Diffuse large B-cell lymphoma - tumour characteristics on RNA and protein level associated with prognosis

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To the heroes; our patients

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ABBREVIATIONS

ABC	Activated B-cell like lymphoma
CD	Cluster of differentiation*
DLBCL	Diffuse large B-cell lymphoma (diffust storcelligt B-cellslymfom)
GC(B)	Germinal center (B-cell) derived lymphoma
GEP	Gene expression profiling
IPI	International Prognostic Index
OS	Overall survival
OTS	Ordinary tissue sections
TAM	Tumour associated macrophages
TILs	Tumour infiltrating lymphocytes
TMA	Tissue microarray
WTS	Whole tissue sections (=OTS, used in paper III)

*standard nomenclature for naming surface proteins on lymphocytes, e.g. CD40

ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I Linderoth J, Jerkeman M, Cavallin-Ståhl E, Kvaløy S, Torlakovic E, Immunohistochemical expression of CD23 and CD40 may identify prognostically favourable subgroups of diffuse large B-cell lymphoma: A Nordic Lymphoma Group Study. Clin Cancer Res 2003; 9(2): 722-8.

II Linderoth J, Ehinger M, Jerkeman M, Bendahl P-O, Åkerman M, Berglund M, Enblad, G, Erlanson M, Roos G, Cavallin-Ståhl E,
CD40 expression identifies a prognostically favourable subgroup of diffuse large B-cell lymphoma. Leuk Lymphoma 2007; 48(9): 1774-9.

III Linderoth J, Ehinger M, Åkerman M, Cavallin-ståhl E, Jerkeman M,
Tissue microarray is inappropriate for analysis of BCL6 expression in diffuse large B-cell lymphoma. Eur J of Haematology 2007; 79(2): 146-9.

IV Linderoth J, Edén P, Ehinger M, Jerkeman M, Berglund M, Enblad, G, Erlanson M, Roos G, Cavallin-Ståhl E,
Genes associated with the tumor microenvironment are differentially expressed in cured versus primary chemotherapy-refractory diffuse large B-cell lymphoma. Br J Haematol, 2007, in press.

INTRODUCTION

Malignant lymphomas

The malignant lymphomas are tumours originating from transformed lymphoid cells and comprise a heterogeneous group of tumours. The recent WHO classification defines these malignancies according to known or postulated lineage and categorizes the lymphomas into three main groups: B-cell neoplasms, T and NK cell neoplasms, and Hodkgin lymphoma[1]. Functionally the B- and T-cell neoplasms can be divided into three categories: indolent, aggressive and very aggressive lymphomas [2]. Although simplified, this division is useful in the clinic. In general, indolent lymphomas are not curable, but on the other hand characterized by a slowly progressive clinical course during which spontaneous remissions sometimes can be observed. The aggressive and very aggressive lymphomas have a more rapid clinical course, but are potentially curable with modern chemotherapy.

DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL)

Clinical characteristics, treatment and prognosis

DLBCL is the most frequent lymphoma subtype and encompasses the majority, ~ 60-70% of the aggressive lymphomas. In Sweden 450 new cases are diagnosed annually. The median age at diagnosis is approximately 70 years [3]. DLBCL is slightly more common in males than in females.

According to the WHO classification there are several morphological variants: centroblastic, immunoblastic, T-cell/histiocyte-rich, anaplastic, plasmablastic, lymphomatoid granulomatosis type, and a variant expressing full length ALK (anaplastic large cell lymphoma kinase protein) [1]. Mediastinal (thymic) B-cell lymphoma, with morphological and genotypical resemblances with Hodgkin lymphoma [4,5], and intravascular B-cell lymphoma, are considered as separate subtypes of DLBCL. The centroblastic morphology has in some studies been associated with a favourable, and the immunoblastic variant with an unfavourable, outcome [6,7]. Other studies have identified similar trends, although not statistically significant [8,9], whereas yet another study could not identify any such prognostical differences [10]. There are today no convincing data supporting that categorization into the different morphological variants could be used for other purposes than for diagnosis.

Clinically DLBCL often presents with rapidly growing lymph nodes but extra-nodal involvement, e.g. gastrointestinal tract, skin, CNS, bone, testis, is not uncommon and occurs in approximately 40%, [11]. Staging is based on the Ann Arbor classification [12], with ~25% presenting with early localized disease (stage I), ~25 % with loco-regional disease (stage II), and 50% with disseminated disease (stage III-IV) [3].

Patients with stage I disease receive either combined modality treatment, i.e. three courses of anthracycline-containing chemotherapy, CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) followed by involved field radiation in the range of 40 Gy, or full chemotherapy. The prognosis is good, with a 5-year overall of approximately 70% [3].

Patients with stage II-IV disease receive full chemotherapy, i.e. at least 6 courses of CHOP or CHOP-like treatments, during the last few years often given in an accelerated fashion [13,14]. Today, most patients with DLBCL also receive therapy with rituximab, a monoclonal antibody directed against the CD20 antigen that is expressed on B-cells.

With modern anthracycline-containing chemotherapy in combination with rituximab, 70-80 % of the patients respond with complete remission. It is not uncommon however, that patients who achieve complete remission later develop recurrent disease, often with fatal outcome. In addition, a relatively small fraction of patients, 10-20 %, present with a primary chemotherapy-refractory disease, associated with a very dismal prognosis. The estimated overall cure rate for patients with advanced stage DLBCL is approximately 50 % [13-16].

For risk stratification the International Prognostic Index (IPI) is used, and the result has implications for choice of therapy. The IPI is based on five clinical features (age, stage of the disease, level of lactate dehydrogenase, performance status and number of extranodal lymphoma sites) and is the best clinical discriminator of prognosis to date [17-19]. It is however not an ultimate instrument, since a considerable proportion of patients classified as low or low-intermediate risk eventually show therapeutical failure.

Biological characteristics and prognosis (Table I)

Several different features on DNA, RNA, and protein level have been connected with prognosis. Much evidence today indicate that factors involved in the regulation of the cell cycle and the apoptotic pathways are the most important determinants of chemotherapy sensitivity, and thereby of prognosis[20]. Of great biological interest is also the "cell-of-origin concept", achieved through gene expression profiling, which indicates a difference in prognosis according to which stage of B-cell differentiation the malignant counterpart correspond, i.e. germinal center B-cell derived (GC) or activated B-cell derived (ABC) (*vide infra*).

Major chromosomal aberrations

The most common cytogenetic aberration in DLBCL is the translocation of the proto-oncogene *BCL6*, at 3q27, to the Ig heavy chain region, at14q32, resulting in the up-regulation of *BCL6*. Other fusion partners can also be involved. The t(3;14) occurs in approximately 30-40% of the DLBCL [21-25]. *BCL6* can also be up-regulated due to gene amplification [25,26], and due to gene mutation in regulatory segments [27]. *BCL6* promotes cell proliferation and blocks differentiation, acting as a transcriptional repressor [21,22]. *BCL6* rearrangements have been associated with a favourable prognosis [28]. Furthermore, fusion to IgH has been associated with better outcome than fusions with non-Ig partners [29,30]. Other studies however, have not been able to show these prognostic differences [31-35].

The second most frequent aberration is the translocation of the anti-apoptotic *BCL2* gene to the Ig heavy chain region, t(14:18)(q32;q21). This translocation is the hallmark of follicular lymphoma but occur also in approximately 20-30% of DLBCL [33,36]. BCL2 can also be up-regulated due to gene amplification [26,37] and possibly via NFκB activation, as indicated by in vitro studies [38,39]. Earlier studies could not associate the t(14;18) with

inferior outcome [33,36,40]. More recent studies, taking the cell-of-origin concept into account, identifies t(14;18) only within the germinal center derived DLBCL cohort, but data concerning the prognostic implication of t(14;18) in this subgroup are equivocal [41,42].

The translocation of MYC to the Ig heavy chain locus, t(8;14)(q24;q32), a characteristic rearrangement of Burkitt´s lymphoma [43] occurs in 5-10% of DLBCL [33,44]. Gene amplification has also been shown to cause the up-regulation of MYC [26]. MYC activates genes that promote cell-cycle progression and suppresses inhibitory proteins. The presence of the MYC translocation, t(8;14), alone [44-46], or with concurrent chromosomal aberrations, e.g. t(14;18) [47,48] has been associated with adverse prognosis.

Cell cycle regulation

Abnormalitites involving the tumour suppressor gene *TP53* located on 17p have been detected in 15-20 % of DLBCL [49,50]. *TP53* is a tumour suppressor gene that initiates cell cycle arrest in response to DNA damage. If the damage is too extensive, the cell is directed towards apoptosis [51]. Mutations of *TP53* have been associated with worse prognosis in some studies [52-54], but not in others [55,56]. Wild-type *TP53* is usually not detected by immunostaining, but *TP53* mutation stabilizes the protein, making it detectable by immunohistochemistry.

Cell-cycle progression to S-phase is promoted by regulatory D cyclins (*CCND1, CCND2*, and *CCND3*) and cyclin-dependent kinases. High expression of *CCND2, CCND3*, or the loss of regulatory proteins, such as *CDKN2* (p16), has been associated with adverse prognosis [57-60].

Expression of other cell cycle regulators, such as *CCNB1* and *CCNE1*, has also been associated with adverse prognosis [61,62]. Concerning the prognostic impact of CDKN1B (p27, Kip1), a cyclin-dependent kinase (CDK) inhibitor, data are equivocal [63,64].

