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Prognostic Markers and Mechanisms of Chemotherapy Resistance in Diffuse Large B-cell Lymphoma

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Prognostic Markers and Mechanisms of Chemotherapy Resistance in Diffuse Large B-cell Lymphoma

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DOCTORAL DISSERTATION

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Title: Prognostic Markers and Mechanisms of Chemotherapy Resistance in Diffuse Large B-cell Lymphoma

Abstract:

Diffuse large B-cell lymphoma (DLBCL) is an aggressive and heterogeneous disease. Identification of prognostic and predictive markers is important to design individualised and effective treatment strategies. This thesis describes different approaches to search for molecular prognostic markers and an attempt to overcome chemotherapy resistance in DLBCL.

In the first study it was investigated whether immunohistochemical expression of CD40, previously associated with a favourable prognosis in DLBCL, had any prognostic impact after the addition of rituximab to anthracycline-based chemotherapy. Results showed better prognosis in CD40-positive patients receiving this combined treatment. An inflammatory stromal process was observed in CD40-expressing tumours when using gene expression profiling, possibly contributing to a better prognosis in CD40-positive patients.

A study about the effect of inhibited protein prenylation on the response to CHOP therapy, using an in vitro cell-line-based model, showed that treatment with geranylgeranylation inhibitors in DLBCL cell lines had a chemo-sensitising effect. This indicates that inhibition of geranylgeranylation may prove a useful strategy for overcoming CHOP resistance in DLBCL.

A recombinant antibody microarray was used to search for prognostic protein profiles in plasma from DLBCL patients in an attempt to identify new candidate markers for the prognosis and treatment response in DLBCL and to provide a basis for future investigations and validation.

The results of this work indicated that the tumour microenvironment and host immune response contribute to the prognosis in DLBCL, and suggest a potential novel strategy to overcome chemotherapy resistance. Further molecular profiling is important for improved treatment of patients with DLBCL.

Key words: Diffuse large B-cell lymphoma, prognosis, CD40, tumour microenvironment, host immune response, chemotherapy resistance, prenylation inhibitors, plasma proteome

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Still confused, but on a higher level
Enrico Fermi

Contents

Thesis at a glance

Paper	Question	Method	Results and Conclusions
I	Does CD40 retain its prognostic impact in DLBCL after the addition of rituximab to chemotherapy?	Paraffin-embedded samples from DLBCL patients previously treated with R-CHOP were investigated regarding the expression of CD40 using immunohisto-chemistry.	The prognostic impact of CD40 is maintained in DLBCL after the addition of rituximab to chemotherapy.
II	Can DLBCL cell lines be sensitised for CHOP treatment by the addition of prenylation inhibitors?	A cell-line-based model was used to study the response to CHOP treatment after the addition of prenylation inhibitors.	Geranylgeranylation inhibitors had a CHOP-sensitising effect in DLBCL cell lines, suggesting a potential novel strategy for overcoming chemotherapy resistance in DLBCL.
III	What are the under-lying mechanisms for the prognostic impact of CD40 in DLBCL?	Gene expression analysis of tumour tissue from CD40-positive and -negative patients was performed using an oligonucleotide microarray.	Gene expression profiling indicated a stromal inflammatory process in CD40-positive tumours that may contribute to the favourable prognosis in CD40-positive patients.
IV	Can protein profiles in plasma from DLBCL patients be associated with clinical variables and prognosis?	Plasma samples from DLBCL patients, previously included in a prospective phase II clinical trial, were analysed using a recombinant antibody microarray.	Protein profiling of plasma revealed novel insights into the biology of DLBCL and provided the basis for further investigations of predictive and prognostic markers.

List of papers

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

I. CD40 is a Potential Marker of Favourable Prognosis in Patients with Diffuse Large B-cell Lymphoma Treated with Immunochemotherapy

***Rydström K**, Linderoth J, Nyman H, Ehinger M, Joost P, Bendahl PO, Leppä S, Jerkeman M*

Leuk Lymphoma. 2010 Sept; 51(9):1643-8

II. Inhibition of Geranylgeranylation Mediates Sensitivity to CHOP-induced Cell Death of DLBCL Cell Lines

*Ageberg M, **Rydström K**, Lindén O, Linderoth J, Jerkeman M, Drott K*

Exp Cell Res. 2011 May; 317(8):1179-91

III. Gene Expression Profiling Indicates that Immunohistochemical Expression of CD40 is a Marker of an Inflammatory Reaction in the Tumour Stroma of Diffuse Large B-cell Lymphoma

***Rydström K**, Joost P, Ehinger M, Edén P, Jerkeman M, Cavallin-Ståhl E, Linderoth J*

Leuk Lymphoma. 2012 Sept; 53(9):1764-8

IV. Plasma Immunoprofiling of Patients with High-risk Diffuse Large B-cell Lymphoma; a Nordic Lymphoma Group Study

Pauly F, **Fjordén K***, Leppä S, Holte H, Björkholm M, Fluge Ø, Møller Pedersen L, Eriksson M, Isinger-Ekstrand A, Borrebaeck C, Jerkeman M**, Wingren C***

* joint first authorship ** joint last authorship
Manuscript, pending submission to Blood

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Related publications

1. Prognostic Impact of Protein Kinase C beta II Expression in R-CHOP-treated Diffuse Large B-cell Lymphoma Patients

   *Riihijärvi S, Koivula S, Nyman H, **Rydström K**, Jerkeman M, Leppä S*

   Mod Pathol. 2010 May; 23(5):686-93

2. High Serum Vascular Endothelial Growth Factor Level is an Adverse Prognostic Factor for High-risk Diffuse Large B-cell Lymphoma Patients Treated with Dose-dense Chemoimmunotherapy

   *Riihijärvi S, Nurmi H, Holte H, Björkholm M, Fluge O, Pedersen LM, **Rydström K**, Jerkeman M, Eriksson M, Leppä S*

   Eur J Haematol. 2012 Nov; 89(5):395-402

3. The Histone Deacetylase Inhibitor Valproic Acid Sensitizes Diffuse Large B-cell Lymphoma Cell Lines to CHOP-induced Cell Death

   *Ageberg M, **Rydström K**, Relander T, Drott K*

   Am J Transl Res. 2013; 5(2):170-83

4. Deregulation of COMMD1 is Associated with Poor Prognosis in Diffuse Large B-cell Lymphoma

   *Taskinen M, Louhimo R, Koivula S, Chen P, Rantanen V, Holte H, Delabie J, Karjalainen-Lindsberg ML, Björkholm M, Fluge Ø, Pedersen LM, **Fjordén K**, Jerkeman M, Eriksson M, Hautaniemi S, Leppä S*

   PLoS One. 2014; 9(3); e91031

My contributions to the papers

Paper I
I participated in the design of the study and in the immunohistochemical analysis together with haematopathologists. I was responsible for analysing the data and writing the paper.

Paper II
I participated in the laboratory work and performed Western blot analysis. I participated in analysing the data and writing the paper.

Paper III
I was jointly responsible for analysing the data and was responsible for writing the paper.

Paper IV
I was responsible for collecting the plasma samples from the Nordic countries, I participated in the design of the study and was introduced to the laboratory work. I was jointly responsible for analysing the data and writing the paper.

Selected abbreviations

aaIPI	age-adjusted IPI
ABC	activated B-cell
ADCC	antibody-dependent cell-mediated cytotoxicity
AID	activation-induced deaminase
ASCT	autologous stem cell transplantation
AUC	area under curve
B2M	β2-microglobulin
BCR	B-cell-receptor
BTK	Bruton's tyrosine kinase
CHOP	cyclophosphamide, doxorubicin, vincristine, prednisone
CI	confidence intervals
CNS	central nervous system
DI	DNA index
DLBCL	diffuse large B-cell lymphoma
DLBCL NOS	diffuse large B-cell lymphoma not otherwise specified
EBV	Epstein-Barr virus
FDR	false discovery rate
FFS	failure-free survival
FPP	farnesyl pyrophosphate
FTase	farnesyl transferase
FTI	farnesyl transferase inhibitor
GC	germinal centre
GCB	germinal centre B-cell
GGPP	geranylgeranyl pyrophosphate
GGTase 1	geranylgeranyl transferase 1
GGTI	geranylgeranyl transferase 1 inhibitor
HMG-CoA	hydroxy-methylglutaryl coenzyme A
HR	hazard ratio
IFN-γ	interferon-γ
IgH	immunoglobulin (heavy)
IgL	immunoglobulin (light)
IKK	IκB kinase
IL	interleukin
IPI	International Prognostic Index
JNK	c-Jun N-terminal kinase

LOO	leave-one-out
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
mRNA	messenger RNA
NFκB	nuclear factor-kappa B
NK cell	natural killer cell
OS	overall survival
PFS	progression-free survival
PI3K	phosphatidylinositol 3-kinase
PSMB5	proteasome beta type 5
Rab GGTase	Rab geranylgeranyl transferase
RAG	recombinase-activating gene
R-CHOP	rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone
R-DHAP	rituximab, dexamethasone, aracytine, cisplatin
ROC	receiver operating characteristics
scFv	single-chain fragment variable
SEM	standard error of the mean
SVM	support vector machine
TGF-β	transforming growth factor-β
TLRs 2/4	toll-like receptors 2 and 4
TMA	tissue microarray
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-α
TRAF	TNF receptor-associated factor
uPAR	urokinase-type plasminogen activator receptor

Introduction

Malignant lymphomas originate from lymphoid cells at various stages of differentiation. They are clonal tumours of B-cells, T-cells or natural killer (NK)-cells that have reached different levels of maturation when they transform into malignant cells. The current WHO classification includes more than 70 different variants of lymphoid malignancies [1]. This large number of entities reflects the diversity of the immune system regarding the variety of functions and stages of differentiation.

In the clinic, malignant lymphomas have widely different features and are often roughly divided into indolent, aggressive or very aggressive lymphomas, with Hodgkin's lymphoma considered as a separate category. In general, indolent lymphomas are slowly progressive but not curable diseases, while aggressive and very aggressive lymphomas have a rapid clinical progress with a survival measured in months without treatment. However, the aggressive and very aggressive lymphomas are potentially curable with modern treatment.

Diffuse large B-cell lymphoma (DLBCL) belongs to the aggressive lymphomas and is the most common form of malignant lymphoma in adults. DLBCL arises from different steps of normal B-cell development, and is a heterogeneous disease with considerable variation in molecular pathogenesis, morphology and clinical behaviour. Major efforts have been made and are ongoing, to better understand the biology of DLBCL, allowing us to be more successful in predicting the prognosis and response to different treatments [2-4].

The work in this thesis concentrates on the search for molecular prognostic markers in DLBCL and their potential contribution to the malignant process, together with an attempt to understand and evade chemotherapy resistance in this disease.

The term DLBCL is here used synonymous with the entity DLBCL-not otherwise specified (DLBCL NOS) in the current WHO classification [1].

Normal B-cell development

The differentiation of haematopoietic stem cells into antibody-secreting plasma cells occurs in an ordered maturation and selection process. It includes phases of extremely rapid cell division together with series of somatic recombination and mutation events [5]. The result is an almost unlimited repertoire of antibodies capable of recognising a vast variety of antigens. The genetic modifications essential for normal B-cell development are, however, also a source of DNA damage with the potential to initiate malignant transformation [2].

The process of B-cell development is illustrated in Figure 1. Starting in the bone marrow, haematopoietic stem cells with the capacity to both self-renew and generate the entire immune system, initiate differentiation and branch into several types of progenitor cells, including the common lymphoid progenitor cell. From there, further steps of differentiation lead to the formation of pro-B cells, in which the first B-cell specific genes such as *PAX5*, *CD19*, *CD79A*, and *CD79B* are up-regulated [6, 7].

In pro-B cells, the variable region of the heavy immunoglobulin (IgH) chain gene is assembled through random recombination of the variable (V), diversity (D), and joining (J) gene segments in the IgH locus. Successful rearrangement of the IgH chain allows progression to the pre-B cell stage, where VJ recombination of the variable region of the light immunoglobulin (IgL) chain takes place. To initiate V(D)J recombination, recombinase-activating genes (*RAG1* and *RAG2*) are expressed in pro-B cells and the RAG nucleases produced cause DNA cleavage close to the V, D, and J gene segments. DNA repair processes then resolve the double-stranded DNA breaks resulting in a wide variety of V(D)J segments and variable regions of the Ig chains [8]. Next, successfully rearranged IgH and IgL chains associate with the subunits CD79A and CD79B to form an antigen-specific surface receptor, the B-cell receptor (BCR), and allowing transition into an immature B-cell. However, only 20% of the immature B-cells pass through the following receptor-mediated negative selection where autoreactive B-cells are deleted [7].

The surviving immature B-cells leave the bone marrow and enter the spleen as transitional B-cells. The transitional stage is yet another checkpoint for BCR autoreactivity, before progression to either marginal zone B-cells or naive mature B-cells [9]. Marginal zone B-cells have the ability to become short-lived plasma cells and are an essential component of the early T-cell-independent immune response to blood-borne pathogens [10].

In contrast, the naive mature B-cells can progress into follicular B-cells in a T-cell-dependent manner after antigen binding, and enter the germinal centre (GC) of secondary lymphoid organs [8]. B-cells in the GC divide rapidly and selectively

express BCL6, a transcriptional repressor of cell cycle arrest, apoptosis and differentiation [5, 11, 12]. Activation-induced deaminase (AID) is expressed at high levels, and catalyses both somatic hypermutations of the variable regions of Ig chains for higher affinity to antigen, and class switch recombination of the constant region of the IgH chain to generate different isotypes of antibodies [8]. Positive selection of B-cells with the highest antigen affinity then occurs through interaction with follicular dendritic cells and $CD4^+$ T-cells. Positive selection induce down-regulation of GC transcription factors such as BCL6, and up-regulation of plasma cell lineage transcription factors such as BLIMP-1 and IRF-4, allowing the final differentiation into plasma cells or memory B-cells [5, 13].

Interestingly, DLBCL is thought to arise from B-cells that undergo transformation adjacent to, or shortly after, the maturation steps in the GC [13].

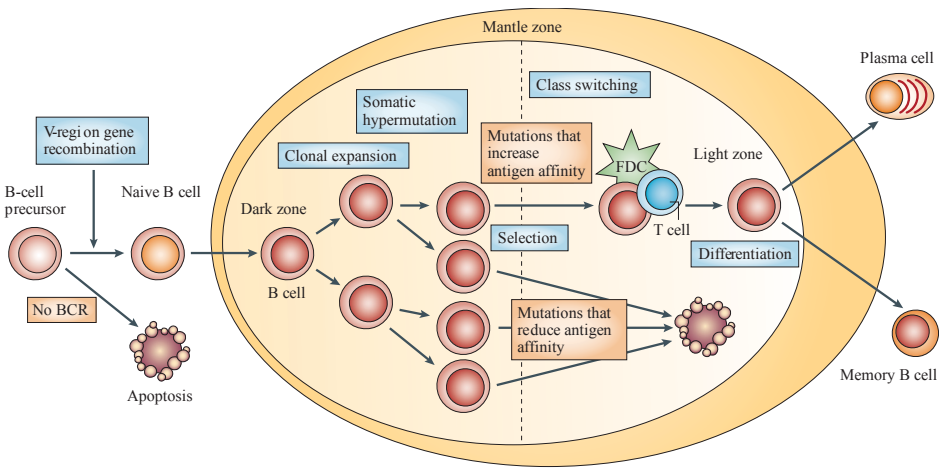

Figure 1. B-cell development. V(D)J recombination of the variable region of the Ig chain genes in precursor B-cells (pro- and pre-B cells) is initiated by RAG1/RAG2. After encountering an antigen, mature naive B-cells enter the GC, surrounded by the mantle zone, in secondary lymphoid organs such as lymph nodes. In the dark zone of the GC, B-cells proliferate rapidly and undergo somatic hypermutations of the variable region of the Ig chain, catalysed by AID. In the light zone, positive selection of B-cells occurs through interaction with follicular dendritic cells (FDC) and T-cells, and class switch recombination is catalysed by AID. After final differentiation, B-cells leave the GC as plasma cells or memory B-cells. *From* [13], *with permission from Macmillan Publishers Ltd.*

Diffuse large B-cell lymphoma

Epidemiology

DLBCL is the most common subtype of lymphoma. In Sweden, the incidence is approximately 5.5 patients per 100 000, corresponding to around 500 new patients each year and representing 25% of all malignant lymphomas in Sweden [14, 15]. DLBCL is slightly more common in males than in females and the median age at diagnosis is approximately 70 years, but it also occurs in young adults [14].

Aetiology

The aetiology of DLBCL remains unknown in most cases and the disease is then considered to arise *de novo* or primary. Secondary or transformed cases from less aggressive lymphomas are seen [1]. Autoimmune disease is a known risk factor [16], as is underlying immunodeficiency, often involving an Epstein-Barr virus (EBV) infection [1]. In immunocompetent patients with DLBCL, the rate of EBV infection is around 10% in Asian and Latin American populations, but < 5% in Western populations [17-19]. Other suggested risk factors include hepatitis C virus, family history of lymphoma disorder, and occupational exposure to certain chemicals [20, 21].

Clinical features

Patients often present with rapidly enlarging single or multiple lymph nodes. Extranodal disease is seen in up to 40% of patients, e.g. in gastrointestinal tract, central nervous system (CNS), testis, and bone [22]. Bone marrow involvement is reported in 11-27% of patients [23-25]. Malignant cells are found in peripheral blood in about one third of the patients with bone marrow involvement [26]. Staging is based on the Ann Arbor classification [27]. Approximately 25% of the patients present with localised disease (stage I), 25% with loco-regional disease (stage II), and 50% with disseminated disease (stages III-IV) [28]. Symptoms often arise over a short period of weeks to months, and are dependent on sites of involvement. Constitutional symptoms including weight loss, night sweats, and fever are frequent [29].

Diagnosis

The most crucial part of lymphoma diagnosis is the histopathological examination with immunohistochemical staining of tumour material, according to the WHO classification [1]. Surgical excision or needle biopsy of a lymph node or a specimen from an extranodal manifestation should provide enough material for formalin fixation and paraffin embedding. Immunohistochemically, DLBCL usually express pan B-cell markers including PAX5, CD19, CD20, CD22, and CD79A. BCL6 is expressed in 60% of DLBCL tumours [30].

Careful clinical examination, laboratory investigations, and medical history are essential at the time of diagnosis. A computed tomography (CT) scan including the thorax and abdomen, and bone marrow aspirate and biopsy, are required for staging. In high-risk patients, a diagnostic spinal tap should be considered [31]. In addition, recent recommendations in the Lugano classification state that positron emission tomography (PET) should be used together with CT for accurate staging of newly diagnosed DLBCL patients [32].

Morphology and classification

DLBCL has historically been morphologically characterised according to the size of the malignant cells. As the name implies, the lymph nodes involved show diffuse proliferation of large lymphoid cells erasing the normal architecture. The nuclei of the malignant cells are traditionally defined as equal to or exceeding the size of a normal macrophage nucleus, or more than twice the size of a small lymphocyte [30]. According to the WHO classification [1] there are three common morphological variants: centroblastic, immunoblastic, and anaplastic, as well as additional rare morphological variants that are together classified as DLBCL NOS. Findings regarding the prognostic impact of the different morphological variants are conflicting and not sufficiently convincing to be used in clinical routine.

T-cell/histiocyte-rich large B-cell lymphoma, primary DLBCL of CNS, primary cutaneous DLBCL (leg type), and EBV-positive DLBCL of elderly are considered to be separate subgroups of DLBCL in the current WHO classification, while primary mediastinal large B-cell lymphoma belongs to the group called “other lymphomas of large B-cells” [1].

Treatment, response and survival

During more than 30 years, the anthracycline-based chemotherapy regime CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) has been the most commonly used chemotherapy for patients with DLBCL, because several attempts

to intensify the chemotherapy have failed to show further benefit [33]. However, addition of the monoclonal CD20-directed antibody rituximab to CHOP about ten years ago resulted in a distinct improvement in outcome and R-CHOP is now considered the standard treatment for patients with DLBCL [34-37].

Despite the improvements seen with R-CHOP, a significant number of patients still die of the disease. The ten-year overall survival (OS) is approximately 40% in patients over 60 years of age with stage II-IV disease [38]. After treatment with R-CHOP, complete remission is achieved in 70-80% of patients [34]. However, 20-30% of these patients will suffer relapse and about 20% of patients are partial responders or refractory to first-line treatment [39].

Patients with relapsed or primary refractory DLBCL represent a major challenge. High-dose therapy with autologous stem cell transplantation (ASCT) is the standard treatment in younger patients with chemosensitive relapse or primary refractory disease [40]. When the two most commonly used regimens for relapses, R-DHAP (rituximab, dexamethasone, aracytine, and cisplatin) and R-ICE (rituximab, ifosfamide, carboplatin, and etoposide) were compared in the CORAL study, no differences in response rates or survival were observed [41]. Worth noting in this report is the poor response rate to salvage therapy in patients previously treated with rituximab compared with rituximab-naive patients (51% vs. 83%, $p < 0.001$), which led to a significant difference in three-year event-free survival, 21% for patients with prior rituximab treatment vs. 47% for rituximab-naive patients [41].

Salvage therapy in elderly patients not suitable for ASCT or intensive chemotherapy represents yet another challenge. Suggested treatments for these patients include rituximab in combination with either bendamustine or gemcitabine and oxaliplatin, as well as inclusion in clinical trials [42-44].

