This avoids the confounding factors arising from the individual staining and assessment of the preparations. TMA-ROM has been produced in Lund, and cut from this robot has been colored in Pathological Clinic, Huddinge.

Flow cytometry results validated by automated quantification of tissue microarrays and re-validated qualitatively by pathologists in whole tissue sections. The aim was that, as in flow cytometry, to quantify the number of cells but, unlike flow cytometry this analysis also allowed localisation in the preparation, and thus distinguish between cells located inside the follicles and outside follicles (follicular and interfollicular cells).

We measured the number and pattern of infiltration of several types of immune cells: cytotoxic (CD8+), regulatory (FOXP3+), suppressive (PD-1+), helper (CD4+) and senescent (CD57+) T cells, macrophages (CD68+) and mast cells (tryptase+), under the hypothesis that the prognosis of FL is dependent on several types of immune cells. Specifically the CD8+ T cells might be associated with good prognosis (due to killing of tumor cells), and FOXP3+ and PD-1+ T cells (due to inhibition of B cell stimulation), whereas CD4+ T cells might be associated with poor prognosis (due to B cell stimulation).

Results: The hypothesis was confirmed: CD8+, PD-1+ and (follicular) FOXP3+ T cells is associated with good prognosis and CD4+ T cells (especially follicular) and interfollicular macrophages with bad prognosis. Moreover, high CD4/CD8-ratio correlated with poor prognosis.

Results from Multivariate analysis can be represented in a 2x2 table 3 as follows:

	Good Prognosis	Poor prognosis cohort
Follicular compartment	T-regulatory cells (FOXP3+)	CD4+ T-cells (FOXP3-, PD1-)
Inter-follicular compartment	Cytotoxic T-Lymphocytes (CTL)	Macrophages

The automated subset quantifications from TMA were verified with corresponding flow cytometry results, showing strong correlations between total-core TMA and flow results in all applicable cases: CD3, CD4, CD7, CD8 and FOXP3/CD3+CD25+. This is the first study which demonstrated that several different immune cell types affect the prognosis independent of each other and of FLIPI.

Thus, the prognosis in FL is affected by several subsets simultaneously at work in the FL microenvironment, rather than being dictated by an individual immune cell subset. Our data confirm that CD8+ cells correlate with good prognosis, regardless of their distribution patterns in the FL nodes, although the vast majority of CTLs are interfollicular. The adverse action of CD4+ cells was especially apparent in the follicular compartment. A high CD4/CD8 ratio in all compartments correlated with poor outcome.

Discussion:

In the present study, a trend towards increased risk of transformation was seen in patients with more follicular CD4+ cells. Tregs, defined as FOXP3+, negatively regulate T-follicular helper and T-follicular helper-dependent B-cell survival and directly suppress B cells. Helper T cells and T-follicular helper cells most proficiently, express CD40L, which protects FL cells from apoptosis through CD40, and other signals stimulating the FL cells to proliferate. The putative mechanism through which macrophages would be adverse in FL is that macrophages possibly prevent CTLs from attacking tumor cells.

We need tools to better predict prognosis for patients with follicular lymphoma, since many patients will never require therapy while others are at high risk for rapid death in their illness. It is also of great value to identify the immunological factors that can tell what kind of therapy is best for the individual patient. With our research, we hope to learn more about the key components of the micro-environment and its importance in modern therapy.

GENERAL CONCLUSIONS

Understanding the biology of B-cell lymphoma provides basis for improving our understanding of tumor biology in general and classification system for lymphomas in particular. The consequence of these efforts is also towards discovery of routine laboratory markers of prognostic value and development of targeted therapy approaches for lymphoma patients, enabling them a better quality of life. For example, targeting TCL1A oncogene by highly specific chemical inhibitors could be a useful strategy in the treatment of lymphomas.

Future:

In ongoing studies, I am experimenting to relate our previous findings with TCL1 oncogene and Cannabinoid Receptor -1(CB-1) with cytogenetic abnormalities in Chronic Lymphocytic Leukemia. We are using immunohistochemistry and FISH as a combination of approaches on patient samples to achieve our objective and also to understand clonal evolution in different lymphoid compartments in CLL patients. We would like to see if high TCL1 expression or certain pattern of TCL1 expression in different compartments, specifically in the spleen, is correlated to 11q or 17p deletion clones in CLL.

We still don't know enough how the milieu is different in spleen, lymph nodes, blood and bone marrow; however, observations show the clonal picture to be similar in blood and bone marrow while that in lymph nodes and spleen homing multiple clonal types or diverse clones.

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