Ki-67 (MIB1) is a nuclear antigen expressed by dividing cells. The prognostic effect in DLBCL is controversial [53,64-66].

Apoptosis

Over-expression of the anti-apoptotic BCL2 protein has been associated with chemotherapy resistance and adverse prognosis reflected in shortened disease-free survival [36,40,66-69].

In a recent report over-expression of the BCL2 protein caused by the t(14;18) in GC-like DLBCL was not correlated to inferior outcome, whereas BCL2 protein expression in the ABC-group was associated with adverse prognosis. In the ABC-group, BCL2 expression strongly correlated with chromosomal gains at 18q21 and high levels of BCL2 mRNA [70].

Some retrospective studies suggest that the addition of rituximab to chemotherapy

may overcome the adverse effects of BCL2 [71,72].

Another potential mechanism for the BCL2 expression in the ABC-group could be a downstream effect of a constitutive activated NFκB, which has been found in ABC-like DLBCL cell lines [38]. Results from in vitro studies also indicates that rituximab may inhibit the NFκB-pathway and thereby cause down-regulation of BCL2 [73].

Other proteins involved in inhibition of apoptosis, as survivin and X-linked inhibitor of apoptosis (XIAP), have been associated with inferior prognosis [74-76].

Cell-of-origin concept

Through molecular profiling important biological insights have been gained of which the "cell-of-origin concept" is now a widely accepted theory on the biology and origin of DLBCL. Two main groups of DLBCL have been identified: tumours expressing genes characteristic of germinal center B-cells, GC-like DLBCL, and tumours expressing genes normally induced during in vitro activation of peripheral blood B-cells, ABC-like DLBCL [77,78]. The GC-profile was associated with a superior outcome compared to the ABC-profile. In a large study verifying this "cell-of-origin concept", patients with a germinal center B-cell genotype (GC) had a five-year survival rate of 60% versus 35% for patients with an activated B-cell genotype (ABC). Patients with non-classifiable tumours, "type 3" had a five year survival of 39% [78]. Interestingly this division in GC and ABC has helped to sort out some of the oncogenic events described above. For instance the *BCL2* translocation has only been reported in the GC-group, perhaps further defining a subset of this group [41,42,79], whereas chromosomal gains at 18q21 may be responsible for expression of the BCL2 protein in ABC-like tumours [70].

However, the separation into a genotypic GC or ABC profile does not occur spontaneously, in an entirely unsupervised way (vide infra "microarray technology"), neither in the original studies[77,78] nor in the subsequent [48,80-83]. The GC or ABC related genes described [77,78,84] have to be used in order to get this subdivision. Still, several studies using platforms provided by Affymetrix, clustering on the genes originally described by Alizadeh and Rosenwald have not been able to show significant differences in overall survival [80,81,83].

Several groups, including our own, have with different results sought to identify a protein pattern corresponding to the genotypic GC-like and ABC-like tumours by combining immunohistochemical markers associated with different stages of B-cell development[85-92]

CD10, BCL6 and MUM-1 are the most frequently used and commented on separately below. The most successful algorithm was proposed by Hans *et al*, using an arbitrarily set cut-off value of 30% for all three markers [88]. Regarding the genotypic division as reference method, this method could correctly classify 87% of GC-like and 73% of ABC-like tumours. The five-year survival for patents with a GC phenotype (GC) was 76% versus 34% for patients with a non-GC center phenotype, comprising ABC-like and type 3 tumours [88].

Recent data indicates, that the addition of rituximab to chemotherapy might eliminate the prognostic value of immunohistochemically defined GC and non-GC phenotypes [93].

BCL6 is necessary for germinal center formation [94] and the protein is expressed in the nuclei of normal germinal center B-cells[95], as well as in the nuclei of the neoplastic cells of DLBCL [96,97], where the expression can be caused by the t(3;14), gene amplification or by mutations in regulatory segments (vide supra). In DLBCL, BCL6 expression is seen in approximately 50% of the cases, using a cut-off level for positivity at 30% [86,88,92]. BCL6 protein is used as a marker of a germinal center origin. Interestingly however, one third of patients in the phenotypically defined non-GC group express the BCL6 protein [35,88]. Protein expression and/or over-expression of bcl-6 mRNA have been associated with favourable prognosis [35,86,88,98,99].

CD10 or CALLA (common acute lymphoblastic leukaemia antigen) is an established marker of GC derivation [100] and is immunohistochemically expressed on 20-50% of DLBCL [88-90,92,101,102]. In the algorithm suggested by Hans *et al.* (vide supra) CD10 status is of greater importance than BCL6 status for defining GC vs. non-GC phenotype. Regarding the prognostic value of CD10 as an individual marker, data are conflicting [90,92,101-103].

MUM1/IRF4 is a transcription factor which besides being a marker for postgerminal center stage is thought to be involved in activation of T-cells, lineage commitment of lymphocytes and in Fas-dependent apoptosis[178]. Data are equivocal concerning the prognostic value of MUM1 alone [86,88,90], but as a part of the GC-non-GC algorithm proposed by Hans *et al.*, MUM1 is useful [86,88,89].

HGAL is an interleukin-4-inducible gene with unknown function [104]. Expression of the protein is almost exclusively seen in GC-derived lymphomas [105]. High mRNA expression of HGAL is an IPI-independent factor of good prognosis [104].

CD40

The cell surface molecule CD40, a member of the tumor necrosis factor-receptor family, plays a central role in the immune system. The receptor is expressed on all stages of B-cell development but also on monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, and some epithelial cells [106]. The natural ligand of CD40, CD40L (CD154) is mainly expressed on activated helper T-lymphocytes but can also be found on most cells of myeloid lineage, endothelial cells, smooth muscle cells, and epithelial cells. The expression of CD40L is in general non-constitutive, but can rapidly be induced after activation [107].

CD40 signalling is essential for T-cell dependent B-cell activation. When helper T-cells recognize antigen-presenting B-cells, they ligate and CD40L expression is induced. The CD40-CD40L interaction stimulates the antigen presenting ability of the B-cell, and further guides the B-cells through their differentiation program, including rescue from apoptosis, differentiation into germinal center cells, isotype switching, and maturation into memory cells [106,107]. Patients carrying a mutation in the CD40L gene develop an

immunodeficiency characterized by the absence of germinal centers and the unability of mounting a T-cell dependent humoral response [108].

CD40 activation is also of importance for other parts of the immune system. The CD40-CD40L interaction is involved in the generation of a cytotoxic T-cell response. This is mainly mediated via CD40 expressing antigen presenting dendritic cells, which become more efficient in their antigen presenting function after ligation with CD40L on activated T-lymphocytes [109-112]. Furthermore, the activation of the CD40 receptor on antigen presenting macrophages may result in increased production of proteolytic enzymes [113].

Immunohistochemical expression of CD40 is also found in malignancy, both in lymphoproliferative diseases and in solid tumours [114]. The fraction of DLBCL expressing CD40 varies between 38-76% in different reports [89,92,115-117]. Immunohistochemical expression correlates with mRNA level [117] and with a superior prognosis [89,92,117].

Drug resistance

The multidrug resistance gene (*MDR1*) and its product, the P-glycoprotein (Pgp), a member of the ATP-dependent transporter (ABC) family, is associated with resistance to anthracyclins, epipodophyllotoxins, and vinca alkaloids in vitro [118,119]. Immunohistochemical expression of Pgp, detected in 37-66 % of DLBCL, has in some studies been associated with adverse prognosis [120,121], however not found in others [66,122,123].

Lung resistance protein (LRP) or Major Vault Protein has in vitro been shown to confer resistance to doxorubicin, vincristine, carboplatin, cisplatin, melphalan, etoposide, and paclitaxel[124,125]. LRP has by immunohistochemical methods been detected in 23-68% of DLBCL, and was in these studies associated with lower response rates to chemotherapy and worse prognosis [120,126].

The glutathione family may be involved in resistance to alkylating agents (e.g. cyclophosphamide), steroids, but also to anthracyclins [127]. The protein over-expression of glutathion-S-transferase (GST-π) has been associated with inferior complete responses rates [127].

Recent gene expression data confirms the adverse prognostic effect of the *MDR1* gene, and identifies a member of the glutathion family associated with inferior outcome, glutathione peroxidase 1 (*GPX1*) [128].

Angiogenesis

In a recent study investigating the immunohistochemical expression of several of the members of the vascular endothelial growth factor (VEGF) family, expression of VEGF-A was

detected in 77% of DLBCL and associated with inferior overall survival [129]. In addition, low serum levels of VEGF have been associated with prolonged overall survival [130]. By molecular profiling, VEGF was found to be over-expressed in a therapy-resistant cohort of DLBCL[80].

Other biomarkers

CD5 is expressed on normal T-cells. Expression is also found in mantle cell lymphoma, lymphocytic lymphoma, and in a subset of DLBCL, approximately 5-10%, where it has been associated with inferior survival [92,131-133].

The up-regulation of the *PKC-β* gene, a protein kinase involved in B-receptor signalling, was first identified in a therapy-resistent cohort of DLBCL [80], and a recent retrospective analysis of global gene expression data suggests that elevated gene levels are connected to a worse prognosis [134]. By immunohistochemical methods PKC-β was detected in 22% of DLBCL and associated with worse prognosis [58].