Clinical prognostic factors and their limitations

During the past 20 years, the International Prognostic Index (IPI) has been the primary model for stratifying risk and predicting the outcome for patients with aggressive lymphomas. Four distinct prognostic subgroups are identified based on the number of clinical risk factors at diagnosis (age > 60 years, stage > II, elevated lactate dehydrogenase, performance status > 1, and extranodal sites > 1). An age-adjusted IPI (aaIPI) has also been proposed for patients younger than 60 years [45]. The IPI remains prognostic also after the addition of rituximab to chemotherapy [46], but the differences in outcome between the IPI risk groups have narrowed, patients with identical IPI scores show marked variability in outcome, and the ability to identify patients with the worst prognosis is limited after the introduction of rituximab. Therefore, additional clinical factors have been suggested to influence the prognosis, such as tumour diameter > 10 cm [47], bone

marrow involvement [24], low absolute lymphocyte count [48], high absolute monocyte count [49], and elevated serum-free light chains [50].

Efforts to improve the IPI model have included focusing on elderly patients in the E-IPI [51] and regrouping the original IPI score in the revised R-IPI [52]. Recently, clinical data from the National Comprehensive Cancer Network was used to build the NCCN-IPI, which outperforms IPI by refining the categorisation of age and lactate dehydrogenase as well as identifying extranodal involvement in bone marrow, CNS, liver/gastrointestinal tract, or lung as strong predictors of aggressive disease [53].

However, both the IPI and other suggested clinical risk factors are probably surrogates for an underlying biological heterogeneity between patients. Several attempts have been made to find molecular prognostic markers that are more accurate in identifying patients with different prognosis than the clinical factors included in IPI. So far, only a few of the molecular markers have had an impact on routine clinical practice and the choice of treatment.

Molecular background of DLBCL

During the past decade, the molecular heterogeneity in DLBCL has been revealed through different approaches. Gene expression profiling has identified molecular subgroups representing different stages of lymphoid differentiation at the time of transformation into DLBCL, and progress in sequencing technologies has provided further insights into the molecular complexity of DLBCL. The biological diversity is in accordance with the clinically observed heterogeneity of DLBCL, and has uncovered a number of interesting therapeutic targets in both the tumour cells and components of the tumour microenvironment.

In this section, a selection of recent insights into the biology of DLBCL are described, together with some potential promising novel agents for treatment.

Cell-of-origin concept

Gene expression profiling has identified at least two distinct molecular subgroups derived from different parts of normal B-cell maturation: germinal centre B-cell (GCB) DLBCL and activated B-cell (ABC) DLBCL [54, 55]. These subgroups are indistinguishable using conventional diagnostic tools, but their gene expression profiles differ widely, suggesting that they arise from different stages of B-cell differentiation and are dependent on different oncogenic pathways. In addition, these subgroups provide important prognostic information with a significantly poorer outcome in ABC DLBCL than in GCB DLBCL [56]. Critical oncogenic

pathways and targeting agents under evaluation for patients in these two subgroups are shown in Figure 2.

GCB DLBCL

Several genes specific to the GC reaction are expressed in GCB DLBCL, such as *BCL6, CD10,* and *LMO2* [55]. Further evidence of GC derivation includes ongoing somatic hypermutations of the variable Ig chain gene segments, mediated by the enzyme AID, expressed at high levels in GCB-cells [57].

Regarding oncogenic pathways, about 35% of GCB DLBCL cases harbour a t(14:18) translocation of *BCL2* to the IgH chain locus, resulting in constitutive anti-apoptotic activity of BCL2 [54, 58]. Another deregulated pathway in this subgroup is the phosphatidylinositol 3-kinase (PI3K) signalling pathway, which promotes cell growth, survival, and proliferation. The tumour suppressor gene *PTEN* is the negative regulator of the PI3K pathway [59]. *PTEN* is deleted in 10% of GCB DLBCL, resulting in unregulated activation of PI3K signalling [60]. Furthermore, a gain-of-function mutation of the histone methyltransferase gene *EZH2* is found in 20% of GCB DLBCL, thought to result in enhanced methylation and transcriptional repression of key regulator genes [61, 62].

Novel targeting agents most likely to benefit patients with GCB DLBCL include inhibitors of BCL2, PI3K, and EZH2 [63] (Figure 2). To further support the hypothesis of clinically relevant differences between GCB and ABC DLBCL, patients in the GCB subgroup have been reported to selectively benefit from the addition of etoposide to R-CHOP [64]. Moreover, better results have been reported in relapsed patients in the GCB subgroup than in the ABC subgroup following R-DHAP [65].

ABC DLBCL

ABC DLBCL is thought to be derived from B-cells that have passed through the GC and are in the process of differentiating to plasma cells [54]. They show up-regulation of several genes normally expressed in plasma cells, e.g. *XBP-1*, which is required for terminal differentiation of B-cells to plasma cells [66, 67]. However, full differentiation to a plasma cell is blocked by several genetic abnormalities in ABC DLBCL. Almost 25% of ABC DLBCL cases have inactivating mutations or deletions of the *PRDM1* gene which encodes BLIMP1, a protein needed for final plasma cell differentiation [68, 69]. Another mechanism for down-regulation of BLIMP1 is overexpression of the transcription factor SPIB, in about 25% of these patients, which represses *PRDM1* expression [60, 70].

Regarding oncogenic pathways, constitutive activation of the pro-survival nuclear factor-kappa B (NFκB) signalling pathway is a hallmark of ABC DLBCL. NFκB is a family of transcription factors that are activated by, for instance, BCR signalling and regulate various genes with proliferative and anti-apoptotic

properties [71, 72]. The NFκB transcription factors are maintained inactive by the inhibitory IκB family in the cytoplasm. In normal B-cells, engagement of the BCR by an antigen leads to phosphorylation and degradation of the IκB proteins by IκB kinase (IKK), allowing the nuclear translocation of the NFκB subunits and activation of their target genes [72]. In normal B-cells, IKK is transiently stimulated by a protein complex of CARD11, BCL10, and MALT1 (CBM complex), which is formed as a result of BCR signalling [73]. However, in ABC DLBCL, the NFκB pathway is constitutively active, and the disturbance of NFκB signalling kills ABC DLBCL cells but not GCB DLBCL cells [71, 74].

As shown in Figure 2, molecular sequencing techniques have revealed recurrent mutations of several genes in the BCR pathway in ABC DLBCL. BCR signalling not only initiates downstream activity in the NFκB pathway, but also induces the PI3K and the mitogen-activated protein kinase (MAPK) pathways, all of which are crucial for cell proliferation and survival [75-77]. Mutations of the BCR subunits CD79A/B occur in 20% of ABC DLBCL cases and result in chronic active BCR and NFκB signalling [76]. An activating CARD11 mutation has been found in 10% of ABC DLBCL cases [77]. Inactivating mutations or deletions of the negative NFκB regulator A20 have been reported to be present in 30% of ABC DLBCL cases [75, 78]. Moreover, activating mutations of the gene encoding MYD88, an adapter protein that mediates toll-like receptor signalling, have been found in 30% of ABC DLBCL, also resulting in constitutive NFκB activity [79]. In addition to increased BCR signalling and NFκB activity, the expression of anti-apoptotic *BCL2* is frequently up-regulated due to gene amplification in ABC DLBCL, which is in contrast to the t(14:18) of *BCL2* often seen in GCB DLBCL [60].

Novel targeted therapy suggested for patients in the ABC subgroup includes multiple strategies to inhibit BCR signalling and NFκB activation (Figure 2). Results are awaited from ongoing phase 3 trials in which ibrutinib, bortezomib or lenalidomide, have been added to R-CHOP in first-line treatment of DLBCL. These agents have all shown selective benefits within the ABC subgroup in earlier stage trials [63]. Ibrutinib inhibits Bruton's tyrosine kinase (BTK), which plays a prominent role in the signalling downstream of the BCR [76]. Bortezomib is a proteasome inhibitor that blocks the degradation of the NFκB-inhibitory IκB, thus maintaining the NFκB transcription factors inactive [80]. Lenalidomide has pleotropic effects including immunomodulation and inhibition of NFκB activity, through counteracting the oncogenic effects of MYD88 mutation in the ABC subgroup [81]. Due to the importance of BCR signalling and NFκB activity in the ABC subgroup, additional agents targeting different components of these pathways are under development [63]. Moreover, R-ACVBP (dose-intensive rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone) has been suggested to selectively benefit patients in the ABC subgroup [82].

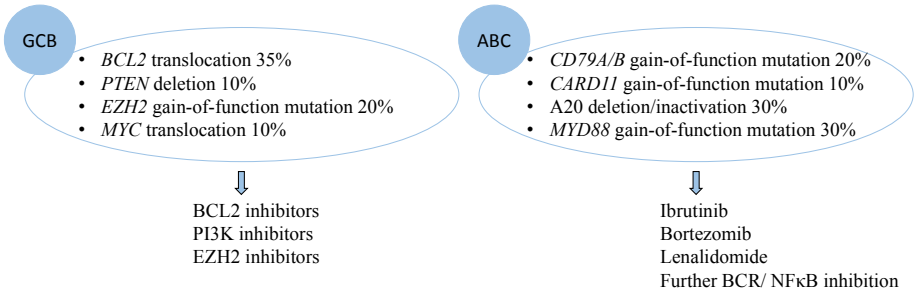

Figure 2. Subgroup-associated genetic alterations and novel agents. A selection of critical oncogenic pathways and targeting agents under evaluation in GCB and ABC subgroups of DLBCL.

Diagnostic challenges concerning the cell-of-origin

Considering the significant differences in oncogenic pathways and novel agents with selective activity within the GCB and ABC subgroups of DLBCL, attempts have been made to find a clinically useful and reliable method of determining the cell-of-origin in newly diagnosed DLBCL patients. Gene expression profiling is not yet routinely performed due to high technical requirements and the need for fresh-frozen tumour tissue. Therefore, efforts have been made to find immunohistochemical-based algorithms that can replace gene expression profiling [83-87]. However, the results regarding the prognostic value of these immunohistochemical classification algorithms have proved inconsistent, with poor reproducibility, especially after the introduction of rituximab [88-90].

Recently, a 20-gene-expression-based assay was shown to be able to robustly and accurately determine the cell-of-origin subgroup with maintained prognostic significance, using formalin-fixed paraffin-embedded tumour tissue [91]. Further validation is required to see if this method can be used in clinical routine.

Additional aspects of molecular heterogeneity

Although a great deal of attention has been paid to the cell-of-origin-derived subgroups in DLBCL during the past decade, gene expression profiling and sequencing techniques have also revealed information on several other molecular features that can distinguish between DLBCL subgroups. Some of these features are associated with alterations in the tumour cells, while others are thought to arise in non-tumour cells in the surrounding microenvironment.

Features of tumour cells

In addition to identification of the cell-of-origin, **gene expression profiling** has been used to detect other biologically interesting subgroups of DLBCL. For example, a gene-expression-based outcome-predictor model has been used to

identify a proliferation signature with poor prognosis after chemotherapy, and a major histocompatibility complex (MHC) II signature that correlated with good prognosis, suggesting that antigen presentation may be involved in the therapeutic response [54]. Moreover, unsupervised clustering of gene expression data from DLBCL tumours defined a subgroup with high expression of genes involved in oxidative phosphorylation, denoted the OxPhos cluster, and another subgroup with a signature of BCR signalling and proliferation. Tumours in the OxPhos subgroup had increased expression of proteosomal subunits and molecules involved in mitochondrial membrane potential and apoptosis. Tumours in the BCR/proliferation subgroup exhibited higher levels of many components of the BCR signalling cascade and cell-cycle regulatory genes. Patients in these two subgroups had a similar 5-year survival, indicating that the subgroups reflect different oncogenic mechanisms rather than predicting prognosis [92].

Use of high-resolution **next-generation sequencing** techniques has redefined the genetic landscape of DLBCL by allowing the identification of novel genetic lesions, providing further potential targets for treatment. Next-generation sequencing has revealed a high frequency of mutations in chromatin-modifying genes in DLBCL, indicating a previously unappreciated disruption of chromatin biology in lymphomagenesis. Over 30% of DLBCL cases display inactivating mutations or deletions of the highly related acetyltransferase genes *CREBBP* or *EP300*, suggested to result in deficient acetylation and impaired regulation of BCL6 and p53 expression. Genomic alterations of *CREBBP* have been identified in both GCB and ABC subgroups [93]. In addition, inactivating mutations of the histone methyltransferase, and supposed tumour suppressor gene, *MLL2* have also been observed in about 30% of DLBCL cases, with no significant difference between the GCB and ABC subgroups [94]. Interestingly, the alterations in *CREBBP/EP300* and *MLL2* are thought to be mutually exclusive, indicating that they represent two alternative mechanisms of achieving a common, and presumably critical, epigenetic regulation in DLBCL [95].

Another set of recently detected genetic lesions in DLBCL concerns genes involved in immune recognition by T-cells and NK cells. Inactivating mutation or deletion of the β2-microglobulin gene (*B2M*) is observed in almost 30% of DLBCL cases, in both GCB and ABC subgroups [96]. The result is loss of MHC I on the cell surface, which is required for recognition by cytotoxic T-cells [97]. Although loss of MHC I is thought to provide immune escape from cytotoxic T-cells, cells that lack MHC I are usually targeted and destroyed by NK cells instead. However, inactivation of *B2M* is often associated with inactivation of *CD58*, encoding a molecule involved in NK and T-cell response [96]. The coordinated inactivation of both *B2M* and *CD58* indicates that these alterations are co-selected to achieve immune escape from both cytotoxic T-cells and NK cells. These findings imply that the escape of immune recognition is an important factor in DLBCL pathogenesis.

In addition, several genetic lesions resulting in deregulated BCL6 activity have been detected. *BCL6* encodes a transcriptional repressor expressed in B-cells during GC reaction and is essential for GC formation including affinity maturation during antibody response [98, 99]. Translocation to various fusion partners including the IgH region and up-regulation of this proto-oncogene occurs in about 30% of DLBCL cases [100-103]. Up-regulation of *BCL6* can also be due to mutations in regulatory gene segments [104]. Moreover, frequent mutations in epigenetic regulators of *BCL6* may cause a deregulated BCL6 activity [93, 105].

Features of non-tumour cells

Gene expression profiling has demonstrated that components of the tumour microenvironment can define different subgroups of DLBCL, in some cases with influence on survival. For example, a prognostically favourable lymph node signature after CHOP treatment, with the expression of genes encoding components of the extracellular matrix and genes typically expressed in macrophages and NK cells, has been reported, suggesting a favourable anti-tumour immune response [54]. In addition, a subgroup showing a host response signature without prognostic impact has been observed in a study using unsupervised clustering, with increased expression of genes indicating inflammation and immune response including markers for the infiltration of macrophages and dendritic cells, and activation of T-cells and NK cells [92]. Moreover, a previous study performed by our group, using gene expression profiling to compare cured vs. primary refractory DLBCL patients after chemotherapy, revealed a reactive tumour microenvironment with up-regulated expression of genes coding for proteins involved in proteolysis and inflammatory processes in the cured cohort [106].

Finally, a prognostically favourable stromal-1 signature has been discovered, with expression of components of the extracellular matrix and genes associated with monocytic lineage. In contrast, a stromal-2 signature associated with poor prognosis has been found, with expression of genes including key regulators of angiogenesis and markers of endothelial cells. These signatures reflect characteristics of non-malignant cells that affect prognosis after R-CHOP [56].

The role of MYC and BCL2

A number of studies have reported a poor outcome in DLBCL patients with *MYC* rearrangement [107, 108]. Translocation of the global transcription factor *MYC* occurs in roughly 10% of DLBCL cases, often to the IgH region, leading to up-regulation of the protein [109, 110]. MYC regulates the expression of up to 10% of the genes in the human genome, and plays a central role in cell differentiation, proliferation, and survival [111, 112].

It has been shown that rearrangement of anti-apoptotic *BCL2* influences the impact of *MYC* rearrangement. Approximately 5% of DLBCL cases harbour both *MYC*

and *BCL2* translocations and are so-called double-hit lymphomas, with dismal prognosis after standard treatment with R-CHOP [110, 113]. This adverse outcome has prompted a search for alternative therapies. Based on retrospective observations of the benefits of DA-EPOCH-R (dose-adjusted etoposide, doxorubicin, cyclophosphamide, with vincristine, prednisone, and rituximab) in *MYC*-rearranged DLBCL, a phase 2 trial using DA-EPOCH-R in patients with *MYC*-rearranged DLBCL including double-hit lymphomas has been initiated, showing initial promising results [114]. However, it has been suggested that the partner gene of *MYC* rearrangement affects the prognosis, indicating that not all patients with *MYC* rearrangement need a more aggressive treatment [115].

During recent years, several observations have indicated that high protein expression of MYC together with high protein expression of BCL2 define a subset of DLBCL patients, called dual-expressers, exhibiting a double-hit lymphoma biology with poor outcome after treatment with R-CHOP [110, 113, 116]. High protein expression of MYC and BCL2 has been reported in about 25% of DLBCL patients, thus being more common than rearrangements on the DNA level, and indicating additional mechanisms for overexpression of MYC and BCL2 [110, 113]. Interestingly, while *MYC* and *BCL2* rearrangements are seen mainly in the GCB subgroup, high protein expression of MYC and BCL2 appears more often in the ABC subgroup [117-119]. To obtain more knowledge, and approach a more effective treatment for patients with concurrent MYC and BCL2 deregulation, it has been proposed that all newly diagnosed DLBCL patients should be assessed for translocations and/or protein overexpression of MYC and BCL2 [63].

Background of the current studies

CD40

The cell surface receptor CD40 belongs to the tumour necrosis factor (TNF)-receptor super family. In general terms, members of the TNF-receptor family are involved in diverse biological processes ranging from the promotion of survival and inflammatory response, to the induction of apoptosis and cell death [120].

During previous work carried out by our group, we investigated CD40 as a potential protein marker of GC-derived DLBCL, to replace the gene-expression-based cell-of-origin classification [55]. We found that CD40 could not be correlated to GC-derived DLBCL [121, 122]. However, expression of CD40 was found to be a marker of favourable prognosis after treatment with CHOP [121, 122]. This was an observation not previously described and it motivated us to perform further studies on the clinical and biological relevance of CD40 expression in DLBCL, as reported in **Papers I** and **III**.

CD40 is expressed on B-cells during all phases of normal B-cell development, as well as on dendritic cells, monocytes and non-immune cells such as endothelial cells, fibroblasts, and epithelial cells. CD154, the natural ligand for CD40, is mainly expressed on activated T-helper cells in a transient and regulated manner, but it is also expressed on myeloid cells. In addition, both the CD40 receptor and its ligand have been described in a soluble form [123].

Upon engagement of its ligand, CD40 recruits adaptor proteins of the TNF receptor-associated factor (TRAF) family to the cytoplasmic tail of the receptor. TRAF proteins can then activate several intracellular pathways such as the NFκB and MAPK pathways including c-Jun N-terminal kinase (JNK) and p38, as illustrated in Figure 3 [124, 125]. In addition, the JAK/STAT, PI3K, and ERK MAPK pathways have been shown to be induced by CD40 signalling [125-128]. These pathways are thought to act in concert and to contribute to many of the diverse reported activities of CD40 signalling [129]. CD40 ligation has been shown to have a proliferative effect in normal B-cells and low-grade B-cell lymphoma cell lines [130]. In contrast, CD40 ligation of high-grade B-cell lymphoma cell lines has been reported to result in decreased proliferation and apoptosis [131, 132]. This indicates a disparate outcome of CD40 signalling, ranging from proliferation to apoptosis, partly depending on cell type and the stage

of B-cell maturation at which the transformation occurred. In addition, observations from carcinoma cells suggest that the level of CD40 ligation, as well as whether the CD40 ligand is membrane bound or soluble, can influence the outcome of CD40 engagement [133, 134].

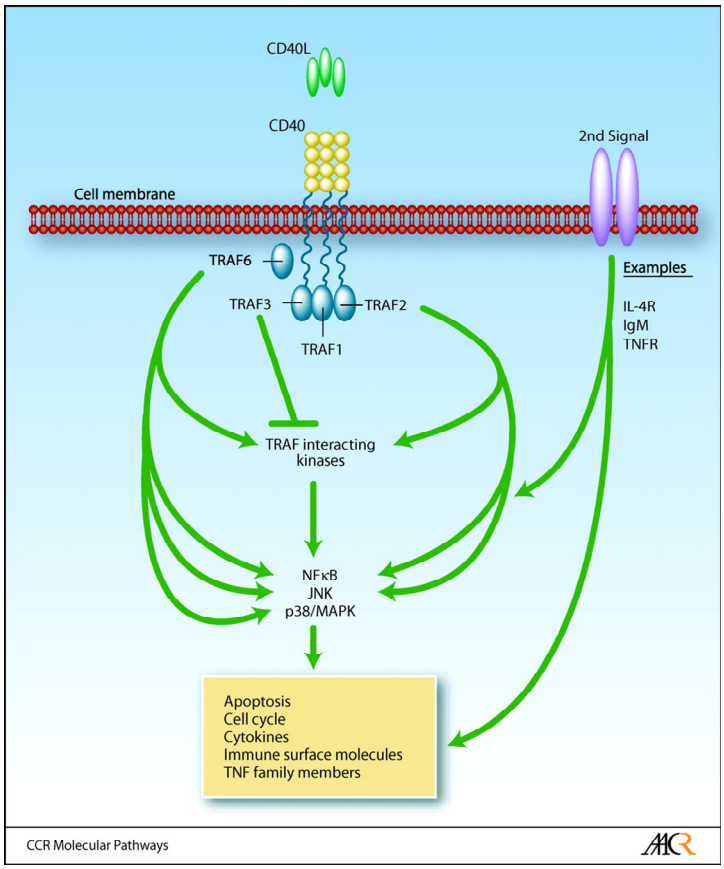

Figure 3. The CD40 signalling pathway. Engagement of CD40 by the CD40 ligand induces recruitment of adaptor proteins of the TNF-receptor-associated factor (TRAF) family to the cytoplasmic tail of the CD40 receptor, followed by modulation of several well-characterised signalling pathways which regulate diverse and critical activities in the cell. Second extracellular signals can cooperate with the CD40 signalling pathway and affect the response. *From* [124], *with permission from AACR.*

CD40 plays a prominent role in regulating the humoral and cellular immunity. The interaction between the CD40 receptor on B-cells and the CD154 ligand on T-helper cells is essential for GC formation and differentiation of B-cells into Ig-producing plasma cells [123, 135]. CD40 signalling is also crucial for the activation of cytotoxic T-cells, through enhanced antigen presentation on dendritic cells after their CD40 interaction with T-helper cells, leading to potent stimulation

of a cytotoxic T-cell response [136, 137]. The key role of CD40 signalling in immune function is exemplified by patients with mutations in the *CD154* gene, who show a severe immune deficiency called X-linked hyper-IgM syndrome, with the inability to produce IgG, IgA, and IgE, resulting in recurrent bacterial and viral infections [138].