Tumour microenvironment

Several studies have shown a favourable impact on prognosis of an increased amount of tumour infiltrating CD4 and/or CD8 positive cells, representing the helper and cytotoxic/cytolytic lymphocyte populations respectively [89,135-139]. However, infiltration of a subpopulation of cytotoxic T-lymphocytes, "activated cytotoxic T-lymphocytes" (CD3+/granzyme B+ or CD3+/perforin+) is associated with worse prognosis [140,141].

Recently, the infiltration of other inflammatory cells, such as mast cells [142], and macrophages (paper IV in this thesis), has been associated with favourable outcome.

Biological marker	Status	Prognostic significance
Cytogenetic changes		
BCL6 at 3q27, translocation to IgH, t(3;14) or other fusion partners	presence	favourable[28-30] no difference[31-35]
BCL2 t(14;18)	presence	no difference[33,36,40]
- as a part of GC subtype	presence	equivocal data[41,42]
MYC t(8;14) - alone	presence	unfavourable[44-46]
- with other chrom. aberr.	presence	unfavourable[47,48]
Cell cycle regulation		
TP53 mutation	presence	equivocal data[52-56]
Cyclins D2, D3, B1 and E	presence	unfavourable[57-59,61,62]
p16	loss of expression	unfavourable[60]
proliferation (Ki-67)	not defined	equivocal data[53,64-66]
Inbibitors of apoptosis		
BCL2:	presence	unfavourable[36,40,66-69]
-in GC subtype	presence	no difference[70]
-in ABC subtype	presence	unfavourable[70]
Survivin	presence	unfavourable[74,75]
XIAP	presence	unfavourable[76]
B-cell differentiation		
Germinal center (GC) subtype, RNA or protein	presence	favourable[48,77,78,82,85-89]
CD10	presence	conflicting data[90,92,101-103]
BCL6 mRNA	presence/high levels	favourable[98]
BCL6 protein	presence	favourable[35,86,88,98,99]
HGAL mRNA	presence	favourable[104]
Activated B-cell like (ABC) subtype (=non-GC) RNA or protein	presence	unfavourable[48,77,78,82,85-89]
Non-classifiable, "type 3" (=non-GC) RNA and protein	presence	unfavourable[78,86,88,89]
CD40	presence	favourable[89,92,117]

Drug resistance		
P-glycoprotein (Pgp)	presence	equivocal data[66,120-123]
Lung resistance protein	presence	conflicting data[120,126, (paper IV)]
Angiogenesis		
VEGF serum	low levels	favourable [130]
VEGF mRNA	presence/high levels	unfavourable[80]
VEGF-A protein	presence	unfavourable[129]
Other		
CD5	presence	unfavourable[92,131-133]
PKC-beta, mRNA and protein	presence	unfavourable[58,80,134]
Tumour microenvironment		
CD4+ helper T-lymphocytes	increased infiltration	favourable[89,138,139]
CD8+ cytotoxic T-lymphocytes	increased infiltration	favourable[89,135-137]
- "activated cytotoxic" (CD3+/granzyme B+/ TIA +)	increased infiltration	unfavourable[140,141]
Mast cells	increased infiltration	favourable[142]
Macrophages	increased infiltration	favourable (paper IV)

Table I Prognostic factors on DNA, RNA and protein level in diffuse large B-cell lymphoma

BACKGROUND FOR THIS THESIS

The origin of this thesis was the report by Alizadeh *et al* 2000, presenting the "cell-of origin concept". Our first aim was to adapt this concept on the protein level for clinical usage, applying immunohistochemistry for discriminating GC-like and ABC-like DLBCL.

Secondly, the close collaboration and nearby localization of the Swegene Microarray Resource Center in Lund has given us the opportunity to perform gene expression profiling ourselves, a work facilitated by colleagues in Uppsala and Umeå giving us access to a larger patient material. Our aim in this ongoing project is to identify novel factors associated with prognosis and to verify them with immunohistochemical means.

AIMS OF THE THESIS

To identify a protein pattern that can be used for discriminating germinal center derived (GC) and activated B-cell like (ABC) DLBCL (Paper I).

To confirm the possible prognostic effect of CD23 and/or CD40 expression found in paper I, and to elucidate the underlying mechanisms for this effect (Paper II).

To investigate the impact of different immunohistochemical techniques for the evaluation of BCL6 expression (Paper III).

To identify possible differences in gene expression profiles between two clinically contrasting patient populations, with cured versus primary chemotherapy-refractory DLBCL, in order to get knowledge in mechanisms involved in chemoresistance (Paper IV).

MATERIALS AND METHODS

Methods and materials are described in detail in the papers of this thesis. What follows is a summary.

Paper I

Tumours from 125 patients with de novo DLBCL, stage II-IV, adequately treated with anthracycline-containing regimens (either CHOP or MACOP-B, methotrexate, doxorubicine, cyclophosphamide, vincristine, and prednisone) were investigated by immunohistochemistry (IHC). CD23 was chosen as a marker of pre/early GC-origin, BCL6, CD10 and CD40 as markers of a GC-phenotype, and CD138 as a marker of post-GC origin (i.e non-GC). In addition, protein expression of CD5 was investigated.

Multi-tissue blocks were used, each tumour represented by 2 representative core biopsies with a diameter of 4 mm. The immunostainings were performed using the EnVision method (Dako), which is a standard enhancement method for visualization of immunohistochemical reactions. Results from immunohistochemistry (IHC) were correlated to clinical data.

Papers II-III

Tumours from a new cohort of 125 patients with de novo DLBCL, stage II-IV, adequately treated with anthracycline-containing regimens (CHOP or CHOP-like treatments) were investigated by immunohistochemistry. For CD23 and CD40 ordinary tissue sections (OTS) were used for assessment of immunoreactivity. For investigation of BCL6, CD10, MUM1, CD4, and CD8, the tissue microarray technique (TMA) was applied, each tumour represented by three representative core biopsies with a diameter of 0.6 mm. BCL6, CD10, CD4, and CD8 were also investigated on OTS.

The EnVision method was used for visual detection of all antibodies. For BCL6 additional staining using a conventional labelled streptavidin-biotin method (LSAB, Dako) was also performed. The expression of CD23 and CD40 were correlated to clinical data and to a phenotype defined by CD10, BCL6 and MUM1. CD40 expression was also correlated to the degree of tumour infiltrating CD4 and CD8 positive cells.

Paper IV

Tumour samples from 37 patients, selected from the cohort investigated in paper II, with de novo DLBCL, stage II-IV, either in continuous complete remission (n=24) or with primarily progressive disease (n=13) were examined with respect to gene expression profiles. The 55K oligonucleotide arrays used were produced at the Swegene Microarray Resource Centre, Department of Oncology, Lund University. Data was uploaded and analyzed in BioArray Software Environment (BASE). Some of the findings from gene expression profiling were confirmed on the protein level, using immunohistochemistry performed on TMA in available 33/37 cases.

MICROARRAY TECHNOLOGY - in brief

A microarray consists of a comprehensive array of samples or probes, either deposited or synthesized in situ at discrete locations over a small surface of a solid substrate, e.g. a glass slide. This approach allows the simultaneous investigation of one protein in hundreds of patient samples (tissue microarrays), or a global analysis of thousands of genes from one patient (DNA microarrays).

On order to save tumour material we applied two variants of the tissue microarray technique (TMA). In study I we used multi-tissue blocks. By this method each tumour is represented by 2 representative core biopsies with a diameter of 4 mm. In total, this provided 25 mm^2 for evaluation. In the subsequent studies, II-IV, we apply a more "traditional" technique where each tumour is represented by three representative core biopsies with a diameter of 0.6 mm. In total, this provided 0.85 mm^2 for evaluation.

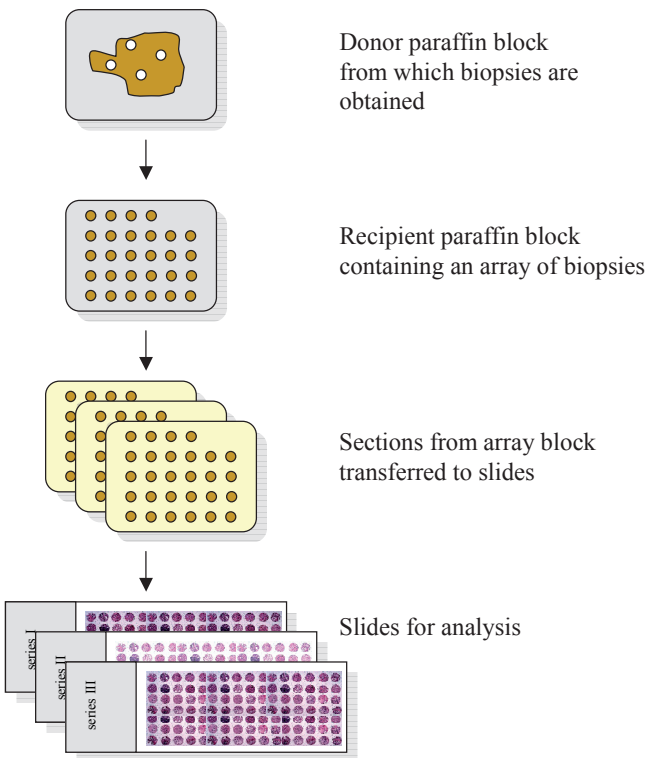


Fig 1 *Schematic presentation of the tissue microarray technique (courtesy of Jacob Engellau)*

The oligonucleotide microarray technique makes it possible to study the expression of mRNA for thousands of genes/the whole genome simultaneously. This, and other similar methods investigating the global expression of mRNA, is often referred as gene expression profiling (GEP) or molecular profiling.