Although the role of CD40 signalling was initially thought to be restricted to the regulation of B- and T-cells, it is now known that CD40 interactions have a wide range of functions. The expression of CD40 on non-immune cells is considered to be involved in the amplification and regulation of inflammatory responses [123]. Pro-inflammatory activity of CD40 signalling has been observed in atherosclerotic vascular disease and in autoimmune disorders [139-141].

The role of CD40 in tumours

CD40 is expressed in the majority of B-cell malignancies [142, 143] and in several non-haematopoietic malignancies such as melanoma, and carcinoma of the bladder, breast, ovary, and cervix [144-148]. Considering the frequent expression of CD40 on malignant cells, it could be expected that CD40 interactions provide an advantage to the malignant cells. However, CD40 signalling has in several ways been recognised as a contributor to an anti-tumour response [129]. Firstly, several reports suggest that CD40 ligation of CD40-expressing malignant B-cells may result in enhanced antigen presentation of MHC I, followed by increased cytotoxic T-cell response to the lymphoma cells [149, 150]. Another anti-tumour effect of CD40 engagement may be anti-growth or pro-apoptotic signalling in CD40-postitive tumour cells, since CD40 stimulation of high-grade lymphoma cells has been associated with decreased tumour proliferation [131, 132, 151]. The mechanisms of CD40-induced tumour growth inhibition and apoptosis are not fully understood. However, CD40-mediated apoptosis through the induction of pro-apoptotic Bax or via TRAF-3 and JNK has been reported in lymphoma cells and carcinoma cells, respectively [132, 152].

In addition, CD40 stimulation of non-tumour cells is considered to have a major impact on the host immune response against tumour cells, irrespective of the CD40 status of the tumour cells [129]. The anti-tumour response from lymphoid cells relies largely on effective tumour antigen presentation, mainly by dendritic cells. Upon CD40 stimulation, dendritic cells up-regulate their antigen presentation on MHC I and II to activate a T-cell response to the tumour cells [136, 137]. Furthermore, intratumoural administration of the CD40 ligand has been associated with the up-regulation of T-helper 1 cytokines such as interferon-γ (IFN-γ) and interleukin (IL)-12, together with reduced levels of T-helper 2 cytokines such as IL-10 and transforming growth factor-β (TGF-β) [153-155]. Thus, CD40 stimulation is suggested to counteract a pro-tumourigenic

microenvironment, including T-helper 2 type cytokines, which may be created by malignant cells and surrounding immune cells [129]. It has also been suggested that CD40-stimulated macrophages can participate in an anti-tumour response, possibly through increased antigen presentation on MHC II and the release of pro-inflammatory cytokines [156, 157]

In accordance with the observed responses of CD40 interactions in tumour and non-tumour cells, initial clinical trials with CD40 agonists have shown clinical activity in patients with lymphoma and solid tumours [158]. The partial-agonist anti-CD40 antibody dacetuzumab has shown modest single-agent activity in patients with relapsed DLBCL, and more encouraging results in combination with rituximab and chemotherapy, which is also supported by *in vitro* data [159-162]. Multiple potential mechanisms of action have been demonstrated in preclinical studies of dacetuzumab, including antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis, but also the induction of apoptosis by signalling through CD40 on malignant cells, and immunomodulation by the activation of CD40-expressing antigen-presenting cells [158, 163, 164]. Further work is ongoing to explore the use of CD40 agonists in tumour treatment, and its combination with chemotherapy, vaccines, or other immunomodulatory drugs has been suggested [158].

Host immune response

The importance of the interactions between host immune response, tumour microenvironment, and the tumour cells in DLBCL has been emphasised in several studies during recent years. Some of the findings have already been mentioned in the sections above, regarding lymphocyte and monocyte counts as prognostic risk factors [48, 49], gene expression signatures with features of non-tumour cells [54, 56, 92, 106, 165], and sequencing data indicating frequent immune escape of the tumour cells [96]. The findings presented in **Papers III** and **IV** further support a role of the tumour microenvironment and host immune response in the pathogenesis of DLBCL.

Tumours are not only masses of mutant tumour cells, but complex tissues composed of multiple cell types such as innate and adaptive immune cells, fibroblasts, and cells forming the vasculature. Although the activation of NK cells and the adaptive immune system with cytotoxic T-cells can result in the eradication of tumour cells, the immune system can also contribute with tumour-promoting inflammatory cells. Subsets of inflammatory cells have been shown to support tumour progression, for instance through the induction of tumour cell proliferation, angiogenesis, tissue invasion, and metastatic dissemination [166, 167].

Macrophages

Macrophages are described as one of several essential components of the tumour microenvironment. Tumour-infiltrating macrophages have been reported to have a prognostic impact in DLBCL, but the results are conflicting and constitute a subject of discussion [168-171]. The divergent results may depend on the diverse functions of macrophages, but probably also reflect the problems associated with small patient cohorts, changes in treatment strategies, and methodological differences between studies.

In general, classically activated macrophages (M1 macrophages) are typically induced by exposure to IFN-γ and described as having a role in anti-tumour immunity. In contrast, exposure to other cytokines such as IL-4, IL-10, or IL-13 induces alternatively activated macrophages (M2 macrophages), shown to suppress adaptive tumour-specific immune response and promote tumour growth by the release of immunosuppressive cytokines [172, 173]. Tumour-associated macrophages often acquire M2-like properties and are associated with tumour progression and poor prognosis in solid tumours [174-176].

Immunoediting

Another way of considering the interactions between malignant cells and host immune response is the process described as cancer immunoediting, in which the immune system is thought to both protect the host against the tumour and shape less immunogenic tumour variants that can evade the host response [177-179]. Cancer immunoediting is suggested to play a role also in lymphoid malignancies, and can be schematically described by means of three phases: elimination, equilibrium, and escape, as illustrated in Figure 4 [177, 180].

In the *elimination phase*, malignantly transformed cells are thought to be recognised and destroyed by components of both the innate and adaptive immune system. Tumour antigens are expressed in the context of MHC molecules, and activating cytokines such as IFN-γ and tumour necrosis factor-α (TNF-α) contribute to an anti-tumour activity of the immune system. Next, in the *equilibrium phase*, tumour cells that have survived the elimination phase are proposed to be held in a state of functional dormancy through a balance of anti-tumour cytokines such as IFN-γ and IL-12 together with tumour-promoting cytokines such as IL-10 and IL-23. Due to a constant immune pressure, genetic and epigenetic changes occur in some tumour cells, resulting in less immunogenic tumour cells with the capacity to resist immune recognition. During the *escape phase*, it is suggested that the immune system fails to restrict tumour outgrowth since some tumour cells can evade immune recognition, and immunosuppressive cytokines such as IL-10 and TGF-β are expressed. In addition, regulatory T-cells

may express inhibitory receptors such as programmed cell death protein (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) that further suppress the anti-tumour response.

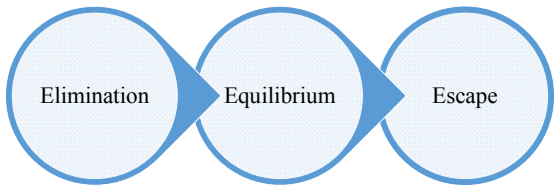

Figure 4. Schematic overview of the concept of immunoediting. Tumour cells are thought to be recognised and destroyed by the immune system in the elimination phase. During the equilibrium phase, genetic alterations that result in less immunogenic tumour cells are suggested to occur, enabling a subset of the tumour cells to resist immune recognition in the escape phase.

The concept of immunoediting probably does not reflect the full complexity of interactions between tumour cells and host immune system, but it provides an interesting view of tumour progression. In "Hallmarks of Cancer" by Hanahan and Weinberg, in which critical underlying principles of transformation and tumour progression are proposed, the ability of tumour cells to achieve immune escape is recognised as one of the hallmarks, indicating an important contribution to the malignant process [167].

Of the cytokines mentioned above, IL-10 and TNF-α have been associated with poor prognosis in DLBCL [181-183]. IL-10 is known as a highly immunosuppressive, but also B-cell-stimulating, cytokine [184, 185]. TNF-α is a potent immunostimulatory cytokine, but is also considered a key regulator of the tumour microenvironment with various tumour-promoting effects [186].

Drug resistance

Despite the advantages of incorporating rituximab into anthracycline-based chemotherapy, a significant number of patients with DLBCL are still refractory to first-line treatment with R-CHOP. Identifying the mechanisms of resistance to conventional therapy and developing novel therapeutic approaches beyond R-CHOP for patients with DLBCL remain major challenges.

Resistance to chemotherapy and molecularly targeted drugs are major problems in the entire field of tumour treatment. Several mechanisms of drug resistance have been suggested, involving pharmacokinetic and pharmacodynamic events together with the characteristics of the tumour cells and the microenvironment, as illustrated in Figure 5. Drug resistance may be intrinsic, indicating a tumour with a

pre-existing ability to resist anti-tumour treatment, or resistance can be acquired during treatment [187].

Pharmacokinetic factors such as absorption, distribution, metabolism, and elimination can prevent adequate amounts of the drug from reaching the tumour cells [187]. The variability in pharmacokinetics between patients may be due to a number of factors, including age, gender, diet, smoking habits, renal and liver function, concomitant disease, and medication. In addition, polymorphism in genes encoding drug-metabolising enzymes can have a major influence on drug dispersal and thus contribute to treatment resistance [188].

At the level of the tumour cell, a diverse range of resistance mechanisms may take place such as the pharmacodynamic effects of increased drug efflux or drug inactivation, while mutations of the target molecule, activation of alternative signalling pathways, repair of drug-induced DNA damage, and deregulation of apoptosis may also take place [188]. For example, increased drug efflux is a well-established cause of drug resistance. Efflux is carried out by members of the ATP-binding cassette transporter (ABC transporter) superfamily, which shows up-regulation in several tumours including lymphoma, and promote the elimination of hydrophobic compounds such as anthracycline and vincristine [187, 189, 190]. Another example of drug resistance emerging from the tumour cell is the deregulation of apoptosis. Most prominent among the anti-apoptotic proteins are the members of the BCL2 family, which are overexpressed in several tumours and have been linked to resistance to both chemotherapy and targeting drugs [187]. Furthermore, mutations of p53 result in the avoidance of apoptosis after DNA damage and are frequently associated with drug resistance, for instance in lymphoma [191]. More recently, the proposed contribution of tumourigenic stem cells and the increasingly recognised genetic heterogeneity within many tumours has added new levels of complexity in drug resistance [192-194].

The tumour microenvironment has also been shown to mediate drug resistance. The concept of so-called stroma-induced drug resistance is considered to be multifactorial, and to include the activation of pro-survival signalling pathways in the tumour cell through interactions with secreted factors or cell adhesion molecules in the tumour stroma [195]. The tumour microenvironment can also provide physiological drug resistance, such as reduced uptake and efficacy of anthracyclines and vinca alkaloids when the tumour stroma has a low pH, which is a hallmark of many tumours [196, 197]. Future preclinical models that can simulate relevant interactions between tumour cells and their surrounding stroma are expected to give further insights into the mechanisms of drug resistance resulting from both the tumour cell and the microenvironment [195].

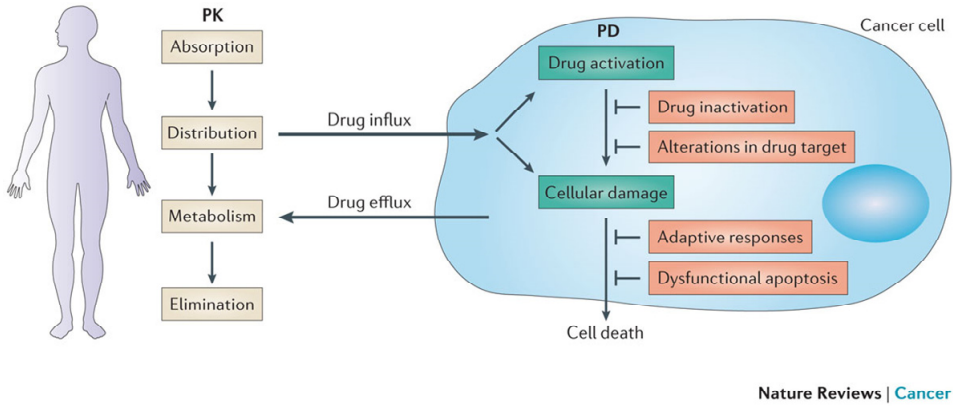

Figure 5. General principles of drug resistance. Pharmacokinetic (PK) and pharmacodynamic (PD) factors can contribute to drug resistance, together with alterations in the tumour cell. In addition, events in the tumour microenvironment can mediate drug resistance (not shown in the figure). *From* [187], *with permission from Macmillan Publishers Ltd.*

The mevalonate pathway

To identify mechanisms involved in chemotherapy resistance in DLBCL, our group has previously searched for differences in tumour tissue gene expression profiles between patients with primary CHOP-refractory disease and patients in continuous complete remission after first-line CHOP treatment. Interestingly, the beta subunit of the prenyl transferase, named Rab geranylgeranyl transferase (Rab GGTase), was found to be significantly up-regulated among the CHOP-refractory patients [106].

A literature survey revealed that Rab GGTase is overexpressed in several tumours and has been suggested to have anti-apoptotic effects in *C. elegans* [198]. Rab GGTase is responsible for post-translational lipid modification of proteins in the Rab family [199], which consists of proteins essential for the regulation of organelle biosynthesis and vesicular transport, used in both normal and oncogenic signalling [200, 201]. Rab proteins have been found to be deranged in several tumours, and are suggested to promote tumour progression and drug resistance [202-204].

The combination of up-regulated gene expression of Rab GGTase in CHOP-refractory patients, and previous reports indicating that Rab GGTase and Rab proteins play a role in tumourigenesis and drug resistance, led us to explore different ways to inhibit the actions of Rab GGTase, as described in **Paper II**. To understand the background and rationale for the work presented in **Paper II**, it is

helpful to be aware of certain metabolites in the mevalonate pathway, and previous efforts to modify this pathway to interfere with oncogenic activity in tumour cells.

The mevalonate pathway is responsible for *de novo* synthesis of cholesterol. Interestingly, some of the intermediate metabolites in this pathway play key roles in essential cellular processes. In particular, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are two lipid isoprenoid intermediates in the mevalonate pathway that are used for post-translational lipid modifications of several proteins with critical functions in both normal and oncogenic signalling. The attachment of FPP or GGPP to certain proteins is referred to as prenylation. Prenylation creates a lipidated hydrophobic domain of the prenylated protein, which is essential for membrane attachment and interactions with other proteins, and is thus important for the biological function of the prenylated protein [205, 206].

Prenylation is executed by three different prenyl transferases: farnesyl transferase (FTase), geranylgeranyl transferase 1 (GGTase 1), and Rab GGTase. Prenylation can be divided into farnesylation and geranylgeranylation. In farnesylation, FTase uses FPP to prenylate certain proteins. In geranylgeranylation, GGTase 1 and Rab GGTase use GGPP to prenylate their target proteins [205].

Inhibition of protein prenylation is considered an attractive strategy in tumour treatment since several oncogenic proteins, including many members of the Ras superfamily of small guanosine triphosphatases (GTPases), require this post-translational lipid modification for their malignant activity [207]. The Ras superfamily includes more than 150 human proteins, which together modulate a wide range of critical cellular processes. Depending on functional similarities, the Ras superfamily is divided into five subfamilies: Ras, Rho, Rab, Ran, and Arf [208]. As illustrated in Figure 6, FTase is responsible for the farnesylation of Ras family proteins, while GGTase 1 catalyses the geranylgeranylation of proteins in the Rho family, and Rab GGTase performs the geranylgeranylation of the Rab protein family [199].

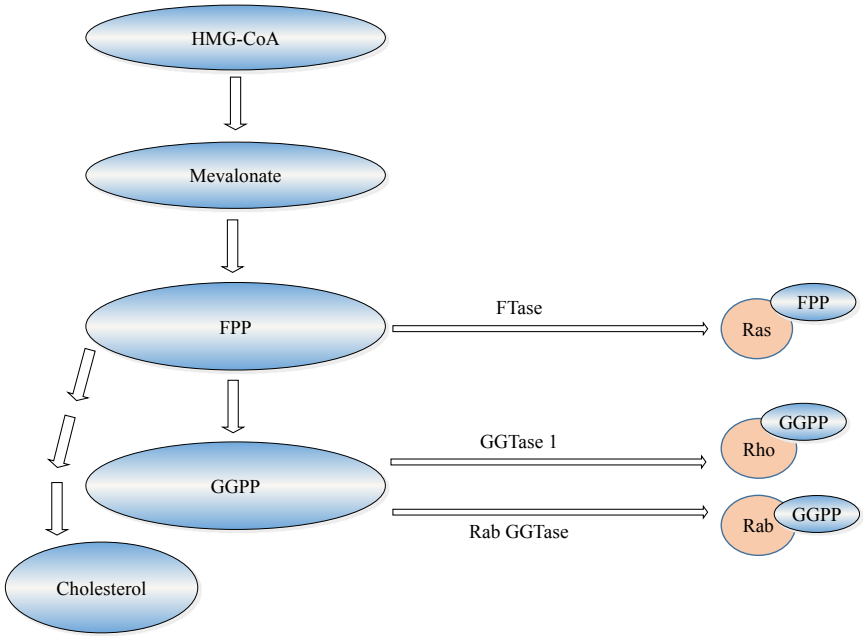

Figure 6. Schematic overview of the mevalonate pathway. The conversion of hydroxy-methylglutaryl coenzyme A (HMG-CoA) to mevalonate by the enzyme HMG-CoA reductase is the rate-limiting step in cholesterol biosynthesis. The intermediate metabolites farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are used for post-translational lipid modifications (prenylation) of several proteins critical for oncogenic signalling. The enzyme FTase is responsible for the farnesylation of the Ras protein family, while GGTase 1 catalyses the geranylgeranylation of proteins in the Rho family and Rab GGTase performs the geranylgeranylation of the Rab protein family.

Specific inhibitors of prenylation

Since members of the Ras superfamily are frequently mutated in tumours, inhibitors directed against their prenylating enzymes have been developed with the aim of preventing prenylation and thus blocking the oncogenic activity of the proteins. Particular interest was initially devoted to inhibitors of FTase, in the hope of blocking farnesylation and the function of the commonly mutated oncogenic Ras proteins in human tumours [209]. Despite high expectations based on preclinical data, farnesyl transferase inhibitors (FTIs) have shown relatively poor efficacy in clinical trials. The discrepancy between preclinical and clinical findings regarding FTIs is thought to be explained by the fact that Ras can be alternatively prenylated by GGTase 1, and thus escape FTI-mediated inhibition. This prompted the development of GGTase 1 inhibitors (GGTIs), which resulted in serious concerns regarding toxicity, probably due to the fact that geranylgeranylated proteins are more numerous than farnesylated proteins in the human body. Moreover, the effect of FTIs was not correlated with the mutational status of the

Ras proteins, indicating that FTIs have other targets than Ras proteins, which may be responsible for the activities seen in preclinical models [207, 210].

An interesting finding during the work on FTIs, further supporting the concept of cross-prenylation between the prenyl transferases and their substrates, indicates that Rab GGTase is an additional target of some FTIs and that loss of Rab GGTase function triggers apoptosis [198]. As a result, inhibitors of Rab GGTase are under development [204, 211].

Statins

The well-known drug family of statins interfere with the mevalonate pathway. Statins were developed as lipid-lowering drugs to inhibit hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase which executes the rate-limiting step of the mevalonate pathway. In addition to preventing the synthesis of cholesterol, statins also block the formation of FPP and GGPP in the mevalonate pathway, resulting in impaired prenylation of important signalling proteins. Anti-proliferative and apoptotic effects have been observed in tumour cells after treatment with statins, and are thought to be mediated by inhibition of geranylgeranylation since the addition of GGPP, but not FPP, restored the statin-induced negative effects on cell viability [212, 213]. Statin use has been associated with the reduced risk of several tumours including lymphomas [214], and has been proposed as a potential anti-tumour treatment [205, 215, 216].

Aims of this work

The general aim of the research presented in this thesis was to search for molecular prognostic markers and to contribute to a better understanding of the tumour biology in DLBCL with the ambition of improving the treatment and prognosis of patients with this disease.