An oligonucleotide probe consists of 60-70 base pairs designed to be representative for a specific target gene. A brief overview of the technology is given in the figure below. The platform we used was produced at the Swegene Microarray Resource Centre, Department of Oncology, Lund University. All arrays contain 27744 probes printed in duplicate on each array, in total 55488 features.

Data obtained from gene expression profiling can be analyzed and presented in many ways. However there are two different concepts of how to approach the analyses.

By unsupervised learning, the samples organize into groups with similar signatures without using any prior knowledge about the samples. This approach could be used for class discovery or identifying new entities. For instance, one can use hierarchical clustering and present results as diagrams seen in Fig 2.

By supervised learning, information about the sample, e.g. clinical data, is included in the analysis. This can be used for identifying sets of genes that discriminate between defined classes. In paper IV we use this approach.

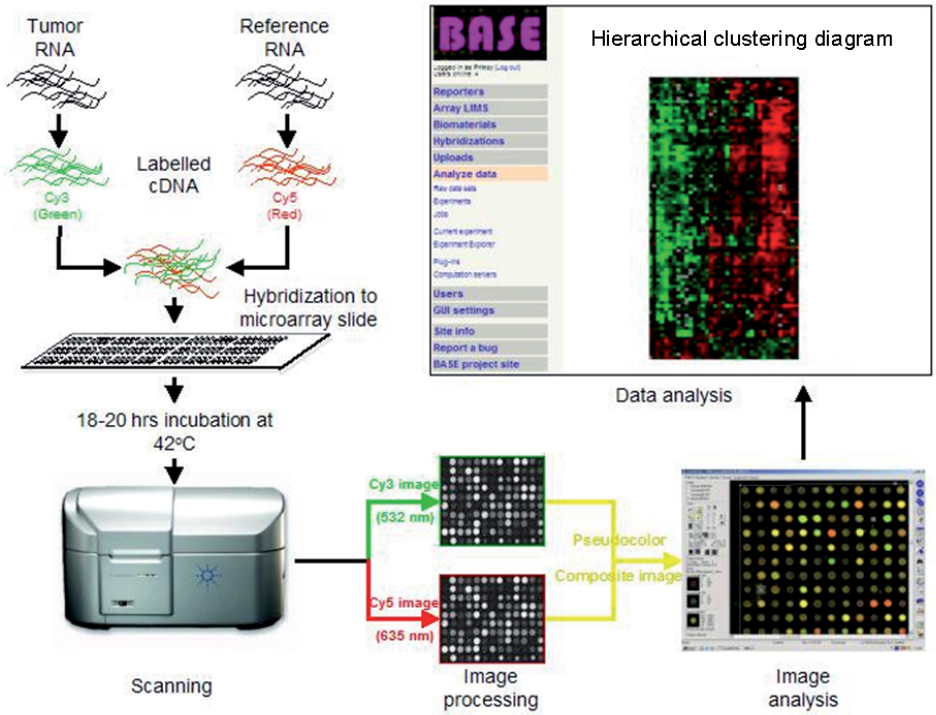


Fig 2 A brief overview of the oligonucleotide technology. RNA extracted from the tumour tissue and reference RNA are converted to cDNA and labelled with different fluorescent dyes (green for tumour and red for reference) after which they are pooled together and hybridized to the microarray slide. After 18-20 hours incubation at 42ºC the slides are scanned in a microarray scanner. The obtained grey-scale images for both fluorescent dyes are then overlaid to generate a composite pseudo-coloured image where the colour of the spot represents the relative abundance of the respective gene in both the samples. Green spots represent up-regulation in the tumour compared to the reference whereas red spots represent down-regulation. Yellow spots are genes with equal expression in both samples. Spot intensities are quantified and the data are then up-loaded into BioArray Software Environment (BASE) for further analyses and interpretation of the results (courtesy of Princy Francis, adapted).

RESULTS

Paper I

CD10 was positive in 51%, BCL6 in 97%, and CD138 in 2% of the tumours. Neither of these markers had any impact on the survival of the patients. CD40 was positive in 76% of the cases, and the expression did not correlate with CD10 expression. However, there was a strong association between expression of CD40 and overall survival (OS) (p=0.007) with a median survival of 108 months for positive cases and 33 months for negative cases. Also in multivariate analysis, adjusting for IPI class, CD40 remained a prognostic factor for OS (RR=0.42, 95% CI 0.22-0.82).

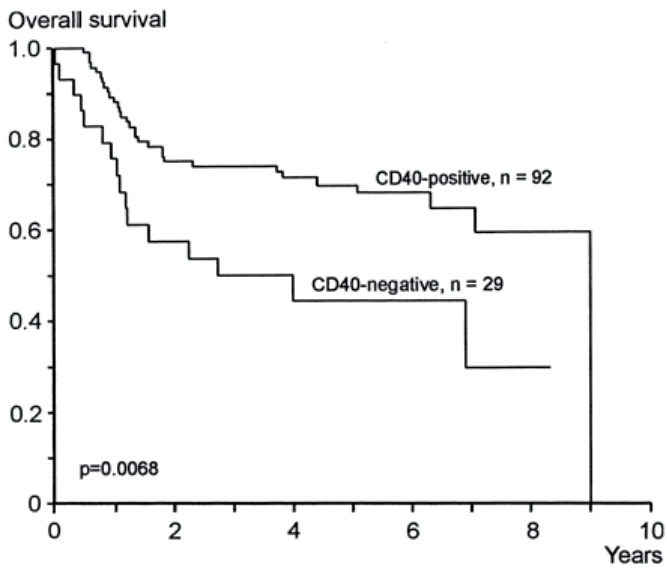


Fig 3 Overall survival in 121 patients with diffuse large B-cell lymphoma in relation to CD40 expression

Sixteen percent of the tumours were CD23 positive. The CD23 positive tumours were all CD5 negative (thus excluding variants of CLL) and CD40 positive. This group showed a tendency for better OS (p= 0.033), with a median survival not reached at 8 years for CD23 positive cases, and a median survival of 85 months for negative cases.

Furthermore CD5 expression was seen in 9/125 (7%) and associated with inferior OS. CD5 positive cases had a median OS of 7 months compared with 108 months for the negative cases (p=0.0008).

In conclusion, no prognostically different subgroups of DLBCL, corresponding to the GC-like or ABC-like tumours, could be identified using CD10 and BCL6 as germinal center markers and CD138 as a post-germinal center marker. The frequency of BCL6 positive tumours was very high, 97%, possibly due to the staining technique. CD40 is probably not a marker of GC origin and the function of CD23 in this context remains unclear. A new finding however, was the positive prognostic impact of CD23 and CD40 expression. The observed association between CD5 positivity and adverse prognosis confirmed earlier reports.

Papers II-III

Categorization in GC- vs. non-GC profiles was performed according to the algorithm proposed by Hans *et al* [88]. By the use of TMA CD10 was positive in 37%, BCL6 in 25% and MUM1 in 42%. Forty percent of the tumours were classified as GC phenotype. Median survival for GC and non-GC cases was 62 and 44 months respectively (p=0.19), i.e. a not significant difference. However, sixty of the 125 tumours investigated were earlier studied regarding GC-profile where the immunohistochemistry was performed on ordinary tissue sections; Berglund *et al* 2005 [86]. In 13/60 cases (22%) there was

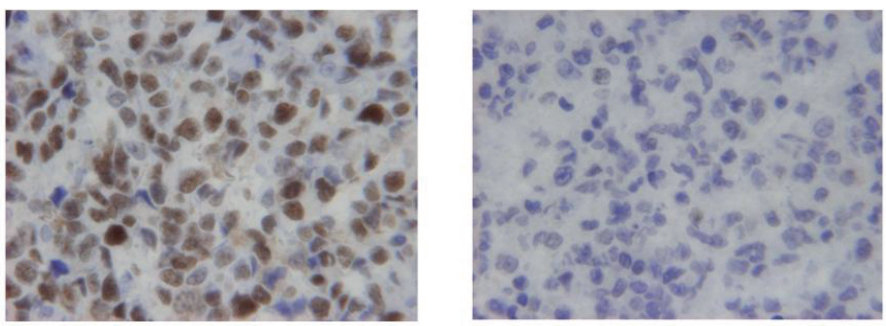


Fig 4 Two different areas (40X magnification) of an ordinary tissue section with heterogenous BCL6 expression. In the left panel more than 80% of the lymphoma cells express BCL6. The area showed in the right panel is almost completely BCL6-negative.

a discrepancy in the results between the earlier and the present study regarding the expression of BCL6 and/or CD10. BCL6 and CD10 were therefore re-investigated on ordinary tissue sections (OTS). This revealed a profound heterogeneity of BCL6 staining within the same tumour, with a high number of false negative cases using TMA (Fig 4).

By the use of OTS, CD10 was positive in 34% of the lymphomas and BCL6 in 52%. A germinal center phenotype was in this setting present in 47% (Fig 5) and significantly associated with superior OS. Median survival for patients with GC vs. non-GC derived DLBCL was 121 and 32 months respectively (p=0.006, log-rank).