The specific aims were:

- To determine whether the immunohistochemical expression of CD40, previously found to be a prognostic marker in DLBCL, continues to have a prognostic impact after the addition of rituximab to chemotherapy (**Paper I**)
- To evaluate the effect of protein prenylation on the response to CHOP therapy, by using a cell-line-based model for response to CHOP treatment in DLBCL (**Paper II**)
- To explore the underlying mechanisms behind the prognostic impact of protein expression of CD40 in DLBCL, using gene expression profiling (**Paper III**)
- To search for prognostic protein profiles in plasma samples from DLBCL patients, using a recombinant antibody microarray (**Paper IV**)

Patients

Paper I

The study population consisted of 101 patients with *de novo* DLBCL, stages I-IV, treated during 2002-2006 at the University Hospitals of Lund, Sweden and Helsinki, Finland, with tumour material available for evaluation. Patients with primary CNS involvement, primary mediastinal large B-cell lymphoma, or transformation from low-grade lymphoma were not included. All patients received stage-adequate treatment with a combination of anthracycline-based chemotherapy and rituximab. The protocol for the study was approved by the institutional review boards at the relevant organisations in Sweden and Finland, and the Finnish National Authority for Medicolegal Affairs.

Paraffin-embedded pre-treatment tumour samples from the patients had previously been analysed with immunohistochemistry for the expression of CD10, BCL6, and MUM1 to determine the GCB or ABC-phenotype according to the Hans algorithm [86, 217]. In addition, 97 samples had previously been analysed immunohistochemically for the expression of BCL2 [218]. In the present study, the paraffin-embedded samples were analysed immunohistochemically for the expression of CD40. Five patients were excluded because of inadequate lymphoma tissue, and one patient because of indeterminate staining.

Paper III

The study population consisted of 98 patients with *de novo* DLBCL, stages I-IV, treated during 1990-2002 at the University Hospitals of Lund, Umeå, and Uppsala in Sweden. Patients with primary CNS involvement, primary mediastinal large B-cell lymphoma, or transformation from low-grade lymphoma were not included. Thirteen patients did not receive stage-adequate treatment, and were not included in the survival analysis. However, tumour samples from these patients were used in the gene expression analysis, since the intention of this study was not to determine survival, but to explore what an immunohistochemical expression of CD40 represents in *de novo* non-treated tumours. The remaining patients (n=85) all received stage-adequate treatment with anthracycline-based chemotherapy. Rituximab was not included in the recommendations for standard treatment during this period. The local ethics committee approved the conduct of the study.

The inclusion criteria were that both gene expression profiling and immunohistochemical definition of the CD40 status in the tumour had been performed. These analyses were performed in connection with previous studies by our group, using frozen tumour tissue samples taken at diagnosis for gene expression profiling [106] and paraffin-embedded pre-treatment tumour samples for immunohistochemical assessment of CD40 expression [121]. The immunohistochemical expression of CD4, CD8, CD68, and the urokinase-type plasminogen activator receptor (uPAR) had been analysed previously in 84, 84, 85, and 83 cases, respectively [106, 121].

In the present study, gene expression data were correlated to the immunohistochemical CD40 status of the tumours. Immunohistochemistry was then used to confirm some of the major results from the gene expression profiling on the protein level. For this purpose, tissue microarray (TMA) blocks of pre-treatment tumour samples were used to analyse the immunohistochemical expression of biglycan and integrin αV. Of the 98 included patients, 86 patients had tumour material available in TMA blocks. However, due to earlier usage of the material, only 62 (biglycan) and 69 (integrin αV) cases were available for proper evaluation.

Paper IV

The study population consisted of 126 patients with *de novo* DLBCL or follicular lymphoma grade III, included in a prospective phase II clinical trial of the Nordic Lymphoma Group during 2004-2008 [219]. The patients were treated at hospitals in Sweden (four sites), Norway (five sites), and Finland (three sites). Inclusion criteria were age 18-65 years, stage II-IV, aaIPI 2-3, and performance status < 4. Patients with primary CNS involvement, transformed lymphoma, post-transplantation, or Burkitt-like lymphoma were not included. Treatment consisted of six cycles of R-CHOEP-14 followed by systemic CNS prophylaxis with one cycle high-dose cytarabine and one cycle high-dose methotrexate. The study protocol was approved by the medical agencies and ethics committees in Sweden, Norway, Finland, and Denmark.

In the present study, patients were selected based on available plasma samples taken at diagnosis as a baseline sample (BL, n=116), after cycle 3 (Cy3, n=61), and/or after the final cycle 8 (Cy8, n=58). In addition, age and gender matched healthy controls (n=40) were included. Plasma samples were analysed using recombinant antibody microarray.

Methods

Tissue microarray

The tissue microarray (TMA) technology was introduced in the late 1990s for research purposes and allows cost-effective and tissue-saving high-throughput analyses of tumour markers [220]. The technology is now well established and commonly used for immunohistochemical analyses of the expression of multiple tumour markers in a large number of patients [221].

TMA blocks are constructed by retrieving core biopsies from representative areas of the primary paraffin-embedded tumour material and transferring them to a recipient paraffin block in an array-based coordinate system for easy identification. The diameter of the core biopsies usually varies from 0.6 to 2.0 mm. Despite the relatively small amount of tumour tissue in each core biopsy, several studies have demonstrated good concordance between results from TMA blocks and whole-tissue sections. The general consensus is that two core biopsies of 0.6 mm adequately represent the staining seen on a whole-tissue section [221]. However, when analysing markers with pronounced heterogeneity within a tumour, or variable expression depending on localisation in the tumour, multiple and larger core biopsies are preferred for reliable assessment [222, 223].

TMA technology was applied in the study presented in **Paper III**. Whole-tissue sections from the primary paraffin block were first stained with haematoxylin-eosin to identify and delineate areas of representative and non-necrotic sites. Three core biopsies with a diameter of 0.6 mm were then punched out of these representative areas using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA), and transferred to the recipient paraffin block. The procedure is illustrated in Figure 7.

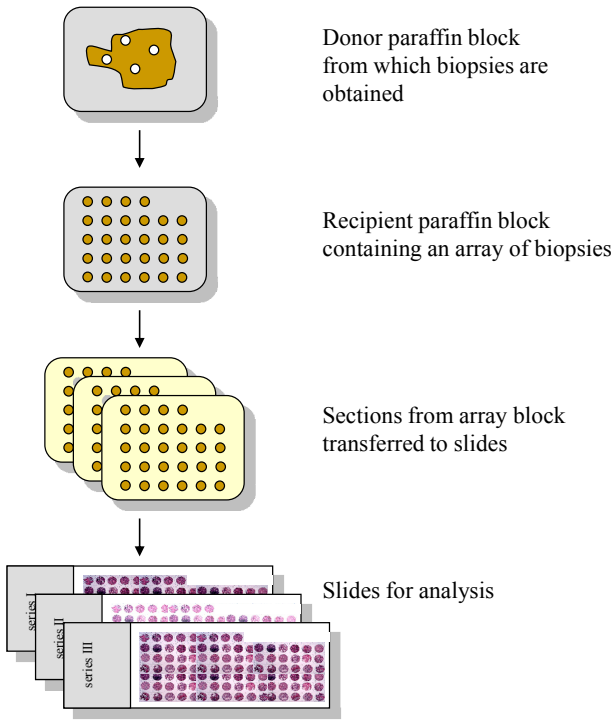

Figure 7. A schematic overview of the tissue microarray technology. *Courtesy of Jacob Engellau*.

Immunohistochemistry

Immunohistochemistry is a widely used technique to analyse the expression of specific proteins in tumours. The technique provides information about the localisation and quantity of the protein in the tumour sample. A primary antibody directed against the protein of interest is first added to the sample. A second antibody with an attached label and affinity for the primary antibody is then added for detection.

In the studies described in **Papers I** and **III**, paraffin blocks were cut in 4-6 µm thin sections and dried over night at 60ºC before being deparaffinised in xylene. The sections were then rehydrated through graded alcohol in water and boiled in EDTA buffer (pH 8.9) in a microwave oven (800 W for 7 min and 300 W for 15 min) to retrieve the antigen. After boiling, the sections were cooled at room temperature for 20 min and rinsed with water, before being placed in tris-buffered saline for 5 min. The primary antibodies were incubated for 25 min at room temperature. Peroxidase blocking solution, provided in the EnVision kit, was used to block the endogenous peroxidase for 25 min, after which the slides were rinsed

with tris-buffered saline. Immunodetection was performed using a Tech-Mate instrument (Dako) and the EnVision method (Dako) according to the manufacturer´s instructions.

In the study described in **Paper I**, the immunohistochemical expression of CD40 was determined from whole-tissue sections taken from paraffin blocks containing pre-treatment tumour samples, using the CD40 antibody (NCL-CD40; Novocastra at a dilution of 1:50). The samples were analysed independently by two haematopathologists (Mats Ehinger and Patrick Joost). Disagreements were resolved by joint review using a multi-headed microscope. The expression of CD40 was determined according to the percent of positive-stained tumour cells of the total number of tumour cells. Three different cut-off levels were used: $\geq$ 10% CD40-positive tumour cells (level 1), $\geq$ 30% CD40-positive tumour cells (level 2), and $\geq$ 90% CD40-positive tumour cells (level 3).

In the study presented in **Paper III**, the immunohistochemical expression of biglycan and integrin αV was determined on sections cut from TMA blocks constructed from pre-treatment tumour samples, using the biglycan antibody (BGN; Atlas Antibodies/Sigma at a dilution of 1:400) and the integrin αV antibody (CD51; Atlas Antibodies/Sigma at a dilution of 1:100). Attempts were also made to evaluate commercially available antibodies against lumican and versican, but these antibodies resulted in non-specific staining and could not be used for further analysis. The samples were analysed by one haematopathologist (Patrick Joost). To be consistent with our previous analyses of markers correlating to, or representing tumour-infiltrating cells, the cut-off level for biglycan was set to 5% of the total number of cells. Similarly, the cut-off level for CD51, which represents integrin αV on the tumour cells, was set to 10% of the total number of tumour cells, to be consistent with our previous analyses of CD40, which also is a marker on the tumour cells.

Gene expression profiling

Genetic information is stored in the DNA sequence in the cell nucleus. After transcription of nuclear DNA into messenger RNA (mRNA), the genetic information is carried to the ribosomes in the cytoplasm for translation into proteins. The expression level of a gene can be estimated by the level of mRNA transcripts from the gene in the tumour tissue. Several techniques can be used to measure mRNA levels for a single gene, such as Northern blotting and quantitative PCR. However, these techniques suffer from practical limitations when a large number of genes are considered for analysis. In 1995, Schena et al. described a new approach, DNA-based microarray technology, to simultaneously measure the expression levels of a large number of genes [224]. The microarray

technology rapidly evolved into a tool for whole transcriptome analysis of all types of cells. More recently, RNA-based massive parallel sequencing (RNAseq) has emerged as an even more powerful high-throughput method than DNA microarrays for transcriptional analysis of normal and malignant cells.

In the study described in **Paper III**, gene expression analysis was performed using dual-channel oligonucleotide microarray technology, in which RNA from a tumour and a reference sample are labelled with different fluorescent dyes by cDNA synthesis and co-hybridised on a microarray. An intensity ratio between the tumour and reference RNA for a given spot on the microarray reflects the expression level for a given gene in the tumour sample, and may be compared between tumour samples after a data-processing step called normalisation.

A summary of the method and data analysis used in the present study is given below. Further details can be found in **Paper III**.

The current oligonucleotide microarray technology

The total RNA was extracted from frozen tumour tissue. After evaluation of RNA integrity and determination of RNA concentrations, the tumour RNA and reference RNA were converted into single-stranded fluorescently labelled cDNA. During cDNA synthesis, the tumour and reference cDNA were labelled with different fluorescent dyes, green for tumour and red for reference cDNA. The procedure is illustrated in Figure 8.

Co-hybridisation of tumour and reference cDNA was performed on oligonucleotide arrays produced at the Swegene Microarray Resource Centre, Department of Oncology, Lund University. An oligonucleotide sequence, also called a reporter, consists of 60-70 base pairs designed to represent a specific target gene. The single-stranded oligonucleotide sequences were deposited on the surface of a glass slide as individual features (spots). Each spot on the microarray represents a unique clone of oligonucleotide sequences. All microarrays contain the same human oligonucleotide set, consisting of 27 744 unique oligonucleotide sequences printed in duplicate on each microarray, resulting in a total of 55 488 features.

After hybridisation, unbound tumour and reference cDNA were removed from the slides by a series of washes before image analysis, in which the intensity ratio (tumour/reference, Cy3/Cy5) was extracted. The intensity ratio provides a relative measure of the amount of mRNA in the tumour sample, and can be compared with intensity ratios for the selected mRNA in other samples. The quantified data from the image analysis, including intensity ratios, fluorescence signal intensity of the background, feature size, and feature position, were used for further processing and analysis.

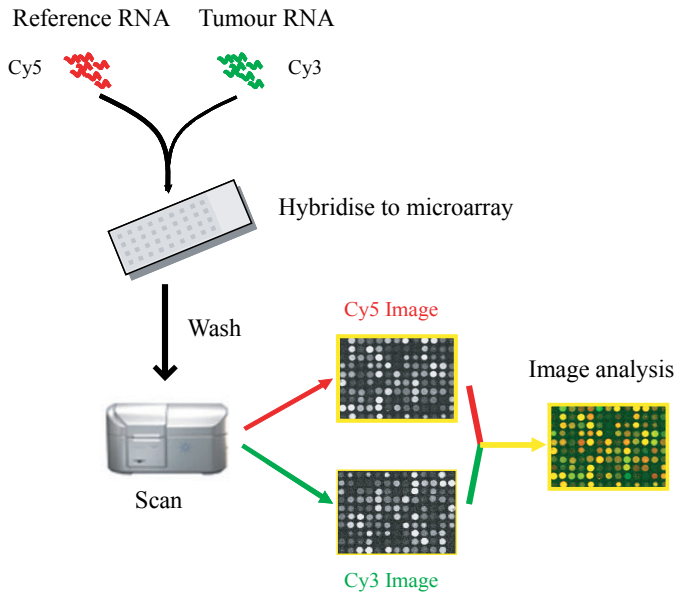

Figure 8. A schematic overview of the oligonucleotide microarray technology. RNA extracted from the tumour tissue and reference RNA are converted to cDNA and labelled with different fluorescent dyes (green for tumour and red for reference) prior to co-hybridisation on the array. After scanning of the microarray slide, the fluorescence signal from each dye is recorded before the individual dye images for each reporter are overlaid in image analysis software for extraction of the intensity ratio (tumour/reference, Cy3/Cy5) which reflects the relative amount of mRNA in the tumour sample.

Data pre-processing

Pre-processing of the microarray data can be divided into three main stages: background correction, filtering of low-quality spots, and data normalisation. The purpose of **background correction** was to decrease the contribution of non-specific factors, such as optical noise from the equipment or potential deposition of foreign items on the slide, to the observed intensity from each spot. **Filtering** of low-quality spots was then performed to remove spots flagged as missing, spots with a non-positive intensity, and spots with 10% or more saturated pixels in either channel. Moreover, reporters annotated to be unspecific and reporters without a known annotation (gene symbol) were removed. **Normalisation** was then performed to adjust for effects derived from technical variations rather than biological differences between the tumour samples. Technical variations can be due to sample quality, dye properties, fluorescent labelling efficiency, spatial hybridisation effects, or scanner properties.

After normalisation, the 2-logarithm of the intensity ratio was used as a measurement of gene expression, and all measurements were assigned an

uncertainty based on the signal-to-background variation in both channels [225]. All reporters were printed in duplicate on the microarray, and these duplicates were now merged in a weighted fashion, taking each spot uncertainty into account [226]. Similarly, reporters representing the same gene symbol, according to the UniGene 181 build, were combined in a weighted merge. Reporters with more than 10% missing values among the 98 assays were removed, as were reporters for which the standard deviation of expression among the samples was less than 0.4.

Data analysis

The (absolute value of the) Fisher score was used to rank the reporters by differential expression. Given the mean m and variance V of expression for a given reporter within each group (CD40-positive or CD40-negative group), the Fisher score F is given by: $F= (m_1-m_2)/ (V_1+V_2)^{1/2}$. The false discovery rate (FDR) [227] was estimated with a permutation test. The FDR can be defined as the expected proportion of false positives among the declared significant results.

The discriminatory gene lists generated in the previous step were subject to gene ontology classification in order to functionally classify the genes and enable a biological interpretation of the findings.

Recombinant antibody microarray

Proteins catalyse and control essentially all biological processes in our body, and the complete set of proteins expressed in an organism is referred to as the proteome. The proteome is inherently complex, partly due to the large number of protein coding genes in the human genome, approximately 22 000, but also due to processes such as mRNA splicing and a wide variety of different post-translational modifications of the proteins.

In general terms, proteomics refers to the large-scale analysis of the proteome. Unlike DNA or RNA sequences, proteins do not have obvious complementary binding partners, making the analysis of the proteome rather challenging. So far, efforts within proteomics have included protein separation techniques such as two-dimensional gel electrophoresis [228], together with mass spectrometry-based approaches [229]. These methods have highlighted technological limitations due to sample complexity and low sensitivity and/or specificity, resulting, for example, in repeated identification of mainly high-abundant proteins. This has led to the development of novel proteomic methods such as recombinant antibody microarrays [230].

In the study described in **Paper IV**, recombinant antibody microarrays were used to analyse plasma proteins in DLBCL patients. This method is based on affinity proteomics, meaning that the binding properties of antibodies to selected proteins are utilised. The recombinant antibody microarray technology used in this study was designed and developed at the Department of Immunotechnology, Lund University, during the past decade [231, 232]. Several key technological issues have been assessed to ensure the reliability of the antibody microarrays, including the choice of affinity probes, the design and production of the arrays, the handling of clinical sample formats, and methods of detection before data processing [230]. The result is a proteomic technique with the potential to detect both high- and low-abundant proteins in different biofluids, providing protein expression profiles that reflect the composition of the proteome. The intention is to search for protein expression profiles that may be associated with clinical variables, such as a specific disease or the progression of a disease. So far, disease-specific signatures have been observed for several solid tumours and different inflammatory conditions by targeting mainly immunoregulatory proteins [233-237]. In addition, a recent pilot study using plasma samples from DLBCL patients revealed a protein profile of 23 plasma proteins with the potential to differentiate the patients into two subgroups with significantly different OS [238].

A summary of the method and data analysis used in the present study is given below. Further details can be found in **Paper IV**.

The current recombinant antibody microarray technology

In the study presented in **Paper IV**, fragments of antibodies, called single-chain fragments variable (scFvs), were used as affinity probes for the selected proteins. These fragments are composed of the variable region of both the heavy and light immunoglobulin chains, joined together by a peptide linker. The scFvs were selected from a phage-display library harbouring more than 10^{10} unique scFv clones, which are all based on the same framework to retain stable on-chip properties, but differ in their antigen biding sites and thus target different proteins [239]. The approach used was to target mainly immunoregulatory proteins thought to be involved in the response of the immune system to tumours, but scFvs with affinity for enzymes and signalling molecules of potential importance for survival of the tumour cell were also included. In total, 283 scFvs were used, which together had affinity for 97 different plasma proteins.

The scFvs were printed out onto the microarray in an ordered pattern, so that each spot on the microarray represented a unique scFv clone. The procedure is illustrated in Figure 9. Each scFv was printed in three replicates. After labelling the plasma proteins with biotin, in order to enable detection, the plasma samples were added to the microarray and the scFvs were allowed to capture their selected

protein. After washing the slides, fluorophore-coupled streptavidin was added to the microarray to detect and visualise any captured biotinylated proteins. The signal intensities from the subsequent scanning procedure were then quantified and the intensity values from each spot provided a measure of the relative amount of the selected protein in the plasma sample.

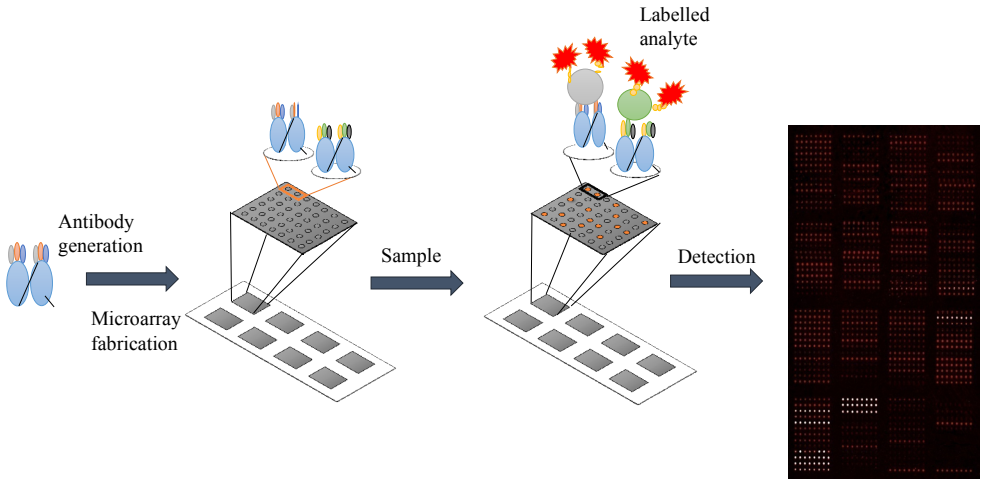

Figure 9. Schematic overview of the recombinant antibody microarray technology. Fragments of antibodies (single-chain fragments variable, scFvs) targeting mainly immunoregulatory proteins were printed out onto the microarray, so that each spot on the microarray represented a unique scFv clone. Biotin-labelled plasma protein samples were added to the microarray. Fluorophore-coupled streptavidin was used for detection. Signal intensities from each spot provided a measure of the relative amount of the selected protein in the plasma sample. *Courtesy of Frida Pauly*.