Furthermore different staining techniques were evaluated. By use of a conventional biotin-avidin method (LSAB), BCL6 was positive in 12% on TMA sections. Using a more sensitive technique, EnVision, and the same TMA-blocks, BCL6-positivity was twice as high, 25%.

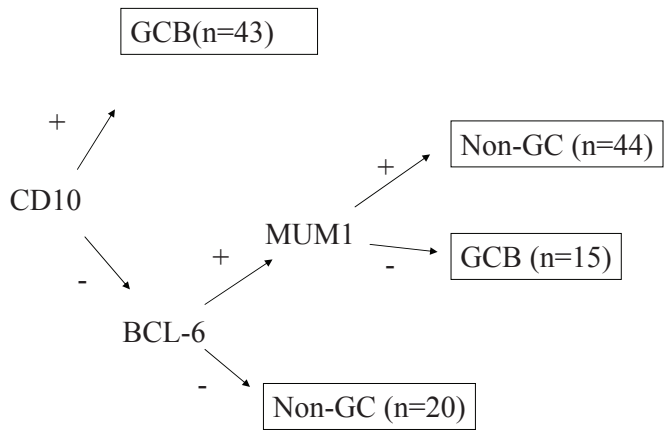


Fig 5 Categorization in germinal center (GC) and non-germinal center (non-GC) profiles according to the algorithm proposed by Hans et al[88]

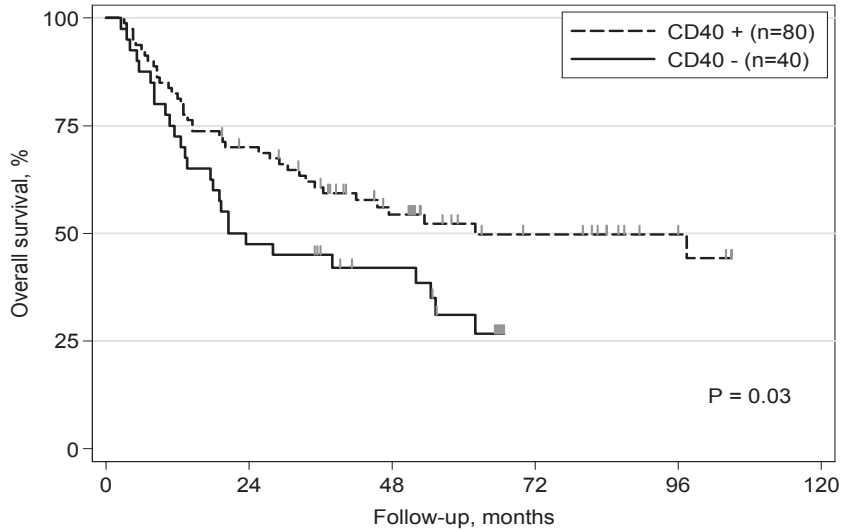


Fig 6 Overall survival in 120 patients with diffuse large B-cell lymphoma in relation to CD40 expression

The favourable prognostic impact of CD40 expression was confirmed (Fig 6). CD40 expression was seen in 67% and associated to a prolonged overall survival (OS), with a median survival of 62 months for positive cases versus 20 months for negative cases (p=0.03, log-rank). The effect of CD40 on OS was approximately the same in univariate Cox regression analysis, HR=0.58 (p=0.032, 95% CI 0.35-0.96) and in multivariate analysis adjusting for IPI class, HR=0.62 (p=0.079, 95% CI 0.36-1.06).
There was no significant correlation between tumors expressing CD40 and tumors expressing a germinal center phenotype (r=0.15, p=0.10). In multivariate anlysis, the prognostic effect of CD40 on OS was independent of GC-phenotype, HR=0.59 (p=0.045, CI 0.35-0.99).

The degree of tumor infiltrating CD4 and CD8 positive cells was estimated. Fifty-five % of the tumors showed more than 5% infiltrating CD4 positive lymphocytes. The corresponding figure for CD8 was 70%. CD4 and CD8 expression correlated significantly, r=0.42 (p=0.004), and there was a trend for better OS in patients with more than 5% infiltrating CD4 or CD8 positive cells (Fig 7 a+b). CD40 expression did not correlate with CD4 (r=-0.04) or CD8 expression (r=-0.12).

CD23 expression was seen in 12 cases. All but one co-expressed CD40. There was no significant correlation between tumors expressing CD23 and tumors expressing a germinal center phenotype, r=0.13(p= 0.15). CD23 expression did not correlate with patient outcome.

In conclusion, the prognostically advantageous effect of CD40 expression was confirmed. The biological mechanism for this is not obvious and not due to association

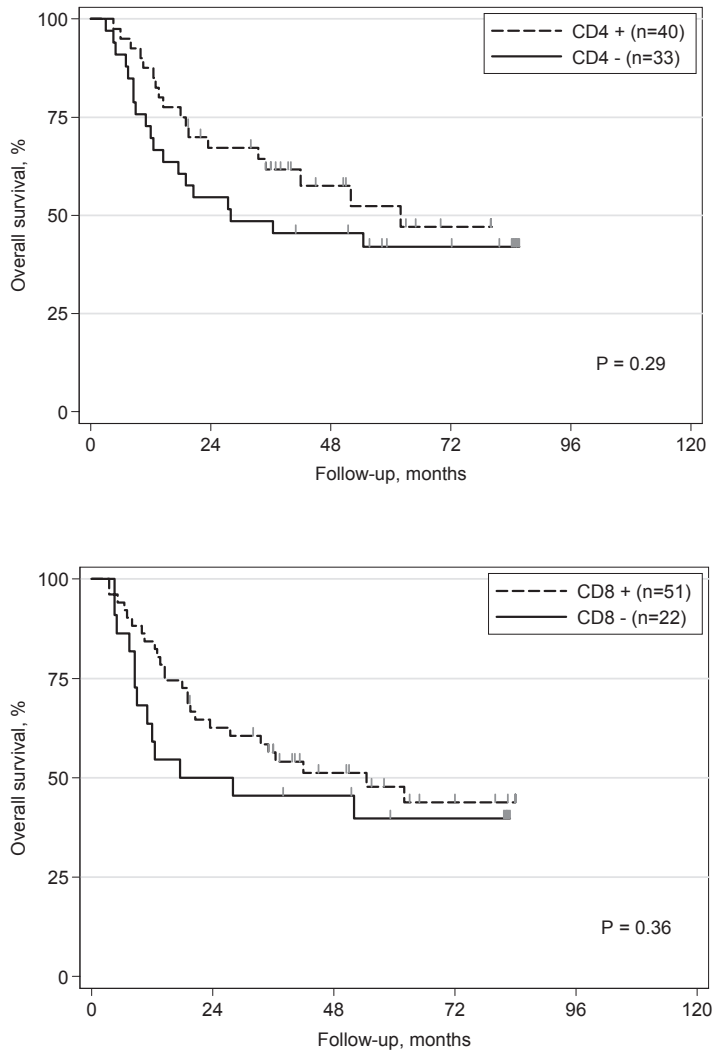


Fig 7 a +b Overall survival in 73 patients with diffuse large B-cell lymphoma in relation to tumor infiltrating CD4 and CD8 positive lymphocytes respectively. CD4+ and CD8+ indicates >5% infiltrating cells.

with the GC-phenotype or an enhanced autologous tumour response, as detected by tumour infiltrating helper and cytotoxic T-lymphocytes. The prognostic effect of a GC versus non-GC phenotype according to Hans was confirmed. Finally, the TMA technique showed to be unreliable for immunohistochemical detection GC vs. non-GC phenotypes, mostly due to difficulties interpreting BCL6 status.

Paper IV

The top 86 genes that best discriminated between the cured and refractory cohorts were chosen for further analysis. Of these, the majority was up-regulated in the cured cohort and coded for proteins involved in proteolytic activity, cytokine signalling, remodelling of extra cellular matrix, inflammation, and antigen presentation. Major groups of genes are listed in Table II. All 86 genes are listed in "supplemental data" in Paper IV.

Lysosomal enzymes	CD68 lysozyme/muramidase cathepsins Z, B, D and C prosaposin glucosamine(n-acetyl)-6-sulfatase matrix metallopeptidase 12/ macrophage elastase
Cytokine signaling	plasminogen activator urokinase receptor/U-PAR/CD87/ /monocyte activation antigen/Mo3 plasminogen activator urokinase/ PLAU chemokine C-C Motif receptor 1/CCRI endothelial cell growth factor 1 platelet derived/ ECGF1 signal transducer and activator of transcription 1/STAT1 suppressor of cytokine signalling 3/ SOCS 3
Remodelling of extra cellular matrix and inflammatory processes	tissue inhibitor of metalloproteinase 1/TIMP-1 tissue inhibitor of metalloproteinase 2/TIMP-2 galectin-3 peptidylglycine alpha-amidating monooxygenase/ PAM TGFβ
Antigen presentation and immunological reaction	MHC class I molecules (HLA A, B, F and G) intercellular adhesion molecule1/ICAM-1/CD54 CD3 NK transcript 4

Table II Genes up-regulated in 24 cured diffuse large B-cell lymphoma, listed according to the biological function of their protein counterparts.