Data pre-processing

Although the technical issues of a microarray method often receive considerable attention, the different steps included in the subsequent data processing are equally important for the accurate analysis of the samples and their potential biological variation. Several options are available for normalisation and further analysis of the data, and no universally accepted approach has yet been established. The pre-processing of the data obtained from the current recombinant antibody microarray is similar to that described above regarding gene expression profiling, and includes correction for local background, the removal of poor-quality spots, and normalisation.

The signal intensity values were first corrected for the local background around each spot, and the average value of the three replicates was calculated. Spots of poor quality and scFvs with a signal intensity below the limit of detection were removed. The data were then normalised using a two-step procedure to minimise

the influence of technical variations due to day-to-day variations, or array-to-array differences between the samples.

Data analysis

A support vector machine (SVM) analysis was performed to determine whether the patients could be differentiated from healthy controls [240]. SVM analysis is a supervised learning method, meaning that information regarding the identity (patient or healthy control) is used by the method, and differences between the groups are identified based on the given information. When using SVM, samples are typically divided into a training set (to first build a model for classification) and a test set (to evaluate the performance of the classification model). However, when relatively small cohorts are analysed, it is difficult to create sufficiently large training and test sets. Therefore, a procedure called leave-one-out (LOO) cross-validation was used, in which all the samples except one are included to construct a model for classification and the excluded sample is used to test the model. This is repeated until all the samples have been left out once. The sensitivity and specificity of the classification method can then be displayed through a corresponding receiver operating characteristics (ROC) curve, and the given area under curve (AUC) can be used to describe the power of the classifier to distinguish between the two groups, using no specific cut-off. An ROC AUC value of 1 indicates perfect separation between the two groups, while a value of 0.5 means that the classifier is no better than flipping a coin. SVM LOO cross-validation analysis was also used to classify patients with different clinical characteristics and different outcomes.

In contrast, unsupervised hierarchical clustering was performed to evaluate a previously found prognostic protein profile [238]. Here, differences between the patients were identified based solely on the information contained in the dataset. Patients were grouped together in branched trees, or dendrograms, based on the similarities in the dataset. Corresponding heat maps revealed which proteins that were up- or down-regulated for each patient, and allowed viewing of the complex data [241].

In vitro model

In the study presented in **Paper II**, an *in vitro* cell-line-based model of the response to CHOP treatment was developed. This model included five human DLBCL cell lines with varying degrees of cell death in response to CHOP treatment.

A cell line is a population of immortalised cells that can be maintained in culture over an extended period of time. To become immortalised, and thus have an indefinite lifespan, the cells usually undergo a process of transformation resulting in an altered genetic repertoire compared to their *in vivo* ancestor. It is important to bear in mind these additional genetic alterations when working with cell lines, since they may have an inappropriate influence on the observed results.

Cell lines

In order to cover a broad spectrum of tumour characteristics, five different cell lines with different genetic and immunophenotypic properties were used in the study presented in **Paper II**. The WSU-NHL cell line was established from a patient with initially follicular lymphoma that showed an aggressive course despite several chemotherapy regimens, indicating a transformation to DLBCL. The other cell lines were established from patients with primary DLBCL, although named differently at the time of diagnosis due to changes in the designations of lymphoma entities during the past decades. The cell lines SU-DHL-5, SU-DHL-8, Karpas-422, and WSU-NHL were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The ULA cell line was kindly provided by Dr Berglund (Uppsala University, Uppsala, Sweden).

Reagents

Cells were seeded at a concentration of 0.5-1 x 10^6/ml and treated with selected reagents for 48-72 h. The reagents used included CHOP and four different prenylation inhibitors: simvastatin, farnesyl transferase inhibitor (FTI-277), geranylgeranyl transferase 1 inhibitor (GGTI-298), and a combined farnesyl and Rab geranylgeranyl transferase inhibitor (BMS1). CHOP and the different prenylation inhibitors were given alone or in combination. In addition, rituximab was used in the ADCC assay.

The CHOP regimen used in the current model was determined by the sensitivity of the included cell lines to each of the components in CHOP: cyclophosphamide monohydrate, doxorubicin hydrochloride, vincristine sulphate, and prednisolone. For each substance, the concentration at which the most intermediately responding cell line showed an IC50 (half maximal inhibitory concentration) was added to the final combined CHOP regimen, denoted CHOP 100%. For the determination of IC50, the inhibitory effect was defined as decreased cell viability compared to un-treated control cells.

Cell viability and apoptosis analysis

Cell viability was assessed with trypan blue exclusion which is one of the earliest but still widely used methods for measuring cell viability. It is based on the fact that viable cells have an intact cell membrane and therefore exclude the trypan blue dye. Dead cells, on the other hand, no longer have an intact cell membrane and take up trypan blue, appearing blue under the microscope [242]. After visualising and counting the cells under a microscope, the percent of viable cells in a population was calculated. However, trypan blue exclusion does not differentiate between cells that have died by activation of programmed cell death (apoptosis) or by physical or chemical insult (necrosis). Therefore, to identify apoptotic changes in the dead cells, annexin V analysis was performed with flow cytometry and the expression of cleaved caspase-3 was investigated with Western blot analysis.

An early and critical event in apoptosis involves inside-out inversion of the cell membrane, after which phosphatidylserine molecules are exposed on the cell surface, facilitating the recognition of apoptotic cells by phagocytes. Phosphatidylserine can be identified by annexin V and quantified in flow cytometry analysis as a marker for early apoptosis. However, the translocation of phosphatidylserine to the cell surface is not unique to early apoptosis, since it also occurs in necrotic cells. Thus, to determine whether binding of annexin V to the cell surface is connected to apoptosis, necrotic characteristics such as lost membrane integrity and cell swelling must be excluded [243].

In addition, activation of the apoptotic pathway was investigated by Western blot analysis of the expression of cleaved caspase-3 protein, which is one of the key effector caspases of apoptosis [244].

Western blot analysis

Western blot is a powerful method for the immunodetection of proteins, especially those in low abundance. The principle of Western blot is to separate proteins according to their molecular weight using gel electrophoresis and then transfer the proteins to a membrane to allow subsequent identification and quantification of a selected protein [245].

In the study described in **Paper II**, the membrane was first probed with a primary antibody targeting the selected protein. Primary antibodies directed against the cleaved caspase-3 protein, against the enzyme Rab GGTase, and against several proteins that are subject to prenylation were used. The membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody that is reactive towards the primary antibody. The antibody binding could then be visualised with enhanced chemiluminescence detection, providing a measure of the expression of the selected protein.

Cell cycle analysis

Cell cycle analysis reflects the distribution of the entire cell population between the major phases of the cell cycle. Cell cycle analysis by flow cytometry is based on the differences in DNA content during the pre-replicative G0/G1 phase, the replicative S phase, and the post-replicative G2/M phase. DNA content measured with flow cytometry is often referred to as the DNA index (DI). Un-replicated cells in G0/G1 are considered to have DI=1 while replicated cells in the G2/M phase have DI=2, and S phase cells with ongoing replication have an intermediate value, $1 < DI < 2$. In addition, apoptotic cells can be identified as cells with fractionated DNA and $DI < 1$, considered to be in a sub-G0/G1 phase [246].

The DNA content was measured by labelling cells with a fluorescent DNA-binding dye and then presenting them, one cell at a time, to the laser beam in the flow cytometry equipment. The fluorescent dye emits light at an intensity that is proportional to the amount of DNA in each cell, thus providing information on which phase of the cell cycle the cell was in at that moment.

It should be noted that proliferation cannot be measured by cell cycle analysis alone. This is partly due to the fact that a prolonged regulatory signal is required to accumulate enough cells in a certain phase of the cell cycle to enable detection by flow cytometry, but also because prolongation of the entire cell cycle may not be visualised by flow cytometry if the fraction of cells in each phase of the cell cycle remains unchanged. Proliferation can instead be analysed, for example, by cell counts in a Bürker chamber or by using a cell proliferation assay detecting DNA synthesis as a marker of proliferation.

ADCC assay

Rituximab is a crucial component of the first-line treatment of DLBCL, and ADCC is a critical mechanism of action for this CD20-directed antibody [247]. To investigate whether the presence of prenylation inhibitors could disturb the ability of rituximab to lyse CD20-positive cells in the presence of NK cells, an ADCC assay was performed. Since CHOP treatment was excessively toxic to the isolated NK cells, CHOP was excluded from this assay.

In the study presented in **Paper II**, tumour cells were either treated with prenylation inhibitors or left untreated. Rituximab and NK cells isolated from peripheral blood were then added, to determine whether the prenylation inhibitors affected the amount of dead tumour cells. The dead cells were visualised by staining with the DNA-binding fluorescent 7-AAD followed by flow cytometry analysis. To verify that the dead cells were actually tumour cells, and not NK cells, the tumour cells were initially labelled with PKH26 which is a general cell membrane label.

Statistics

Paper I

Associations between categorical and/or categorised prognostic factors were evaluated using the chi-square test. The Kaplan-Meier method was used to estimate and graphically illustrate the survival rates. To avoid misinterpretation of the unreliable right-hand part of the survival curves, the Kaplan-Meier curves continued as long as at least five patients remained at risk [248]. The log-rank test was used to test for differences in survival. The prognostic effect of each factor, expressed as a hazard ratio (HR) and associated 95% confidence intervals (CI), was estimated using Cox regression analysis. Univariate Cox regression was used to compare survival in different subgroups, and multivariate Cox regression to adjust for confounding effects of different prognostic markers. OS was defined as the time in months from diagnosis until last follow-up or death from any cause, and progression-free survival (PFS) as the time in months from diagnosis until disease progression, relapse or death from any cause. All tests were two-sided and p-values less than 0.05 were considered to indicate statistical significance. SPSS 16.0 (SPSS Inc. Chicago, IL, USA) was used for the statistical calculations and Stata 11.0 (StataCorp LP, College Station, TX, USA) for designing the Kaplan-Meier graphs.

Paper II

Data were plotted as means ± standard error of the mean (SEM). The SEM reflects the accuracy of the estimated mean. Unpaired t-test was used to evaluate significant differences between two observed results. All tests were two-sided and the significance levels used were 0.05* and 0.01**. GraphPad Prism 5.0a (GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft Excel (Version 12.2.7) were used for statistical analysis.

Paper III

Associations between categorical and/or categorised patient characteristics were evaluated using the chi-square test, while the trend version of the test was used for variables with more than two ordered categories. The log-rank test was used to test for differences in survival. OS was defined as the time in months from diagnosis until last follow-up or death from any cause. All tests were two-sided and the significance level was set to 0.05. Stata 12.0 (StataCorp LP) and SPSS 17.0 (SPSS Inc.) were used for statistical analysis.

Paper IV

Significantly up- or down-regulated proteins in different groups of patients were identified using the Wilcoxon rank-sum test (Mann-Whitney), resulting in a p-value indicating the significance, and a fold-change indicating the direction of the deregulation of the protein. To perform longitudinal analysis, paired t-tests were used to identify significantly up- or down-regulated proteins in individual patients between different points in time. In order to correlate the total change in expression of each protein over different times to selected clinical variables, response feature analysis was performed. In this analysis, the repeated measurements of one protein from one patient were replaced with a single AUC value, as the response feature. The significance level was set to 0.05 and p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method [249].

Survival curves were plotted according to the Kaplan-Meier method, and based on our recent finding in the pilot study [238], differences between the curves were evaluated using a one-tailed log-rank test. Uni- and multivariate Cox regression was performed to estimate the prognostic effect, expressed as the HR, of each factor. OS was defined as the time in months from registration date until last follow-up or death from any cause. Failure-free survival (FFS) was defined as the time in months from registration date until documented progression, or lack of response, first relapse, death from any cause, or discontinuation/change of therapy due to toxicity, whichever occurred first. Statistical analysis was performed in R (www.r-project.org).

Statistical analyses of microarray data

The statistical analyses directly related to the microarray data derived from the oligonucleotide array (**Paper III**) and the antibody microarray (**Paper IV**) have been described in connection with each method.

Methodological considerations

Paper I

The limitations of the study described in **Paper I** include the retrospective study design and the relatively small number of patients. The use of immunohistochemical analysis is associated with several well-known reservations regarding technical aspects and individual interpretations that can influence the

results [250]. So far, the CD40 antibody used in this and previous studies has been considered robust by our haematopathologists, and the cut-off level of 10% CD40-positive tumour cells has provided prognostic information in repeated studies [121, 122].

Paper II

Several limitations are important when interpreting the results given in **Paper II**. In addition to the genetic alterations of the tumour cells resulting from immortalisation, the cells have also lost their interactions with host factors such as the tumour microenvironment and the drug metabolism. A limitation of the CHOP regimen used in this work is that cyclophosphamide is a pro-drug that requires activation of hepatic cytochrome P450 to generate cytotoxic metabolites. However, some spontaneous activation of cyclophosphamide can also be expected *in vitro,* and in our hands, the utilised concentration of cyclophosphamide resulted in a cell viability of 70-80% after three days of treatment. In future studies using *in vitro* models, the replacement of cyclophosphamide with novel derivatives that do not need hepatic activation should be considered [251].

The hypothesis tested and the design of the study described in **Paper II** emerged from observations of CHOP-refractory patients. In future work, it would be advisable to look for mechanisms contributing to R-CHOP resistance, since R-CHOP is the current standard treatment.

Paper III

One of the limitations in the study described in **Paper III** is the mixture of malignant and non-malignant cells in the whole-tissue samples used for gene expression analysis, meaning that up- or down-regulated mRNA levels could be derived from tumour cells or cells in the microenvironment. However, this mixture provides a broader view of the tumour biology, allowing potential deregulation in both the tumour and stroma cells to be investigated.

The use of the TMA blocks may also pose certain limitations, regarding the ability to reflect the entire distribution of infiltrating inflammatory cells and extracellular matrix, as seen on whole-tissue sections. However, the TMA blocks used in this study consisted of three core biopsies of 0.6 mm from each tumour, which is considered to represent the findings on a whole-tissue section [221].

Paper IV

The high-risk DLBCL patients included in the final study (**Paper IV**) were homogeneously treated, and clinical variables were well characterised throughout the phase II clinical trial carried out by the Nordic Lymphoma Group [219], and were thus a suitable cohort for the search for prognostic markers.

A limitation in the study described in **Paper IV**, is the fact that ongoing work to select antibodies (scFvs) with high specificity and stable on-chip functionality so far has enabled us to target no more than 97 plasma proteins. To allow a more highly resolved view of the complex plasma proteome, it will most likely be necessary to include a wider range of targeted proteins.

Results

The role of CD40 in DLBCL – Papers I and III

The purpose of the study described in **Paper I** was to examine the prognostic value of CD40 expression in DLBCL patients treated with R-CHOP. In the study presented in **Paper III**, the intention was to explore the underlying mechanisms of the prognostic impact of CD40 expression.

CD40 expression is associated with a better prognosis

The median follow-up time for the survivors was 38 months (**Paper I**). The 5-year OS for all patients (n=95) was 72%. Using a cut-off level of 10% positive tumour cells, 77% of the patients were considered CD40-positive. Immunohistochemical staining of CD40 is shown in Figure 10.

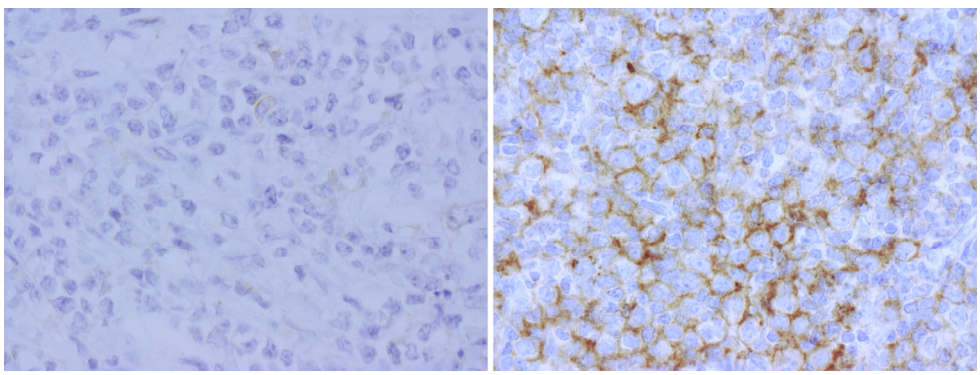

Figure 10. Immunohistochemical staining of CD40 in DLBCL tumour tissue. (Left) Staining defined as CD40-negative, with < 10% positive stained tumour cells. (Right) Staining defined as CD40-positive with a cut-off level of 30% positive stained tumour cells.

The expression of CD40 was not associated with the IPI score, sex, or immunohistochemical features such as the expression of BCL2 or GC phenotype according to the Hans algorithm.

Using a cut-off level of 10%, patients with CD40-positive tumour cells had a better 5-year OS than CD40-negative patients (OS 77% vs. 54%, p=0.02) (Figure 11).

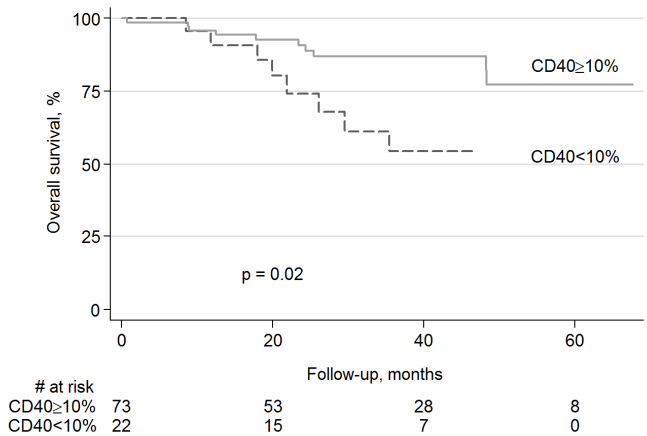

Figure 11. Overall survival stratified by level of CD40 expression using a cut-off level of 10%.

When adjusted for IPI in multivariate analysis, CD40 expression was not an independent prognostic factor, although the HR remained at the same level as in univariate analysis (Table 1). Expression of CD40 using a cut-off level of 10% was associated with improved 5-year PFS (PFS 73% vs. 49%, p=0.04). In multivariate analysis, CD40 expression had no significant prognostic impact on PFS after adjustment for IPI score.

Table 1. Univariate and Multivariate Cox regression analysis of OS

Analysis	Variable	HR	95% CI	p	n
Univariate					
	CD40 positive vs. negative[1]	0.35	0.14-0.88	0.03	95
	IPI low risk vs. high risk[2]	0.30	0.11-0.81	0.02	90
Multivariate					
	CD40 positive vs. negative	0.39	0.15-1.04	0.06	90
	IPI low risk vs. high risk	0.31	0.12-0.82	0.02	

[1] 10% cut-off level.
[2] IPI low risk includes IPI score 0-2 and IPI high risk includes IPI score 3-5.

When using the higher cut-off levels of 30% or 90% CD40-positive tumour cells, no significant difference in OS was observed between CD40-positive and CD40-negative patients.

CD40 expression may reflect an inflammatory stromal reaction

Analysis of the data obtained from the oligonucleotide arrays (**Paper III**) resulted in 3211 reporters with an acceptable missing value rate and overall variation. Of these, 28 reporters had a FDR of ≤ 10%. The expression of the *CD40* gene itself was down-regulated in CD40-negative tumours and was ranked no. 36 of the 3211 reporters, indicating that low protein expression of CD40 is associated with relatively low levels of *CD40* mRNA.

Functional analysis of the top 28 genes revealed that CD40-expressing tumours were characterised by an up-regulated expression of genes coding for proteins involved in cell-matrix interactions: collagens, integrin αV, proteoglycans, proteolytic enzymes, and also antigen presentation with up-regulation of the proteasome beta type 5 (*PSMB5*) gene (Table 2).

Table 2. Genes up-regulated in CD40-positive DLBCL tumours

Biological function	Up-regulated genes
Structure of ECM[1] and cell communication	Collagen type VI α1/2
	Integrin αV (*CD51*)
	Biglycan
	Lumican
	Versican
Proteolysis	Urokinase-type plasminogen activator receptor (*uPAR*)
	Matrix metallopeptidase 2 (*MMP-2*)
Antigen presentation	Proteasome beta type 5 (*PSMB5*)

[1] Extracellular matrix.

The intention of this study was to use immunohistochemical analysis to confirm the results obtained from the gene expression profiling on the protein level. However, due to the shortage of commercially available antibodies and technical difficulties with non-specific staining, only biglycan and CD51 (a marker of integrin αV) could be immunohistochemically evaluated. Expression of biglycan was mainly seen in the cytoplasm of macrophages and stromal cells (Figure 12) while CD51 was found on the surface of the tumour cells.

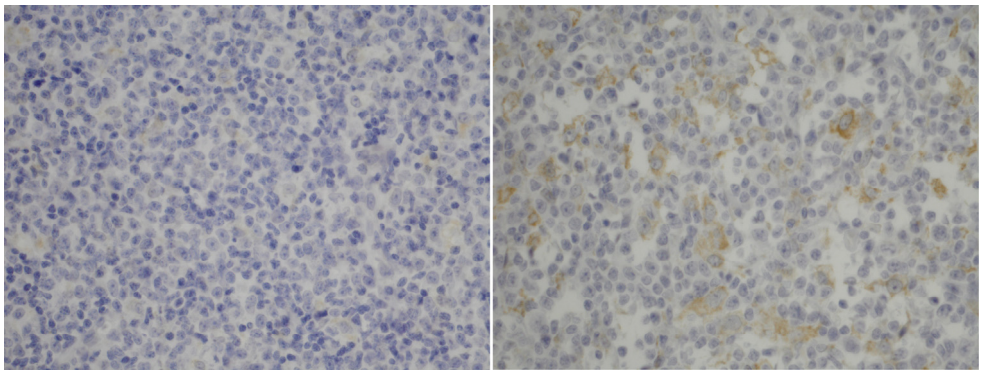

Figure 12. Immunohistochemical staining of biglycan in DLBCL tumour tissue. (Left) Staining defined as biglycan-negative, with < 5% positive stained cells, of the total number of cells. (Right) Staining defined as biglycan-positive with a cut-off level of 5% positive stained cells.