There was a correlation between RNA and protein levels. Several of the genes that were up-regulated in the cured cohort were also more strongly expressed on the protein level. Immunohistochemical analyzes revealed a higher frequency of tumours expressing of lysozyme, Cathepsin D, UPAR, STAT 1 and galectin 3 in the cured cohort than in the refractory. The localization of these proteins was predominantly in non-tumour cells, mainly macrophages (Fig 8).

Further, an increase infiltration of tumour associated macrophages (TAM) was observed in the cohort, correlating to an up-regulation of the CD68 gene transcript.

As the finding of up-regulated MHC class 1 molecules and ICAM-1 in the cured cohort indicated an enhanced antigen presenting ability, tumour infiltrating T-lymphocytes and

ICAM-1 expression were investigated. An increased infiltration of CD4 positive helper and CD8 positive cytotoxic T-cells was observed in the cured compared to the refractory lymphomas. Immunohistochemistry also showed an increased expression of ICAM-1, localized on the reactive cells, in the cured lymphomas.

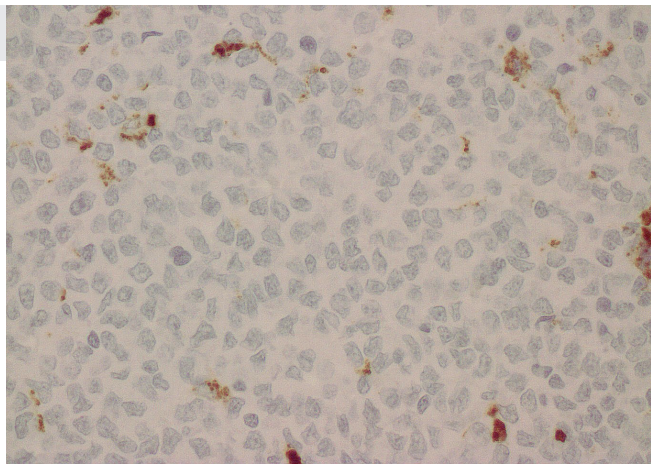
Lung resistance protein (LRP), also named major vault protein (MVP), a protein that has been associated with chemotherapy resistance in DLBCL, was over-expressed in the cured cohort. The localization was mainly in macrophages and follicular dendritic cells.

Only 7 of 86 genes were over-expressed in the refractory cohort, among them rab geranylgeranyltransferase and DNA polymerase epsilon, both potential targets for drug intervention.

In conclusion: The findings suggest that a reactive microenvironment, including tumour infiltrating T-cells and macrophages, might have an impact on outcome of chemotherapy in DLBCL.

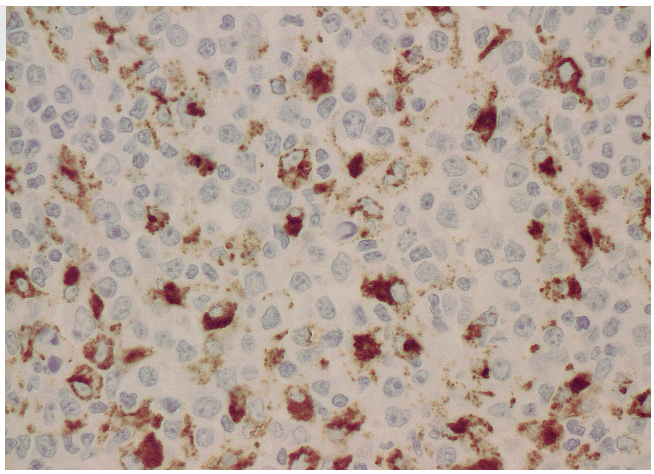
CD68 (negative)

a



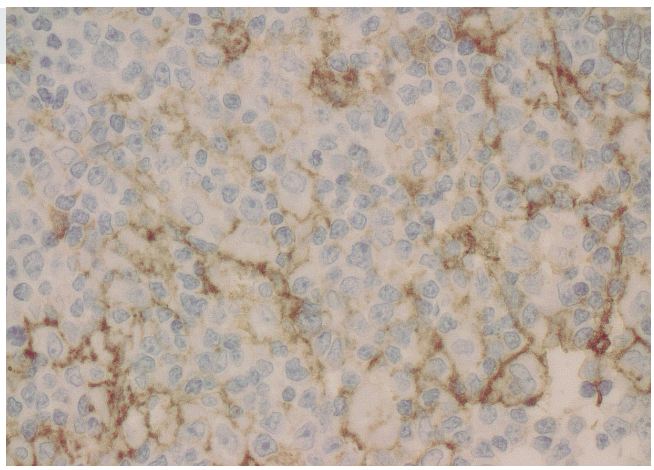
CD68 (positive)

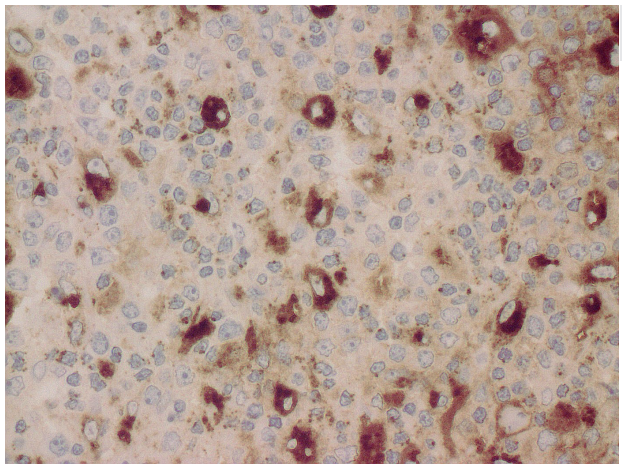
b



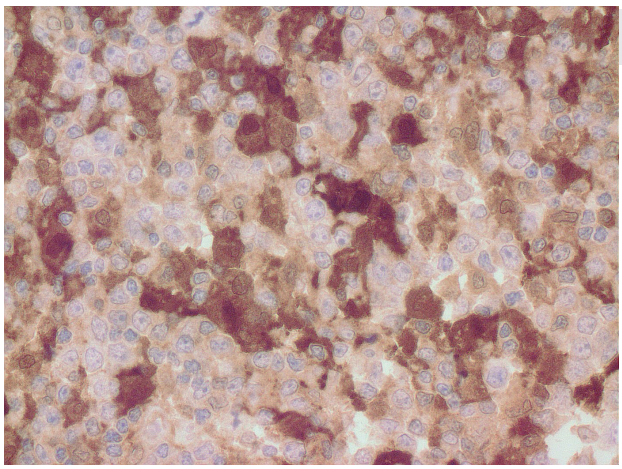
ICAM-1

c

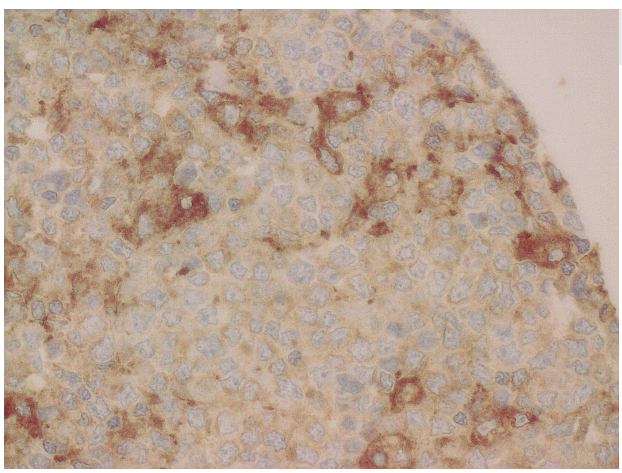




d cathepsin D



e galectin-3



f MVP/LRP

Figure 8 Immunohistochemical expression of proteins over-expressed in a cohort of cured DLBCL. Positivity for all epitopes is seen on the reactive cells, i.e. mainly macrophages.

GENERAL DISCUSSION

Papers I-III

In an, in this context, early study on 125 patients with de novo DLBCL, we performed an immunohistochemical study in order to identify GC-like and ABC-like tumours, but could not, on the basis of established GC markers as CD10 and BCL6 identify any prognostically different groups (Study I). CD10 expression did not give any prognostic information. BCL6 was positive in almost all cases, prohibiting a meaningful survival analysis, and CD138, positive in only 2%, did not serve as an adequate marker for a non-GC origin.

Our suspicion that the immunohistochemical technique used, EnVision method, per se might contribute to a higher frequency of BCL6-positivity was later confirmed (Study III).

Other groups also had difficulties in identifying GC-like and ABC-like DLBCL by immunohistochemical means [90,91] whereas others were more successful [85,87]. The most robust algorithm for categorization of DLBCL with different prognosis on the basis of CD10, BCL6 and MUM-1 was first identified by Hans *et al.* [88], and soon confirmed by others [86]. The cut-off values chosen for all markers, 30%, are quite arbitrarily set but have proved to be efficient in discerning lymphomas with good versus poor prognosis.

In study II, we used this algorithm for categorization in GC and non-GC tumours but found no significant survival differences between the two groups. Immunohistochemistry was performed on TMA, and re-examination on OTS revealed a profound heterogeneity of BCL6 staining within the same tumour, with a high number of false negative cases using TMA, leading to reclassification in 29% when assessed on OTS. CD10 was more stable, but still reclassified in 8% (Paper II, III).