In accordance with the findings from the gene expression profiling, results confirmed that CD40-positive tumours co-express the pro-inflammatory proteoglycan biglycan ($p=0.005$) on the protein level. Considering that the gene expression signature indicated increased inflammatory activity in the CD40-positive tumours, we chose to investigate the relationship between biglycan expression and other possible inflammatory parameters, available to us, in the tumour microenvironment. Protein expression of biglycan was found to be significantly correlated with the expression of the proteolytic enzyme uPAR and with the increased infiltration of CD68-positive macrophages, CD4-positive T-cells, and CD8-positive T-cells, in the tumour.

To summarise, immunohistochemical expression of CD40 on the tumour cells has a potential impact on prognosis also after the addition of rituximab to chemotherapy. Gene expression profiling suggests that a stromal inflammatory process may contribute to the favourable prognosis in CD40-positive patients.

The role of prenylation in CHOP resistance – Paper II

The purpose of the study presented in **Paper II** was to evaluate the effect of protein prenylation on the response to CHOP therapy, by using a cell-line-based model for response to CHOP treatment in DLBCL.

Simvastatin sensitised for CHOP-induced cell death

Simvastatin prevents the synthesis of cholesterol, but also inhibits the formation of metabolites in the mevalonate pathway that are needed for prenylation of

important signalling proteins. Therefore, simvastatin was used as a prenylation inhibitor in the first step of this study, to investigate whether simvastatin could sensitise DLBCL cells to the cytotoxic effects of CHOP. The results showed that four of the five DLBCL cell lines included in the study had reduced cell viability after co-treatment with CHOP and simvastatin, compared to CHOP treatment alone. However, Karpas-422, a cell line considered rather resistant to CHOP treatment alone and also relatively resistant to single-agent treatment with simvastatin, was not affected by co-treatment with CHOP and simvastatin (Figure 13).

To further investigate the sensitising effect of simvastatin and other prenylation inhibitors on CHOP treatment, the cell line WSU-NHL was used. This cell line showed a marked resistance to CHOP treatment alone, with only a 10% decrease in cell viability, while adding simvastatin had a potentiating effect, demonstrated by a 40% decrease in cell viability.

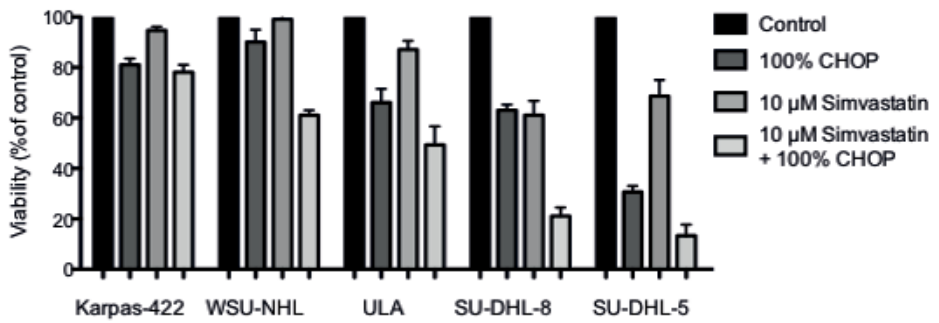

Figure 13. Sensitivity of DLBCL cell lines to CHOP and simvastatin separately and together. Cell viability was assessed with trypan blue exclusion and normalised to untreated control cells.

Geranylgeranylation is important for cell survival

The sensitising effect of simvastatin to CHOP-induced cell death suggested that a deranged mevalonate pathway, and thus disturbed prenylation, may play a role in cell survival. Prenylation of proteins can be divided into farnesylation and geranylgeranylation. To further investigate whether farnesylation or geranyl-geranylation was involved in the decrease in cell viability observed after co-treatment with CHOP and simvastatin, FTI-277 and GGTI-298 were used as these are specific inhibitors of FTase and GGTase 1, respectively. In addition, BMS1 was used as an inhibitor of both FTase and Rab GGTase.

It was found that FTI-277 alone had no effect on cell viability, and in combination with CHOP had no effect on cell viability or proliferation of WSU-NHL cells. However, treatment with GGTI-298 alone resulted in a dose-dependent decrease in both cell viability and proliferation, and these effects where enhanced in combination with CHOP. Similar results were observed for BMS1, as demonstrated by reduced cell viability and proliferation when used as a single agent in WSU-NHL cells, and by potentiating effects when used together with CHOP. Taken together, these results indicate that geranylgeranylation, but not farnesylation, plays a role in cell survival of WSU-NHL cells.

Furthermore, it was established that the increase in cell death observed after treatment with GGTI-298 or BMS1, with or without CHOP, was the result of apoptotic activation. This was demonstrated by increased numbers of annexin-V-positive cells in flow cytometry analysis and increased levels of cleaved caspase-3 protein in Western blot analysis, after treatment with GGTI-298 and BMS1 either alone or in combination with CHOP in WSU-NHL cells.

In addition, treating WSU-NHL cells with either of the two geranylgeranylation inhibitors (GGTI-298 or BMS1), with or without CHOP, had apparent effects on the cell cycle distribution, showing an increased sub-G0/G1 population consistent with apoptosis.

The effect of adding the metabolite used for geranylgeranylation

The impact of GGTI-298 and BMS1 on cell viability suggested that geranylgeranylation and/or Rab geranylgeranylation plays a role in the survival of WSU-NHL cells. The substrate used for geranylgeranylation is GGPP, an intermediate metabolite in the mevalonate pathway. GGOH, which metabolises to GGPP in the cells, was added to investigate if an increased amount of intracellular GGPP could rescue the cells from GGTI-298- and BMS1-induced cell death.

GGTI-298 is considered a competitive inhibitor of GGTase 1 in regard to GGOH, and thus, the addition of GGOH could be expected to reduce the GGTI-298 induced cell death. However, our results showed that the addition of GGOH to GGTI-298-treated WSU-NHL cells did not rescue the cell viability to the extent that would be expected if a GGTase-1-targeted protein was involved in the GGTI-298-induced cell death. Furthermore, the addition of GGOH did not reduce the BMS1-induced cell death in WSU-NHL cells, which may suggest that BMS1 is an uncompetitive inhibitor of Rab GGTase with respect to GGOH.

In contrast to the geranylgeranylation inhibitors (GGTI-298 and BMS1), simvastatin is thought to inhibit protein prenylation by blocking the synthesis of intermediate metabolites in the mevalonate pathway, including the synthesis of GGPP. As expected, given the site of action of simvastatin in the mevalonate

pathway, it was found that the addition of GGOH to simvastatin-treated WSU-NHL cells had a positive effect on cell viability, further strengthen the hypothesis that geranylgeranylation plays a role in survival of WSU-NHL cells.

Sustained effect of rituximab in the presence of prenylation inhibitors

A potential future addition of geranylgeranylation inhibitors to the current standard treatment in DLBCL will depend on a persistent effect of rituximab, as this is a crucial component of R-CHOP. Since ADCC is one of the critical mechanisms of action of rituximab, an ADCC assay was performed to investigate the effect of rituximab and NK cells on WSU-NHL cells, in the presence or absence of GGTI-298 and BMS1. A sustained rituximab-mediated ADCC of WSU-NHL cells was observed in the presence of GGTI-298 or BMS1.

From the results presented above, it can be concluded that the inhibition of protein geranylgeranylation may provide a novel strategy to overcome chemotherapy resistance in DLBCL.

The use of protein profiling in DLBCL – Paper IV

The purpose of the study described in **Paper IV** was to search for prognostic protein profiles in plasma from DLBCL patients, by analysing the plasma proteome with recombinant antibody microarrays.

Differentiation of patients from healthy controls

The supervised learning method, SVM LOO cross-validation, showed that plasma protein profiles could distinguish newly diagnosed DLBCL patients from healthy controls, with a ROC AUC value of 0.90. The number of significantly deregulated proteins in baseline samples taken at diagnosis vs. samples from healthy controls was 58. The top 15 most significantly deregulated proteins were mainly up-regulated in the plasma samples from patients, including MAPK2, BTK, and the T-helper 2 cytokine IL-13.

Massive changes in the plasma proteome during treatment

The paired t-test was used to analyse the longitudinal samples taken at baseline (BL), after cycle 3 (Cy3), and after cycle 8 (Cy8). Large changes in the plasma proteome were observed upon start of treatment; the number of significantly

deregulated proteins being 48 at BL vs. Cy3, compared to only 1 when comparing Cy3 with Cy8, and 0 at BL vs. Cy8. In the case of BL vs. Cy3, the top 15 deregulated proteins were all up-regulated at Cy3, and included both T-helper 1 and 2 cytokines, and the monocyte chemotactic protein-1 (MCP-1).

Proteins with possible connection to clinical variables

SVM LOO cross-validation was used to investigate whether protein profiles at the time of diagnosis could classify the patients according to selected clinical variables, such as stage and B-symptoms. ROC AUC values of 0.53-0.64 were obtained, indicating that patients could not be classified according to the selected clinical variables, based on their protein profile at diagnosis. However, biologically relevant and differently expressed proteins were observed when comparing patients with different aaIPI or different FFS. For example, cyclin-dependent kinase 2 (CDK-2) and BTK were observed to be up-regulated in patients with higher aaIPI and in patients with shorter FFS.

Deregulated proteins in patients with progressive disease

In order to correlate the total change in expression of each protein over the three time points (BL, Cy3, and Cy8) to selected clinical variables, response feature analysis was performed. When patients were divided based on the event of disease progression or no progression, seven significantly deregulated proteins were identified, including IgM, MCP-1 and cytokines such as IL-4, IL-6, and IL-12.

Protein profiles and specific proteins with impact on survival

Based on plasma samples taken at diagnosis and the protein signature of 23 plasma proteins with prognostic impact found in a previous pilot study from our group [238], patients in the present study could be divided into two distinct subgroups, denoted DLBCLa and DLBCLb, by unsupervised hierarchical clustering. The proteins in the profile included immunoregulatory proteins such as T-helper cytokines, chemotactic proteins, and complement factors. Survival analysis of the generated subgroups showed a tendency towards different survival in the two groups (Figure 14), supporting the findings of the pilot study regarding the prognostic impact of the protein signature.

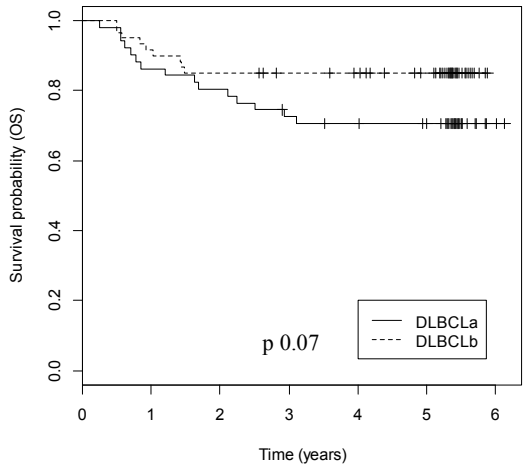

Figure 14. Overall survival according to the two DLBCL subgroups (DLBCLa and DLBCLb) generated from plasma protein profiles.

Furthermore, IL-10 and TNF-α were found to be significantly up-regulated in patients with short OS, defined as OS < 12 months compared to OS > 12, 24, 36, 48, or 60 months. Using multivariate Cox regression analysis of OS, the prognostic impact of IL-10 was shown to be independent of aaIPI score (HR 3.7, 95% CI 1.1-12.3, p=0.03).

To conclude, protein profiling of plasma revealed novel insights into the biology of DLBCL. New candidate markers for treatment prediction and prognostication were identified.

Discussion and future perspectives

This thesis describes different approaches to characterise tumour cells and the host immune response in DLBCL. The intention was to expand our knowledge of various aspects of tumour biology in DLBCL and connect the biology to prognosis and clinical variables, with the ultimate goal of developing rational treatment strategies for individual patients or subgroups of patients.

As outlined in the discussion below, the findings presented in **Paper I**, **III**, and **IV** indicate that the tumour microenvironment and the host immune response contribute to the prognosis for DLBCL, in accordance with several previous reports [48, 49, 54, 56, 106, 165]. In addition, the findings reported in **Paper II** suggest a potential novel strategy to overcome resistance to first-line treatment of DLBCL.

The importance of CD40 in DLBCL

Interest in CD40 as a potential marker of GC-derived DLBCL was awakened in our group several years ago. Despite the importance of CD40 signalling for B-cell maturation in the GC, we were unable to correlate the protein expression of CD40 to other immunohistochemical markers of GC reaction [121, 122]. However, CD40 was found to be a marker of favourable prognosis after treatment with CHOP [121, 122, 252]. Since other immunohistochemically defined prognostic markers in DLBCL have shown inconclusive results after the addition of rituximab to chemotherapy [3, 253], the aim of the study described in **Paper I** was to examine the prognostic value of CD40 in patients treated with both chemotherapy and rituximab. Results indicated a potential importance of CD40 for the prognosis in DLBCL also after the inclusion of rituximab to standard treatment and thus encouraged us to explore the mechanisms behind retained or lost expression of CD40 on tumour cells.

To search for possible biological events connected to the retention of CD40 expression on tumour cells, we have analysed, but not found, any correlation between expression of CD40 and immunohistochemical markers such as GC phenotype, BCL2, or tumour-infiltrating T-cells [121, 122]. Nor was any correlation found between CD40 and clinical variables such as IPI score or sex in

the present work (**Paper I**). Another approach to elucidate the mechanisms behind the prognostic impact of CD40 was the gene expression profiling presented in **Paper III**, which indicated that CD40-positive tumours have up-regulated expression of genes involved in antigen presentation and in interactions between cells and the extracellular matrix.

Antigen presentation and cytotoxic T-cell response

The expression of *PSMB5* was up-regulated in CD40-positive tumours. PSMB5 is a component of the intracellular antigen processing machinery that is necessary for the generation of antigenic peptides presented by MHC I on the cell surface [254]. Tumour cells can induce an anti-tumour response from cytotoxic T-cells by presenting tumour antigens derived from transformed or deregulated proteins on their MHC I molecules [255]. However, dysregulation of the antigen presenting machinery have been found in several solid and haematological tumours, with correlation to worse clinical outcome [256].

Interestingly, components of the antigen processing machinery, including *PSMB5*, have shown up-regulated mRNA expression after CD40 and IL-4 stimulation in malignant B-cells [257], which may be consistent with observations of enhanced antigen presentation on MHC I after CD40 ligation of malignant B-cells [149, 150]. Thus, the relatively low mRNA expression of *PSMB5* among the CD40-negative patients found in the present work (**Paper III**) may contribute to the worse prognosis in this cohort due to impairment of tumour antigen presentation on MHC I, allowing the tumour cells to escape the cytotoxic T-cell response.

This is in agreement with recent reports of frequent inactivation of *B2M* in DLBCL tumours, resulting in the loss of the MHC I on the cell surface and thus indicating a critical role for immune escape in this disease [95, 96]. Moreover, inactivation of *B2M* is often combined with loss of *CD58* to evade an immune response also from NK cells. Taken together, a number of mechanisms may contribute to a selection of tumour cells that can escape immune recognition, and the findings of our work indicate that reduced presentation of tumour antigens from CD40-negative tumour cells may constitute one such mechanism.

Increased inflammatory activity in CD40-positive tumours

In addition to possible differences in antigen presentation between CD40-positive and negative tumours, a disparity was also found in the expression of several genes associated with interactions between cells and the surrounding extracellular matrix. CD40-positive tumours showed an up-regulated expression of genes

coding for pro-inflammatory proteoglycans (e.g. biglycan), proteins associated with proteolysis (e.g. uPAR), collagens, and integrin αV.

Attempts to confirm the results obtained from the gene expression analysis on the protein level were hampered by a shortage of available antibodies and technical difficulties (**Paper III**). However, the pro-inflammatory proteoglycan biglycan was evaluable with immunohistochemistry, providing confirmation that CD40-positive tumours co-expressed biglycan on the protein level; a finding that prompted closer investigation of biglycan. The protein expression of biglycan was correlated to the expression of uPAR and to the amount of tumour-infiltrating macrophages and T-cells. This correlation between protein expression of biglycan and other, for us available, inflammatory parameters in the tumour microenvironment supported the findings of the gene expression analysis, indicating increased inflammatory activity in the CD40-positive tumours.

Biglycan, a small leucine-rich proteoglycan, was found to be a common denominator for the expression of CD40, macrophages, T-cells and uPAR (**Paper III**). Proteoglycans are essential components of the extracellular matrix, initially thought to exclusively regulate the architecture of various extracellular matrices, but now also recognised as important signalling molecules that can influence several cellular functions such as proliferation, motility, and immune response [258, 259]. Following tissue stress or injury, biglycan is considered to contribute to a pro-inflammatory immune response through different mechanisms. For example, biglycan is thought to act as an endogenous ligand of toll-like receptors 2 and 4 (TLRs 2/4), known as important receptors of the innate immune system with potential to cause a rapid antimicrobial immune response [258, 260]. Biglycan signalling via TLRs 2/4 on macrophages and dendritic cells has been suggested to induce synthesis of pro-inflammatory cytokines including chemoattractants for macrophages and T-cells. The newly attracted macrophages can then further enhance the inflammatory response by *de novo* synthesis of biglycan [261]. These findings are supported by reports of high expression of biglycan at sites of inflammation and tissue injury [262, 263]. In malignancies, inconsistent results have been described with a number of studies showing correlation between biglycan expression and tumour aggressiveness in solid tumours, while others report that biglycan has an inhibitory effect on tumour cells [264-267].

Interestingly, biglycan and several other genes found to be up-regulated in CD40-positive tumours such as lumican, *MMP-2*, collagen VI α1, and integrin αV (**Paper III**), are included in the gene-expression-derived stromal-1 signature [56]. The stromal-1 signature has been associated with favourable prognosis in DLBCL and includes the expression of genes related to the extracellular matrix and monocytes [56]. The correlation between genes up-regulated in CD40-positive tumours and the prognostically favourable stromal-1 signature, strengthens our hypothesis of a stromal inflammatory process in CD40-positive tumours, with a possible beneficial effect on patient outcome. Previous studies have revealed

similar favourable gene expression signatures, comprising up-regulation of genes encoding components of the extracellular matrix and inflammatory processes [54, 106]. Collectively, these findings support the importance of considering the tumour microenvironment, and not only the malignant B-cells, in the pathogenesis of DLBCL.

Another similarity between the results presented in **Paper III** and the stromal-1 signature, is the observation that macrophages and cells of monocytic lineage are present together with the pronounced deposition of extracellular matrix, indicating that macrophages may contribute to a better prognosis in DLBCL [56]. Due to contradictious results regarding the prognostic impact of macrophages in DLBCL [168-171], further evaluation of the significance and clinical relevance of various subsets of macrophages in this disease is motivated.

Ongoing and future work regarding CD40

The combination of poor prognosis in CD40-negative patients and *in vitro* observations of CD40-mediated apoptosis and enhanced anti-tumour response in aggressive B-cell lymphomas [132, 149, 150] suggests that it is a disadvantage for a malignant B-cell to express CD40. This motivated continued studies on CD40, and we have hypothesised that restoring CD40 expression in a transformed CD40-negative B-cell may activate pathways for CD40-mediated apoptosis and/or evoke an immune response against the tumour cell. To test this hypothesis, it is necessary to identify the mechanisms responsible for the loss of CD40 expression on the tumour cells.

Since *CD40* mRNA levels were found to be relatively low in tumours with low protein expression of CD40 (**Paper III**), it can be speculated that there is transcriptional down-regulation of the *CD40* gene in CD40-negative patients. This is supported by *in vitro* data obtained by our group, showing low expression of *CD40* mRNA as well as CD40 protein on the cell surface in R-CHOP-resistant DLBCL cell lines (Drott, unpublished data). Transcriptional silencing can be the result of several events, such as deletion, mutation, or epigenetic modification of the *CD40* gene, but may also be due to altered activity of transcriptional regulators, deregulated mRNA stability, or post-transcriptional modulation by microRNAs.

In the search for possible mechanisms causing the loss of CD40 expression, we have studied microarray comparative genomic hybridisation (CGH) data obtained from the patients included in **Paper III**. From this, we concluded that the loss of CD40 protein expression could not be explained by deletion of the *CD40* gene. Furthermore, we have performed DNA methylation analysis, using the mass-spectrometry-based Epityper methodology, of tumour-tissue-derived DNA from patients included in **Paper III**. By doing this, we saw no indications of

deregulated methylation of the *CD40* gene in CD40-negative tumours. However, the methylation analysis also included MIR-503, a microRNA proposed to be a negative regulator of CD40 [268], and results indicated that MIR-503 may play a role in regulating the expression of CD40. Based on these observations (unpublished), we intend to study in more detail the epigenetic and transcriptional regulation of MIR-503 in DLBCL. We also plan to search for possible deregulations of transcription factors controlling the expression of the *CD40* gene. Future work may also include sequencing of the *CD40* gene to search for mutations, and further analyses of epigenetic modifications others than DNA methylation of the *CD40* gene.

An interesting event in relation to our findings concerning CD40 is the ongoing development of CD40 agonists for tumour treatment. Based on preclinical investigations, it has been proposed that there are multiple mechanisms behind the anti-tumour response of CD40 agonists, including induction of apoptosis in CD40-positive tumours, and immune stimulation with the activation of antigen presenting cells. Further work is ongoing to explore the optimal use of CD40 agonists in tumour treatment [158]. It is important to bear in mind the diverse and sometimes paradoxical activities of CD40 signalling reported in different cell types, and further studies are needed to identify subsets of patients most likely to benefit from CD40 agonists.

To conclude, CD40 has a favourable prognostic impact in DLBCL after current standard treatment. Results of gene expression profiling indicate that an inflammatory reaction in the tumour stroma, possibly due to an autologous anti-tumour response, may contribute to a better prognosis in CD40-positive patients. Our intention is now to gain a better understanding of the mechanisms behind the loss of CD40 expression, and to further elucidate the potential advantages that are afforded to a tumour cell when CD40 expression is lost from the cell surface.

A potential strategy to overcome CHOP resistance

The study described in **Paper II** was based on the observation of up-regulated gene expression of Rab GGTase in CHOP-refractory patients [106], and previous reports indicating that Rab GGTase and Rab proteins play a role in tumour development and drug resistance [198, 204]. Using a cell-line-based model to study the response to CHOP treatment, we demonstrated a chemo-sensitising effect of treatment with geranylgeranylation inhibitors in DLBCL cell lines, indicating that the inhibition of protein geranylgeranylation may provide a novel strategy for overcoming chemotherapy resistance in DLBCL.

Inhibition of prenylation

The importance of the post-translational lipid modifications farnesylation and geranylgeranylation, together referred to as prenylation, for proper membrane localisation and function of many oncogenic proteins has led to a particular interest in prenylated proteins and the development of prenylation inhibitors [207]. Despite high expectations that FTIs would block farnesylation and the function of the commonly mutated oncogenic Ras proteins, poor efficacy has been observed in clinical trials, probably due to alternative prenylation of Ras proteins by GGTase 1 in FTI-treated cells. Since the activity of FTIs is not correlated with the mutational status of Ras proteins, FTIs are thought to have other targets than Ras proteins [209, 210]. Consistent with this hypothesis, Rab GGTase has been observed as an additional target of FTIs, and loss of Rab GGTase function has been reported to trigger apoptosis [198]. This led to interest in developing inhibitors of Rab GGTase.

Rab GGTase mediates the geranylgeranylation of Rab proteins. The Rab family includes about 70 different proteins, essential for organising vesicular transport of various proteins from their site of uptake or synthesis to their correct subcellular destination. A wide range of vesicular cargoes have been described, including growth factors, cytokines, nutrients, and integrins. Thus, deregulated function of Rab proteins is thought to be involved in many pathological conditions, including immunodeficiency, diabetes, and tumours [201]. Rab proteins have been found to be deregulated in several tumours, and are suggested to influence tumour invasion, proliferation, interaction with stromal cells, and mechanisms of drug resistance. Consequently, Rab proteins are considered potential targets for anti-tumour drugs [204]. One way to inhibit the function of Rab proteins is to prevent the post-translational modification of geranylgeranylation, mediated by Rab GGTase and essential for the activity of Rab proteins. Rab GGTase has been found to be overexpressed in several tumours, including CHOP-refractory DLBCL [106, 198].

In the present work, a CHOP-sensitising and pro-apoptotic effect of combined treatment with FTase and Rab GGTase inhibitor (BMS1) was seen in DLBCL cell lines (**Paper II**). We also observed that the inhibition of geranylgeranylation, and not farnesylation, probably plays a role in reduced cell viability. Although our study did not provide direct evidence of a causal link between the inhibition of Rab geranylgeranylation and the sensitisation of CHOP-induced apoptosis, we speculate that the chemo-sensitising effect of BMS1 is due to the loss of geranylgeranylated proteins and/or the accumulation of unprenylated proteins, leading to a dysregulation of critical mechanisms for proliferation and survival.

Further work is needed to identify the events and proteins that may connect the inhibition of geranylgeranylation to chemo-sensitisation and apoptosis in DLBCL cells. Identification of the prenylated proteins responsible for the anti-tumour effect of prenylation inhibitors (FTIs, GGTIs, and Rab GGTase inhibitors)

represents a major challenge, but will hopefully result in the design of more specific, and less toxic, inhibitors of selected prenylated proteins [269]. So far, relatively few Rab GGTase inhibitors have been developed, and current efforts are focused on more selective and potent variants of inhibitors [204, 211].

Statins

Another approach to the inhibition of prenylation is to interfere with processes higher up in the mevalonate pathway, and deplete the supply of the metabolites used for prenylation (i.e. FPP and GGPP). Statins interfere with the mevalonate pathway and block the formation of FPP and GGPP, resulting in impaired protein prenylation. In accordance with the findings reported in **Paper II**, treatment of tumour cells with statins has resulted in anti-proliferative and apoptotic effects, thought to be mediated by the inhibition of geranylgeranylation, since the addition of GGPP, but not FPP, restored the statin-induced negative effects on cell viability [212, 213]. Furthermore, statin treatment has been shown to overcome drug resistance and act as a chemo-sensitising agent in haematologic tumour cells [270, 271].

So far, concomitant use of statins during R-CHOP treatment has not been correlated with clinical effects in DLBCL [272-275]. Therefore, attention has been directed towards more specific inhibitors of the mevalonate pathway, such as the inhibitors of prenyl transferases. Moreover, preclinical observations have indicated that the use of statins may reduce the efficacy of rituximab due to statin-induced cholesterol depletion, causing a conformational change in the CD20 epitope, which results in impaired binding of rituximab [276]. Although several clinical studies have shown that concurrent use of statins with R-CHOP does not confer a worse prognosis [272-275], it was suggested in a recent study that the use of statins during R-CHOP treatment had a negative impact in the ABC subgroup of DLBCL [277], indicating that subgroups of patients may respond differently to prenylation inhibitors. This further underlines the need to identify the prenylated proteins crucial for the malignant process. In the present work, rituximab-mediated cellular toxicity was unaffected in the presence of geranylgeranylation inhibitors (**Paper II**), which is in accordance with a previous report [276], and indicates that geranylgeranylation inhibitors may have a role in future treatment of DLBCL patients.

Future work regarding prenylation inhibitors

The findings described above indicate that inhibition of protein geranyl-geranylation may help to overcome chemotherapy resistance in DLBCL. Further efforts are required in the field of prenylation inhibitors and the modulation of the

mevalonate pathway for improved treatment of DLBCL. Future strategies will probably depend on the identification of critical prenylation substrates, not only for the design of more specific and less toxic inhibitors, but also for the discovery of predictive markers that can identify subgroups of patients more likely to benefit from prenylation inhibition. Small interfering RNAs or overexpression of selected prenylated proteins may be useful approaches to learn more about the prenyl transferases and their substrates.

Moreover, inhibition of the mevalonate pathway has been connected to a stress response in the tumour cells due to reduced protein prenylation and suggested to ultimately evoke an immune response against the markedly stressed tumour cells, further increasing the interest in the mevalonate pathway [205]. Thus, inhibiting the mevalonate pathway may result in both an anti-proliferative effect on the tumour cells, and an enhanced immune response against the tumour. In addition, several immunoregulatory proteins are known to be prenylated [278]. Therefore, future studies should also involve the impact of prenylated proteins on the response of host immune system to the tumour.

The plasma proteome in DLBCL

The purpose of the final study was to search for protein profiles in plasma from DLBCL patients, and determine whether they could be correlated to clinical risk factors and prognosis (**Paper IV**).

Plasma is an attractive sample format due to its wide content of proteins representing virtually the whole body; including not only typical plasma proteins, but also secretion products and tissue leakage products from all tissues perfused by the blood [279]. In addition, plasma sampling is a minimally invasive procedure, causing little discomfort to the patient.

Proteins are attractive molecules to analyse as they represent the biological and functional endpoint of many alterations at the DNA or RNA level in tumours. Recent developments in large-scale profiling of the genome and transcriptome have provided important data on the underlying mechanisms of tumour initiation and progression. However, these methods do not provide complete information regarding the post-transcriptional regulations and post-translational modifications that contribute to a proteome with significantly greater complexity than the corresponding encoding genome. The complexity of the proteome poses a considerable challenge in large-scale analyses, in which a broad but, at the same time, highly sensitive and quantitative characterisation of proteins is desired. However, recent progress has been made in proteomic techniques; recombinant antibody microarray being one example [230]. The hope is now to find clinical applications for proteomic technologies, especially in the field of biomarkers, for

the detection, treatment prediction, and prognostication of malignancies and other diseases.

Based on the growing evidence of the importance of the tumour microenvironment and host immune response in DLBCL pathogenesis, the approach adopted in the present study was to target mainly immunoregulatory plasma proteins, in an attempt to reveal biologically and clinically relevant insights. The proteins studied included interleukins, interferons, TNF-α, chemotactic proteins, and complement factors. In view of the complex interactions between different cell types and signalling molecules in the immune system, it is appealing to study the so-called immunoprofiles of several proteins, rather than the isolated effect of a single protein. Some proteins associated with the proliferation and survival of tumour cells were also included and considering a limit of detection in the sub-picomolar range in the current antibody microarray, these potentially tumour derived proteins were expected to be detected although their abundance in the plasma may be very low [231, 232].

So far, only a few studies addressing the plasma or serum proteome in DLBCL have been performed [280-282]. In a recent pilot study, our group showed that plasma protein profiling with recombinant antibody microarray could be used to differentiate patients with DLBCL into two subgroups with significantly different survival, based on a panel of 23 plasma proteins [238]. This encouraged us to expand the protein profiling of plasma from DLBCL patients, as described in **Paper IV**.

Protein profiles at diagnosis and during treatment

In the present work, protein profiles could successfully distinguish newly diagnosed DLBCL patients from healthy controls (**Paper IV**). The fact that the expression of several immunoregulatory proteins differed in these two groups may not be surprising, considering that an immunoreaction of some kind can be expected in newly diagnosed lymphoma patients. However, it is interesting to study some of the differently expressed proteins more closely, such as the B-cell-stimulating T-helper 2 cytokine IL-13 [283], which was up-regulated in DLBCL patients compared to controls. High levels of IL-13 were also reported in a recent study on DLBCL patients, compared to healthy controls [284]. Furthermore, BTK was up-regulated in the plasma from DLBCL patients. BTK has attracted a great deal of interest during recent years due to its prominent role in BCR signalling in the ABC subgroup of DLBCL [76], and the promising clinical results obtained with the BTK-inhibitor ibrutinib in patients within this subgroup [285]. According to the present study, higher levels of BTK in plasma may be associated with a higher aaIPI score and shorter FFS. In future studies, it will be of interest to further explore the relevance of the BTK protein in the plasma of DLBCL patients,

including its cellular source, relation to the ABC subgroup, and potential impact on sensitivity to BTK-inhibitors.

Interestingly, soluble CD40 was also found to be up-regulated in the plasma from newly diagnosed DLBCL patients. Circulating levels of soluble CD40 have previously been reported in haematologic malignancies, with correlation to poor prognosis, and it has been suggested to have a role in modulating the anti-tumour response [286]. Considering the wide expression of CD40 on different cell types and the diverse effects of CD40 signalling depending on cell type, further studies are necessary to investigate the potential function of soluble CD40 in newly diagnosed DLBCL patients.

The protein profiles obtained in the present work indicated massive changes in the plasma proteome upon the start of treatment. Several immunoregulatory proteins showed differences in expression when comparing samples taken at diagnosis with samples taken after cycle 3. Again, this was not an entirely unexpected observation, since patients are treated with chemotherapy, cortisone, rituximab, and granulocyte colony-stimulating factor (G-CSF) during this period, and often experience regression or progression of the tumour burden. The substances used for treatment can all be expected to affect the plasma levels of immunoregulatory proteins. The protein levels were found to be more stable during the course of treatment, i.e. when comparing samples taken after cycle 3 with samples taken after cycle 8, and samples taken four weeks after the final cycle (8) showed protein levels close to the levels at diagnosis. Although it is difficult to draw any firm conclusions from the changes in the plasma proteome seen during treatment, the dynamics are interesting and provide a basis for future studies in the quest of clinically useful information.

Protein profiles associated with response to treatment and prognosis

Another approach adopted in this work (**Paper IV**) was to sum up the total change in expression for each protein over time (from samples taken at diagnosis/after cycle 3/after cycle 8) in individual patients, and then correlate this total change to clinical variables. Significant differences were found in the expression of seven proteins among patients whose disease progressed, compared with patients whose disease did not. The deregulated proteins included IL-6, MCP-1, and IgM, which have all been associated with the pathogenesis of aggressive lymphomas. IL-6 has previously been correlated to worse prognosis in DLBCL, and is considered to promote tumour progression through multiple actions on both tumour cells and tumour microenvironment [287-290]. The chemotactic protein MCP-1 has been correlated to the amount of tumour-associated macrophages and early relapse in solid tumours [291-294]. Aggressive lymphomas have been reported to have higher levels of MCP-1 than indolent lymphomas [295]. Furthermore, IgM-

secreting DLBCL has been suggested to constitute a distinct subset of DLBCL, originating from activated B-cells and showing an aggressive clinical course [296]. The correlation between progressive disease and the deregulation of certain proteins will hopefully be a subject of future studies, to validate and determine the direction in which these proteins change during the treatment of patients with and without disease progression. This may help us in the early evaluation of response to treatment, allowing quick changes in the treatment of patients not responding adequately to first-line treatment.

The results obtained in the present work also showed that the findings in our previous pilot study [238] could be reproduced, and that the current patient cohort could be subdivided into two subgroups, based on a panel of 23 mainly immunoregulatory plasma proteins. The subgroups generated showed a different trend regarding survival, but this was not statistically significant, as was observed in the pilot study. This may be partly explained by differences in the study design and the procedure of patient selection in these studies. However, the present results indicated that plasma protein profiles of newly diagnosed DLBCL patients contained useful prognostic information. Additional studies will hopefully show whether it is possible to refine the protein profile used here, and provide a clinically useful method. The 23 proteins included in the present protein profile were mainly up-regulated in the subgroup of patients showing a trend towards worse prognosis, and included IL-6, MCP-1, and IgM which are discussed above. In addition, IL-10 and TNF-α were included in the protein profiles. IL-10 is considered to be an immunosuppressive, but also B-cell stimulating, cytokine frequently expressed by tumour cells [184, 185]. TNF-α is a potent immunostimulatory cytokine with also tumour-promoting effects [186]. Overexpression of IL-10 in plasma or serum, and TNF-α in plasma or tumour tissue, has previously been correlated to worse prognosis in DLBCL [181-183]. This is in accordance with the present results, showing that high levels of IL-10 or TNF-α at diagnosis were markers of worse prognosis. One question arising from this observation is that of the cellular source of IL-10 and TNF-α in DLBCL patients. Interestingly, these two cytokines are reported to be produced by different immune cells, but also by malignant cells, including lymphoma cells [183, 185, 297].

Future work regarding protein profiling in DLBCL

Protein profiling of plasma from high-risk DLBCL patients revealed novel insights into the biology of DLBCL, and has the potential to reflect both the molecular pathogenesis of the tumour cells and alterations in the host immune response. New candidate markers for the prediction of treatment response and prognosis were identified, and provide a basis for future investigations. As this study was aimed for discovery, the findings must be validated in independent patient cohorts to

confirm whether protein profiling of plasma can be developed into a clinically useful tool for the prognostication and the prediction of treatment response in patients with DLBCL.

Conclusions and concluding remarks

Conclusions

Papers I and III

Immunohistochemical expression of CD40 on tumour cells has a favourable prognostic impact in DLBCL also after the addition of rituximab to anthracycline-based chemotherapy. Gene expression profiling indicates an inflammatory reaction in the tumour stroma that may contribute to the better prognosis in CD40-positive patients.

Paper II

Treatment with geranylgeranylation inhibitors has a chemo-sensitising effect in DLBCL cell lines, and indicates the potential of protein geranylgeranylation as a novel strategy to overcome CHOP resistance in DLBCL.

Paper IV

Protein profiling of plasma from high-risk DLBCL patients revealed novel insights into the biology of DLBCL, and has the potential to reflect alterations in the tumour cells and in the host immune response. Potentially useful markers for the prediction of treatment response and prognosis were identified and provide a basis for future studies.

Concluding remarks

The tumour cells in DLBCL demonstrate a wide range of genetic alterations that collectively result in sustained cell proliferation and survival. They are not affected by the restrictions placed on normal cells, and they exploit features such as enhanced cell cycle progression, reduced apoptosis, and escape from immune recognition to sustain persistent growth. The findings presented in this thesis indicate that the tumour microenvironment and the host immune response contribute to the prognosis for DLBCL, and suggest that at least a subset of patients with DLBCL may benefit from treatment strategies that not only target the tumour cells, but also interfere with the tumour-promoting properties of non-malignant cells. In addition, our results indicate that the use of geranylgeranylation

inhibitors may be a potential strategy to overcome chemotherapy resistance in DLBCL patients.

The work presented in this thesis has introduced me to the remarkable molecular heterogeneity in DLBCL, and to a number of methods available for further exploration of this complexity. I expect the insights obtained to be useful in future clinical work on lymphoma, which will hopefully lead to more individualised and effective treatment than we can offer today.

Despite the successful addition of rituximab to CHOP treatment, a significant number of patients have primary refractory disease or relapse with a poor prognosis after R-CHOP treatment [34-37, 39, 298], clearly indicating that R-CHOP is not an adequate first-line treatment for a subset of patients with DLBCL, and that relapsed disease in the rituximab era is a major challenge. Interestingly, two-year event-free survival after treatment with R-CHOP is reported to confer a subsequent overall survival equivalent to that of age- and sex-matched controls [299], highlighting the importance of optimising first-line treatment in DLBCL.

The clinical risk factors included in the IPI have a limited capacity to identify patients with the worst prognosis, and provide no guidance in the choice of treatment other than R-CHOP, although they probably constitute surrogate markers for underlying biological differences. Our ability to detect and understand the molecular heterogeneity of DLBCL will probably be crucial for the identification of not only prognostic but also treatment predictive markers in this disease. So far, only a few of the proposed molecular markers are in use in clinical practice. Currently, patients with *MYC* and *MYC*/*BCL2* rearrangements often receive more intensified first-line treatment than R-CHOP in view of their poor prognosis, but beyond that, the majority of the patients with DLBCL are still treated with R-CHOP up-front.

The discovery of molecular subgroups based on the cell-of-origin concept has attracted a great deal of interest during the past decade, and a number of novel agents are thought to benefit patients differently depending on their cell-of-origin status [54, 55]. However, recent progress in the genomic analysis of DLBCL has revealed further molecular complexity in the tumour biology, indicating that subgrouping of DLBCL should not rely solely on the cell-of-origin concept. Novel genetic lesions have been identified, some of which are selective for the GCB or ABC subgroup while others are shared by the two subgroups [300]. A major challenge is now to identify the genetic alterations that actually drive tumour progression. Large trials with appropriately collected clinical data are needed to define the relations between the genetic landscape and the clinical behaviour of DLBCL. Considering the recent progress in genomic analysis of DLBCL, it can be assumed that DNA sequencing will play a prominent role in the discovery of useful molecular markers. Detection of alterations in mRNA and protein expression will probably also contribute to further insights, although it may be

more challenging to analyse the complex transcriptome and proteome than the DNA sequence.

A number of potential novel agents are currently under development and being tested in clinical trials [63]. Rational selection of new agents from preclinical studies, and extensive molecular profiling in early clinical trials will be of great importance to identify subsets of patients most likely to benefit from specific agents. Otherwise, the potential effect of an agent may be diluted among patients with molecular alterations not responsive to that particular agent. Cooperation between pre-clinicians, clinicians, pathologists, statisticians, and molecular biologists will be crucial to approach a rational use of novel agents and available diagnostic methods. Furthermore, national and international collaborations with large patient cohorts will probably be required to demonstrate a significant improvement compared to R-CHOP for any agent in first-line treatment, as will organisations and structures that allow a large fraction of the patients to be included in clinical trials.

To conclude, selected agents directed against critical oncogenic events in the GCB and ABC subgroups constitute promising contributions to DLBCL treatment. Further insights into the mechanisms of frequent epigenetic deregulations and the importance of immune escape are awaited. In addition, novel antibodies that may target CD20 more effectively than rituximab are being evaluated, as well as multiple antibody drug conjugates. Further molecular profiling to identify markers or panels of markers associated with clinical behaviour and response to treatment is ongoing. These efforts will hopefully allow molecular findings to be translated into clinically relevant treatment strategies for patients with DLBCL.

Populärvetenskaplig sammanfattning

Lymfom är en grupp tumörsjukdomar som uppkommer från de vita blodkropparna i vårt immunförsvar. Diffust storcelligt B-cellslymfom (DLBCL) är en av de mest aggressiva varianterna av lymfom och uppkommer från den typen av vita blodkroppar som kallas B-celler. Varje år drabbas ca 500 personer i Sverige av DLBCL. Typiska symtom är hastigt tillväxande lymfkörtlar samt viktnedgång, feber och svettningar. Sjukdomsförloppet är ofta snabbt och utan behandling dör de flesta patienterna inom några månader. Idag behandlas dock majoriteten av dessa patienter intensivt med en kombination av cellhämmande läkemedel (cytostatika) och så kallad antikroppsbehandling som riktar sig specifikt mot tumörcellerna. Trots detta botas nästan bara hälften av patienterna. DLBCL är en väldigt heterogen sjukdom med stor variation i det kliniska förloppet. Även på cellnivå ses en stor variation och tumörceller från olika patienter uppvisar ofta klart skilda genetiska förändringar som på olika sätt ger överlevnadsfördelar åt tumörcellerna. Det anses viktigt att skräddarsy behandlingen för patienter med DLBCL så att den angriper de avgörande överlevnadssignalerna som är aktiverade i varje unik tumör.