According to the literature one cannot expect 100% concordance between TMA and ordinary sections. Hedvat *et al* [143] reports concordance between 86 and 100% depending on antibody and lymphoma subtype, and Zettl *et al* [144] reports similar results. There are also obvious difficulties in defining cut-off values for the nuclear marker BCL6. Positivity has been defined from less than 1% to 30% positive tumour cells [23,88,98,99] and consensus is lacking.

Based on the results of study II and III, it is obvious that for the immunohistochemical categorization into GC and ABC derived DLBCL, the TMA technique is not ideal, mostly due to the difficulties interpreting BCL6-status. These observations are supported

by the report from the Lunenburg Consortium, who on TMA sections investigated inter-laboratory and inter-observer variability of frequently used antibodies including CD10, BCL6, and MUM1, finding great discrepancies in general, but poor agreement in particular for BCL6. Their recommendation is to centralize the technical procedures as well as the assessment, if categorization into GC vs. non-GC phenotype is to be used in clinical trials [145].

Due to the essential role of CD40 for the germinal center reaction, CD40 was used as a potential marker of GC origin (Paper I). It was however obvious that CD40 did not reflect a germinal center derivation. The expression of CD40 did not correlate with CD10 expression (Paper I) and there was no correlation to the GC phenotype (Paper II). The lack of correlation between CD40 expression and GC markers is supported by gene expression data indicating that CD40 activation may be involved only in the initial and final stages of the GC-reaction, and not during the actual GC-expansion [146].

Another cell surface antigen, CD23, was used as a potential marker for early GC phase since it is expressed on naïve B-cells in this stage [147,148]. However, no correlation with the GC-phenotype was found (Paper II). Expression of CD23 was strongly correlated to CD40 expression (Paper I, II). In vitro studies have shown B-cells to up-regulate CD23 after CD40 activation [106,149] and the concomitant expression of these molecules has recently been reported in primary cutaneous B-cell lymphoma [150]. Co-expression of CD23 and CD40, present in 16% of the tumours in paper I and 10% in paper II, in is not a well-defined feature of DLBCL, and further studies including CD23 may help to elucidate the function of CD40. The finding of a strong association between CD23 and OS in paper I could not be confirmed in paper II, probably due to small numbers and different patient cohorts.

A previously not described observation was the strong association between CD40 positivity and a favourable outcome (paper I, II). In order to elucidate the underlying biological mechanisms for this effect, CD40 expression was investigated in relation to the GC-phenotype and to the degree of tumour infiltrating lymphocytes, TILs (Paper II). Regarding the GC-phenotype, the positive prognostic impact of CD40 expression is not due to association with this prognostically favourable phenotype.

The theory concerning differences in TILs in relation to CD40 status derived from experimental data, showing that both normal and malignant B-cells can function as antigen presenting cells after activation of CD40, leading to expansion of CD8 positive cells [151-154]. This led to the hypothesis that the survival benefit seen for patients with CD40 expressing tumours was associated with the ability of generating an autologous tumour response, which might be reflected in an increased amount of TILs, previously shown to be associated with a better outcome in DLBCL [135-139]. Tumour infiltrating CD4 and CD8 positive T-cells, mainly corresponding to helper and cytotoxic T-cells respectively, were therefore analyzed and categorized in two groups. Patients with more than 5% infiltrating CD4 or CD8 positive cells showed a tendency for better overall survival. However, no correlation between amount of T-cells and CD40 positivity was found in this study, rejecting the hypothesis of an increased number of CD4 or CD8 positive T-

cells in CD40 positive tumours. This finding do not exclude that the survival advantage seen for patients with CD40 expressing tumors is due to differences in immunological reactivity. The CD40L can, besides expression on activated helper T-cells, also be found on other migrating cells: basophils, eosinophils, monocytes, macrophages, dendritic cells, NK cells, B-lymphocytes and mast cells[107], none of which have been examined in this study. It would be of interest to relate the expression of CD40 also to these cells, e.g. to the infiltration of mast cells, which recently was described being associated with good prognosis in DLBCL [142].

Stimulation of the CD40 system through agonistic CD40 antibodies or through administration of recombinant CD40L have been, or are currently used in phase I trials including non-Hodgkin lymphoma [155,156]. Potential biological effects of such treatment are induction of antibody-dependent cellular cytotoxicty, stimulation of pro-apoptotic pathways, and enhancement of autologous T-cell response [155,157,158]. Although an enhanced autologous T-cell response under such conditions is thought to be mediated via antigen presenting dendritic cells [159], it would be of interest to investigate if CD40 expression on tumour cells and responsiveness to therapy correspond. Potentially troublesome in the context of therapy, are pre-clinical data indicating that administration of CD40 ligand can induce resistance to chemotherapy [160] and data indicating that the CD40-CD40L interaction can induce anti-apototic signals when lymphoma cells are exposed to cytotoxic agents[161].

A study investigating the GEP in relation to CD40 expression is ongoing and preliminary data show good correlation between mRNA levels and immunohistochemical expression of the protein. Of the top 28 genes, at a false discovery rate of 10%, discriminating CD40 positive and CD40 negative tumours, 21 are relatively up-regulated in the CD40 positive cohort and code for proteins involved in cell-to-matrix interactions, e.g. collagens (type VI alpha I and 2), proteoglycans (lumican, biglycan, versican), integrin alpha V, and proteolysis (matrix metallopeptidase 2, UPAR, proteasome beta type 5).

In conclusion, we have in two independent studies shown that immunohistochemical expression of CD40 is associated with prolonged overall survival in advanced stage DLBCL, by mechanisms that we so far cannot explain. Although we have not been able to show any differences in autologous tumour response between tumours expressing or lacking this antigen, we still believe this is an issue to pursue in addition to studies investigating the relation between CD40 expression and proteins involved in cell-to-matrix interactions.

Paper IV

The patients in this study were highly selected and chosen to represent the absolute opposites regarding sensitivity for chemotherapy. The central finding, discovered by immunohistochemistry and indicated by differences in gene expression profiles, was the enhanced infiltration of inflammatory cells in the cured cohort. The frequency of macrophages expressing proteolytic enzymes was significantly higher in tumours from the cured cohort, as was the frequency of tumour infiltrating CD8 positive lymphocytes. A similar trend was seen for CD4 positive cells.

The positive prognostic impact of tumour infiltrating CD4 and CD8 positive cells, representing helper and cytolytic/cytotoxic T-lymphocytes respectively, has earlier been shown in several studies. Xu *et al* and Ansell *et al*, using flow cytometry, found that > 20 % CD4-positive cells was associated with significant prognostic advantages, both regarding failure-free and overall survival [138,139]. Lippman *et al.* used immunohistochemistry and found that <6% tumour infiltrating CD8-postive cells was associated with a significant shorter relapse-free survival [135]. The correlation between low numbers of infiltrating CD8 positive cells and adverse prognosis has later been confirmed by Stopeck *et al* and Rimsza *et al*, using a cut-off level of 6% [136,137]. For study II (and IV) a cut-off value of 5% for both CD4 and CD8 was used in order to simplify the visual estimation. Although not significant, there was a trend for better OS in patients with more than 5% infiltrating CD4 or CD8 positive cells (Paper II).

The association between tumour associated macrophages (TAM) and the prognosis of DLBCL has to our knowledge not been reported earlier. In follicular lymphoma, data concerning the prognostic effect of TAM are conflicting[162-165]. In peripheral T-cell lymphoma, data from a recent study indicate that TAM is associated with a superior prognosis [166]. In breast cancer, TAM are generally associated with adverse prognosis [167-169] whereas a study on colo-rectal cancer indicates the opposite [170]. The conflicting data probably reflects the complex behaviour described for macrophages detected in tumours. According to the literature, macrophages classically activated by microbial antigens or IFNγ, type 1 macrophages (M1), contribute to the killing of microorganisms and tumour cells, producing pro-inflammatory cytokines, whereas macrophages under other conditions (e. g. through stimulation via IL-4) can differentiate into type II macrophages (M2) with anti-inflammatory properties, able to promote tissue remodelling and angiogenesis, contributing to tumor growth and progression [171-174]. A subdivision in M1 and M2 macrophages was not performed in the present study, but it was clearly shown that in DLBCL, an enhanced macrophage infiltration, as well as an increased expression of associate proteins, is related to favourable outcome. It thus seems that the tumour microenvironment could be of importance for the curability of DLBCL, but the means by which chemo-sensitivity could be influenced by lymphocytes and macrophages is not obvious.

The seven genes that were up-regulated within tumours of the refractory cohort were not obviously linked to chemotherapy resistance, although rab geranylgeranyltransferase beta subunit, a potential target of farnesyl transferase inhibitors and bisphosphonates [175,176] and DNA polymerase epsilon (POLE), a target for antimetabolite nucleosides [177] might be of interest for further studies.

The choice taken to analyze genes only with a false discovery rate (FDR) of ≤ 5% could mean a risk of not noticing other genes of biological interest since the FDR of the top 500 genes was as low as 21%. In fact, when analysing the top 100 genes up-regulated in the refractory cohort, other genes of interest appeared, most possibly reflecting the tumor cells and not their surroundings. Several genes associated with mitosis were found to be upregulated, as well as the DNA repair genes topoisomeras II-α, thymidylate synthase and MSH2. Further studies are warranted.

In conclusion: based on results from gene expression profiling and immunohistochemical analyses, we found that the presence of tumour infiltrating T-lymphocytes and macrophages seem to be important for the chance of curability in advanced stage DLBCL.

The German Pathologist Rudolf Virchow (1821-1902) was the first to describe the presence of leukocytes in human tumours, and to make a connection between inflammation and cancer. He suggested that the "lymphoreticular infiltrate" reflects the origin of cancer at sites of chronic inflammation.

Concluding summary

In DLBCL, imunohistochemical categorization in different prognostic subgroups based on GC or non-GC origin is feasible. The most robust algorithm, presented by Hans *et al*, uses the combination of CD10, BCL6 and MUM1. According to our experience, the tissue microarray technique is not to recommend for this analysis, mostly due to difficulties interpreting BCL6 status, with a high risk of achieving false negative results.

We have in two independent studies shown that immunohistochemical expression of CD40, a cell surface molecule of importance for immunological responses, is strongly associated with a favourable outcome in DLBCL. The biological mechanism for this effect is so far unknown. Although no differences in tumour infiltrating T-lymphocytes between tumours expressing or lacking this antigen were found, we cannot rule out that there are other differences in immunological response not yet detected. We will in future projects investigate the connection between CD40 positive tumours and tumour infiltrating cells of myeloid lineage known to harbour the CD40 ligand. Furthermore, we intend to study proteins involved in cell-to-matrix interactions, e.g. proteoglycans, as molecular profiling indicates up-regulation of such proteins in CD40 positive tumours.

An unexpected connection between chemotherapy sensitivity and tumour microenvironment was revealed in our last study. The genes that most differed between chemotherapy sensitive and refractory tumours were not expressed by the tumour cells themselves, but coded for proteins in the tumour microenvironment. By immunohistochemistry, we discovered that reactive cells, mainly macrophages, expressing proteins that in other tumours have been associated with local aggressiveness, e.g. UPAR and cathepsin D, were significantly more frequently found in the chemo-sensitive cohort, as was tumour infiltrating cytotoxic T-cells. The means by which chemo-sensitivity could be influenced by macrophages and lymphocytes is not obvious and warrants further study.

Populärvetenskaplig sammanfattning

Maligna lymfom är tumörer om utgår från en del av immunsystemets celler, de vita blodkroppar som kallas lymfocyter. Årligen insjuknar i Sverige ca 1700 personer i malignt lymfom. Det finns många olika slags lymfom, ett fyrtiotal, och denna indelning görs på grundval av vilken slags lymfocyt, och mot vilket mognadsstadium för normala lymfocyter, den elakartade lymfocyten motsvarar. Huvudgrupperna är B-cellslymfom, T-/NK-cellslymfom (tillsammans ofta benämnda non-Hodgkinlymfom) samt Hodgkinlymfom. Funktionellt delar man upp lymfomen i indolenta, aggressiva samt mycket aggressiva (kallades förr låg- resp. högmaligna lymfom). Indolenta lymfom är påverkbara med cytostatikabehandling men i princip inte botbara. Dock lever många patienter med dessa sjukdomar upp till flera decennier. Aggressiva och mycket aggressiva lymfom är på kort sikt dödliga sjukdomar om man inte behandlar med cytostatika. Då finns det å andra sidan gott hopp om bot.

Diffust storcelligt B-celllymfom (DLBCL) är den vanligaste aggressiva lymfomsjukdomen, och drabbar ca 450 personer årligen i Sverige. Medianåldern vid insjuknande är 70 år. Sjukdomen presenterar sig ofta som snabbt tillväxande lymfkörtlar, men primära sjukdomsmanifestationer av organ utanför blod-och lymfsystemet är inte ovanliga. Av patienter med lokaliserad sjukdom botas 70 %, med spridd sjukdom ca 50%.

Man har länge trott att DLBCL motsvarar det mognadsstadium som den friska B-lymfocyten befinner sig i när den passerar lymfkörtlarnas germinalcentra. I germinalcentrum sker en rad viktiga processer som är nödvändiga för att B-lymfocyten ska kunna utmogna till en minnes-B-cell eller en antikroppsproducerande plasmacell. Bakgrunden till arbete 1 i denna avhandling var en rapport där man med hjälp av genuttrycksprofilering av DLBCL funnit att patienter vars tumörer hade tydliga drag av gener aktiverade under germinalcenterfasen (GC) hade betydligt bättre överlevnad än de patienter vars tumörer hade drag av ”aktiverade B-lymfocyter” (ABC). Definitionen av en aktiverad B-lymfocyt är inte helt självklar, men i dessa sammanhang menar man utvecklingsstadiet/stadierna efter germinalcenterfasen. Eftersom genuttrycksprofilering är kostnadskrävande och tekniskt komplicerat ville vi undersöka om man på ett enklare sätt kunde identifiera GC- eller ABC-profiler på proteinnivå med hjälp av immunhistokemi, en metod med vars hjälp man kan studera om och var olika proteiner uttrycks i tumörvävnad.

Delarbete 1

Med hjälp av etablerade germinalcentermarkörer, CD10 och BCL6, och en förmodad postgerminalcentermarkör, CD138, kunde vi inte med hjälp av immunhistokemi identifiera prognostiskt skilda subgrupper i en grupp av 125 patienter som cytostatikabehandlats pga DLBCL. Frekvensen av BCL6-positiva tumörer var ovanligt hög, 97 %, och vi undrade om detta kunde bero på att den immunhistokemiska teknik som använts, EnVision metoden, var känsligare än äldre metoder.

I studien användes också ytterligare en markör för att identifiera germinalcenterfasen, CD40. Utan CD40, som har en viktig roll i aktiveringen av B- och T-celler, får man ingen germinalcenterreaktion. Uttryck av CD40, som sågs i 76 % av tumörerna, var förenat med förlängd överlevnad för patienterna, men hade inget samband med övriga undersökta markörer. Ingen slutsats kunde därför dras huruvida uttryck av CD40 kan anses representera ett germinalcenterursprung.

Delarbete 2

I detta arbete undersökte vi en ny grupp av 125 patienter som cytostatikabehandlats för DLBCL. Vi använde här en immunhistokemisk modell för uppdelning i GC och icke-GC deriverade tumörer som i en tidigare studie visat sig fungera för att dela upp patienter i olika prognosgrupper. Modellen använde CD10 och BCL6 som markörer för GC och MUM1 som markör för postgerminal/icke-GC fas. Modellen fungerade också i vår studie. Patienter med GC-profil hade en längre överlevnad än patienter med en icke-GC-profil.

Vi kunde på nytt visa att CD40, som uttrycktes i 67 % av tumörerna, var en stark prognosfaktor, men kunde konstatera att detta inte berodde på samröre med den prognostiskt gynnsamma GC-profilen.

Eftersom CD40 har stor betydelse för immunförsvaret och bl.a. stimulerar utvecklingen av mördar T-celler, var vår hypotes att de tumörer som uttrycker CD40 på sin yta behållit en förmåga att aktivera kroppens eget svar på tumörer, det s.k. autologa tumörsvaret. Detta skulle i så fall kunna återspeglas in en ökad andel tumörinfiltrerande T-lymfocyter, en reaktion som i flera studier visat sig vara kopplat till en bättre prognos i DLBCL. Så var emellertid inte fallet i vår studie. En ökad andel infiltrerande hjälpar- och mördarceller var associerat med en bättre prognos, men detta hade inget samband med CD40 uttryck.

Vi fortsätter nu bl.a. med att studera sambandet mellan CD40-uttryck och andra möjliga aktörer i det autologa tumörsvaret, d.v.s. andra typer av vita blodkroppar som skulle kunna reagera med och påverka CD40-uttryckande tumörer.

Delarbete 3

I detta arbete bekräftade vi vår misstanke från arbete 1 att bruket av EnVision-tekniken, numera en standardmetod för påvisandet av immunohistokemiska reaktioner, i sig leder till en högre frekvens av BCL6-positivitet än vad användandet av äldre immunhistokemiska metoder gör.

Vidare konstaterade vi att användandet av s.k. tissue microarrayteknik (TMA) inte lämpar sig för att bestämma om tumörer ska klassas som GC eller icke-GC. Detta beror framförallt på att BCL6 färgar in ojämnt, med hög risk för falskt negativa resultat.

Delarbete 4

Här studerades genuttrycksprofilerna, det totala uttrycket av alla ”påslagna gener” i tumörer från två mycket skilda patientpopulationer, botade versus primärt cytostatikarefraktära/resistenta DLBCL. Den botade gruppen bestod av 24 patienter som samtliga svarat med komplett remission på primärbehandling och varit sjukdomsfria i minst tre år. Den refraktära gruppen bestod av 13 patienter som inte svarat på cytostatikabehandling utan progredierat primärt, och där alla var avlidna inom 13 månader. Något överraskande fann vi att de gener som bäst skilde de båda grupperna inte verkade härröra från tumörcellerna själva utan från deras omgivning. Detta bekräftades genom immunhistokemiska analyser, där höga gennivåer motsvarades av ett högre proteinuttryck. I den botade gruppen var andelen tumörer som uttryckte proteiner som är inblandade i olika inflammatoriska processer mycket större än i den refraktära gruppen. Vidare analyser visade att dessa proteiner var lokaliserade i makrofager som, liksom tumörinfiltrerande mördarceller, mycket oftare fanns i tumörer från den botade gruppen.

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