Ökad kunskap om tumörbiologin i DLBCL är avgörande för att identifiera de mekanismer som orsakar tumörcellernas ohämmade tillväxt. Ambitionen är dels att kunna förutse vilka patienter som förväntas ha ett mer aggressivt sjukdomsförlopp och därav kan ha nytta av en mer intensiv behandling. Dessutom är förhoppningen att ökad kunskap också ska resultera i utveckling av nya och effektivare läkemedel som kan eliminera fler tumörceller och därmed bota fler patienter. Målsättningen med arbetena i denna avhandling har varit att söka efter prognostiska markörer, som kan indikera hur aggressivt sjukdomsförloppet förväntas bli, samt att försöka förstå vilken roll dessa prognostiska markörer har i tumörbiologin.

Det första arbetet handlar om CD40, en receptor som återfinns på ytan av flera olika celltyper, inklusive B-celler. Kvarvarande uttryck av CD40 på tumörcellernas yta har i tidigare studier visat sig vara förenligt med en bättre prognos och därmed längre överlevnad hos patienter med DLBCL. I dessa tidigare studier hade patienterna erhållit behandling med endast cytostatika eftersom antikroppsbehandling inte började användas förrän för ca 10 år sedan. Våra resultat visar att uttryck av CD40 på tumörcellerna är förenligt med en bättre prognos vid DLBCL även med dagens aktuella kombinationsbehandling,

inkluderande både cytostatika och antikropp. För att försöka förstå varför patienter med CD40-uttryck på sina tumörceller klarar sig bättre har vi undersökt vilka gener som är påslagna och används mer i CD40-uttryckande tumörer, jämfört med de tumörer som förlorat sitt CD40-uttryck. Resultaten antyder att CD40-uttryckande tumörer har en bevarad förmåga att visa upp sig för vårt immunförsvar och avslöja sig själva som tumörceller. Detta möjliggör ett angrepp från vårt friska immunförsvar mot tumörcellerna i syfte att eliminera dem. Denna hypotes stöds av att CD40-uttryckande tumörer omges av en ökad inflammatorisk reaktion i sin närmiljö, vilket kan indikera att vårt friska immunförsvar här hjälper till att få bort tumörcellerna och därmed ökar chanserna att patienten ska botas från sin tumörsjukdom.

Vi beskriver även en relativt ny teknik som ger oss möjlighet att undersöka kombinationer av flera olika proteiner, så kallade proteinmönster, i blodprover som samlats in från patienter med DLBCL. De aktuella proteinerna är till stor del kopplade till aktivitet i kroppens immunförsvar och valdes ut för att vi skulle kunna undersöka om reaktioner i patienternas friska immunförsvar kan associeras till aggressiviteten i sjukdomsförloppet och till patienternas överlevnadstid. Våra resultat visar att olika proteinmönster i blodproverna kan användas för att urskilja patienter som förväntas svara sämre på given behandling och har en dålig prognos. Ytterligare studier behövs för att öka förståelsen kring immunförsvarets betydelse för sjukdomsförloppet vid DLBCL.

Utöver detta beskrivs en metod för att på laboratoriet behandla DLBCL-celler med olika substanser i syfte att hitta en behandlingsstrategi som kan eliminera de tumörceller som idag överlever och alltså är resistenta mot vår behandling. Resultaten visar på en ny potentiell behandlingsstrategi som hindrar korrekt produktion av vissa viktiga proteiner i tumörcellerna. Detta leder till att proteinerna inte kan utöva sina funktioner i tumörcellerna som därmed dör eller avstannar i sin tillväxt. Fortsatta studier är nödvändiga för ökad kunskap om de nya substanserna och för att utröna om de kan utvecklas till användbara läkemedel som har en hämmande effekt på tumörcellerna utan att patienterna får för mycket biverkningar.

Sammantaget talar våra resultat för att reaktioner i tumörcellernas nära omgivning och aktivitet i det friska immunförsvaret har betydelse för sjukdomsförloppet vid DLBCL. Detta överensstämmer med rapporter från flera andra studier och antyder att friska icke-tumörceller i vårt immunförsvar inte bara är passiva åskådare till tumörcellerna, utan utövar ett komplext samspel med dessa som antingen kan hämma eller underlätta fortsatt tumörtillväxt. Framtida forskning inom lymfomområdet bör därför belysa egenskaper hos både tumörcellerna och de friska cellerna i tumörens närmiljö, med intentionen att hitta relevanta angreppspunkter för effektiv behandling som kan bota fler patienter med DLBCL.

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Paper I

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ORIGINAL ARTICLE: CLINICAL

CD40 is a potential marker of favorable prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy

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Abstract
We have previously shown that expression of CD40 has a favorable prognostic impact in diffuse large B-cell lymphoma (DLBCL) after anthracycline-based chemotherapy. Here we examined the prognostic value of immunohistochemically defined CD40 expression in 95 patients with DLBCL treated with both anthracycline-based chemotherapy and rituximab. Using a 10% cut-off level, 77% of the patients had CD40-positive tumors and showed a superior overall survival ($p = 0.02$ log-rank, hazard ratio 0.35, 95% CI 0.14–0.88, $p = 0.03$ Cox regression). When adjusted for International Prognostic Index in multivariate analysis, CD40 was not an independent prognostic factor (hazard ratio 0.39, 95% CI 0.15–1.04, $p = 0.06$ Cox regression). However, even after the introduction of immunochemotherapy, CD40 has a potential prognostic impact in DLBCL. Additional and larger studies are necessary, regarding the immunohistochemical robustness of CD40 and the biological mechanisms that contribute to the superior prognosis in CD40-expressing DLBCL.

Keywords: *Lymphoma, diffuse large B-cell lymphoma, prognostication, CD40*

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common form of malignant lymphoma, representing 60–70% of the aggressive lymphomas. The highly variable outcome in patients with DLBCL implies that this entity constitutes a heterogeneous group of neoplasms. Although almost half of patients are cured with anthracycline-based chemotherapy and a further improvement is obtained with the addition of rituximab [1], a significant number of patients still die of this disease. There is an obvious need to identify patients with poor prognosis and allow the development of more risk-adapted and targeted treatments.

The International Prognostic Index (IPI) is today the most accepted prognostic model for DLBCL [2]. The subdivision of patients into different risk groups (according to age, Ann Arbor stage, serum lactate dehydrogenase, performance status, and extranodal sites) is predictive for survival rates and used in treatment stratification. However, patients with identical IPI scores show marked variability in survival, suggesting heterogeneity within each risk group. Consequently, large efforts have been made to identify genetic and molecular markers associated with survival and treatment response. So far, none of the proposed prognostic markers has been stable enough to be incorporated in routine clinical practice.

We have previously demonstrated and confirmed that immunohistochemically defined expression of CD40 in DLBCL results in a favorable prognosis [3,4]. In those studies, the patients were treated with anthracycline-based chemotherapy without rituximab. The aim of the present study was to examine

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the prognostic impact of CD40 expression in patients treated with the current standard treatment including both anthracycline-based chemotherapy and rituximab. Further, we wanted to evaluate the prognostic impact of different expression levels of CD40 and whether expression of CD40 was associated with other well-known immunohistochemically defined prognostic markers.

Materials and methods

Patients and treatment

Clinical data and lymphoma samples were retrospectively collected from 101 patients with *de novo* DLBCL, stage I–IV, treated during 2002–2006 at the University Hospitals of Helsinki and Lund. All patients were treated with a combination of anthracycline-based chemotherapy and rituximab. Cases with primary central nervous system (CNS) involvement, primary mediastinal B-cell lymphoma, or transformation from low-grade lymphoma were not included. The protocol of the study was approved by institutional review boards in the units in Finland and Sweden, and the Finnish National Authority for Medicolegal Affairs. Informed consent was not sought, which was decided to be ethically acceptable by the respective institutional review board.

Immunohistochemistry

Pretreatment samples from all patients, taken at the time of diagnosis, had previously been analyzed immunohistochemically for the expression of CD10, BCL6, and MUM1 to determine the germinal center phenotype (GC phenotype), according to the Hans algorithm [5]. In addition, 97 samples were previously analyzed for BCL2 expression, using a cut-off level of 50% positive tumor cells [6].

For immunohistochemical determination of CD40 expression, the paraffin blocks were cut into 4–6 μm thin sections and then dried overnight at 60°C and deparaffinized in xylene. Subsequently the sections were rehydrated through graded alcohol in water and boiled in ethylenediamintetraacetic acid (EDTA) buffer (pH 8.9) in a microwave oven (800 W for 7 min and 300 W for 15 min). After boiling, the sections were cooled at room temperature for 20 min and rinsed with water before 5 min placement in tris-buffered saline. CD40 antibody (NCL-CD40; Novocastra) was incubated for 25 min at room temperature using a dilution of 1:50. Peroxidase block solution, provided in the EnVision kit, was used to block endogenous peroxidase for 25 min, followed by rinsing the slides with tris-buffered saline. Immunodetection was performed using the Tech-Mate instrument (Dako) and EnVision method (Dako) according to the manufacturer's instructions. The samples were analyzed independently by two hematopathologists (M.E. and P.J.) and disagreements were resolved by joint review using a multiheaded microscope. CD40 expression was estimated according to percent positive-stained tumor cells of the total number of tumor cells (0, $<10\%$ CD40-positive tumor cells [Figure 1]; 1, $\geq 10\%$ CD40-positive tumor cells; 2, $\geq 30\%$ CD40-positive tumor cells [Figure 2]; 3, $\geq 90\%$ CD40-positive tumor cells). Normal tonsil tissue was used as a control for staining.

Statistical analysis

Associations between categorical and/or categorized prognostic factors were evaluated using the χ^2 test,

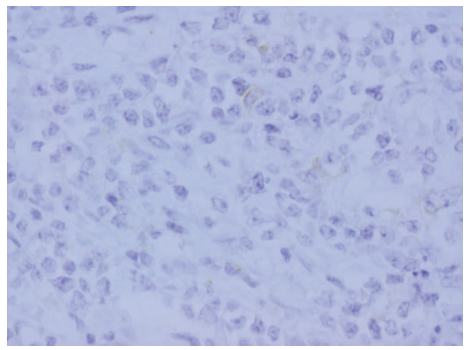

Figure 1. CD40 staining of DLBCL tumor tissue defined as CD40-negative, with $<10\%$ positive-stained tumor cells.

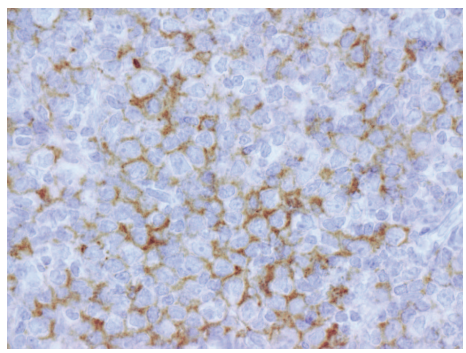

Figure 2. CD40 staining of DLBCL tumor tissue defined as CD40-positive, with a cut-off level of 30% positive-stained tumor cells.

whereas the log-rank test was used to evaluate differences in overall survival (OS) and progression-free survival (PFS). The Kaplan–Meier method was used to estimate and graphically illustrate the survival rates. To avoid misinterpretation of the unreliable right-hand part of the survival curves, all Kaplan–Meier curves were terminated when fewer than five patients remained at risk [7]. OS was defined as the time in months from diagnosis until last follow-up or death for any reason, and PFS as time in months from diagnosis until disease progression, relapse, or death for any reason. Uni- and multivariate Cox regression was used to estimate the prognostic effect, hazard ratio (HR), of each factor. All tests were two-sided and the significance level was set to 0.05. SPSS 16.0 (SPSS Inc., Chicago, IL, USA; 2007) was used for the statistical calculations and Stata 11.0 (Stata-Corp LP, College Station, TX, USA; 2009) for designing the Kaplan–Meier graphs.

Results

Five patients were excluded because of inadequate lymphoma tissue, and one patient because of indeterminate staining. Clinical data from the remaining 95 patients with *de novo* DLBCL stage I–IV were collected retrospectively. The median age at diagnosis was 64 years, with a range from 23 to 84 years. All patients received an anthracycline-based chemotherapy in combination with rituximab. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) was given to 80 patients, R-CHOEP (R-CHOP with addition of etoposide) was given to 13 patients, and other anthracycline-based combination chemotherapy with rituximab was given to two patients. Patients with stage I disease received a minimum of three cycles and patients with stage II–IV disease received at least four cycles. The median follow-up time for the survivors was 38 months. During the follow-up time, 19 patients died and 25 patients had progression/relapse. The 5-year OS and PFS for all patients were 72% and 67%.

As expected, the survival for patients with low IPI scores was significantly (log-rank test) prolonged in comparison to high-risk patients (OS 87% vs. 55%, $p = 0.01$ and PFS 88% vs. 41%, $p = 0.001$). GC phenotype and expression of BCL6 had no impact on OS or PFS (data not shown). Expression of BCL2 was associated with inferior PFS ($p = 0.04$), but no significant difference in OS rates was observed ($p = 0.24$).

Using cut-off levels of 10%, 30%, and 90% CD40-positive tumor cells, 77%, 63%, and 31% of the tumors were considered CD40-positive, respectively. The expression of CD40 using a cut-off level of 10% was not associated with sex or IPI score, neither was it associated with immunohistochemical features such as GC phenotype, BCL2, BCL6, MUM1, or CD10 expression (Table I). Moreover, the higher levels of cut-off for CD40 expression were not associated with the clinical and immunohistochemical features presented in Table I (data not shown).

Expression of CD40 using a cut-off level of 10% was associated with a superior 5-year OS as compared to the absence of CD40 expression (OS 77% vs. 54%, $p = 0.02$; Figure 3). In univariate analysis using a 10% cut-off level, CD40 was shown to be a prognostic factor for OS (HR 0.35, 95% confidence interval [CI] 0.14–0.88, $p = 0.03$ Cox regression; Table II). When adjusted for IPI in multivariate analysis, CD40 was not an independent prognostic factor for OS (HR 0.39, 95% CI 0.15–1.04, $p = 0.06$ Cox regression; Table II). Further, expression of CD40 using a cut-off level of 10% was associated with a superior 5-year PFS (73% vs. 49%, $p = 0.04$; Figure 4). Univariate analysis showed CD40 to be a prognostic factor for PFS (HR 0.42, 95% CI 0.18–0.98, $p = 0.05$ Cox regression; Table III), while no significant prognostic impact of CD40 was shown when adjusted for IPI and BCL2 in

Table I. Patient characteristics.

Characteristic	Patients, *n* (%)	CD40-positive*, *n* (%)	CD40-negative*, *n* (%)	*p*-Value†
	95 (100)	73 (77)	22 (23)	
Sex				
Male	48 (51)	37 (77)	11 (23)	0.96
Female	47 (49)	36 (77)	11 (23)	
IPI				
0–2	54 (57)	43 (80)	11 (20)	0.61
3–5	36 (38)	27 (75)	9 (25)	
Missing	5 (5)			
GC phenotype				
GC	44 (46)	32 (73)	12 (27)	0.38
Non-GC	51 (54)	41 (80)	10 (20)	
BCL2				
Positive	59 (62)	44 (75)	15 (25)	0.47
Negative	32 (34)	26 (81)	6 (19)	
Missing	4 (4)			
BCL6				
Positive	50 (53)	35 (70)	15 (30)	0.08
Negative	41 (43)	35 (85)	6 (15)	
Missing	4 (4)			
MUM1				
Positive	44 (46)	34 (77)	10 (23)	0.89
Negative	46 (49)	35 (76)	11 (24)	
Missing	5 (5)			
CD10				
Positive	32 (34)	23 (72)	9 (28)	0.40
Negative	59 (62)	47 (80)	12 (20)	
Missing	4 (4)			

*10% cut-off level.
†χ^2 test.
IPI, International Prognostic Index; GC, germinal center.

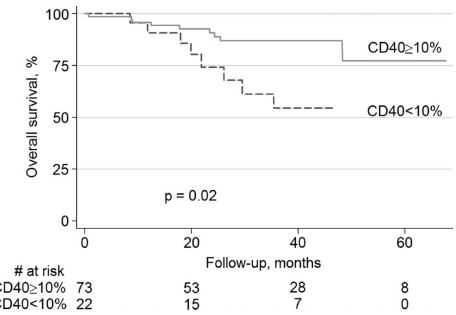

Figure 3. Overall survival stratified by level of CD40 expression using a cut-off level of 10%.

Table II. Univariate and multivariate Cox regression analysis of overall survival (OS).

Analysis	Variable	HR	95% CI	*p*-Value	*n*
Univariate	CD40-positive vs. -negative*	0.35	0.14–0.88	0.03	95
	IPI low-risk vs. high-risk†	0.30	0.11–0.81	0.02	90
Multivariate	CD40-positive vs. -negative	0.39	0.15–1.04	0.06	90
	IPI low-risk vs. high-risk	0.31	0.12–0.82	0.02	

*10% cut-off level.
†IPI low-risk includes International Prognostic Index (IPI) score 0–2 and IPI high-risk includes IPI score 3–5.
HR, hazard ratio; CI, confidence interval.

multivariate analysis (HR 0.48, 95% CI 0.19–1.25, $p = 0.13$ Cox regression; Table III). Considering that neither GC phenotype nor BCL6 expression had any impact on OS and PFS, these variables were not included in multivariate analysis. Similarly, since no significant differences in OS were observed between BCL2-positive and -negative groups, we did not include expression of BCL2 in multivariate analysis for OS.

Using a higher cut-off level of 30% CD40-positive tumor cells, no significant difference in OS was observed ($p = 0.10$), although this cut-off level was associated with superior PFS ($p = 0.03$). Since a 30% cut-off level had no significant prognostic impact on OS, we did not proceed with univariate or multivariate analysis on this level. An even higher cut-off level of 90% CD40-positive tumor cells was not associated with any prognostic impact on OS ($p = 1.0$) or PFS ($p = 0.72$).

Discussion

The aim of the present study was to examine the prognostic impact of CD40 in patients with DLBCL after immunochemotherapy. Our results demonstrate a potential prognostic advantage of CD40 expression even after the incorporation of rituximab to lymphoma therapies. The finding is in accordance with our previous observations regarding the prognostic importance of CD40 in DLBCL [3,4] using a cut-off level of 10%, although in the present series, this was not independent of IPI category.

Other immunohistochemically defined prognostic markers such as GC phenotype, BCL2, and BCL6 have shown inconclusive results after the addition of rituximab to chemotherapy [8–11]. In the present study, no significant difference in survival was seen according to GC phenotype or BCL6 status. Expression of BCL2 was associated with inferior PFS but did not significantly affect OS. As the Lunenburg Lymphoma Biomarker Consortium recently demonstrated, lack of harmonization regarding the procedures for immunohistochemistry may contribute to the variation in prognostic impact seen for several markers [12]. The above mentioned studies regarding GC phenotype, BCL2, and BCL6 highlight the need to re-evaluate and search for new prognostic markers as the treatment of DLBCL evolves.

The cell-surface molecule CD40, considered a critical regulator of both humoral and cellular immunity, is a member of the tumor necrosis factor receptor family expressed on all stages of normal B-lymphocytes as well as on dendritic cells, monocytes, epithelial cells, endothelial cells, and fibroblasts [13]. Moreover, CD40 is expressed in the majority of B-cell malignancies and in 35–100% of solid tumors [14]. CD154, the natural ligand for CD40, is primarily expressed on activated T-helper cells [13]. CD40 activation in B-cells results in proliferation, differentiation, and immunglobulin (Ig) production [13]. CD40 signaling is also involved in the activation of cytotoxic T-cell response, mostly mediated through enhanced antigen presentation after CD40 interaction between dendritic cells and T-helper cells [15]. Trials with CD40 agonists in B-cell malignancies and solid tumors have shown promising results [16]. A possible mechanism is increased antigen presentation followed by enhanced T-cell response directed against tumor antigens [17], although we observed no correlation between CD40-positivity and high amount of tumor-infiltrating T-cells in DLBCL in an earlier study [4].

In normal B-cells and low-grade B-cell lymphoma cell lines, CD40 ligation appears to have a proliferative effect [18]. In contrast, high-grade lymphoma shows a decrease in proliferation both *in vitro* and in a mouse model after treatment with CD40 ligand [19], indicating disparate response to CD40 stimulation, partly depending on the cell type.

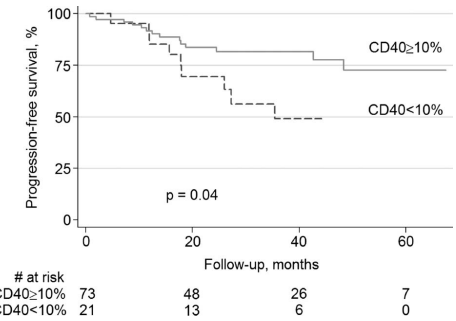

Figure 4. Progression-free survival stratified by level of CD40 expression using a cut-off level of 10%. One patient had a PFS of 0 months and was excluded from the analysis.

Table III. Univariate and multivariate Cox regression analysis of progression-free survival (PFS).

Analysis	Variable	HR	95% CI	*p*-Value	*n*
Univariate	CD40-positive vs. -negative*	0.42	0.18–0.98	0.05	94‡
	IPI low-risk vs. high-risk†	0.21	0.08–0.54	0.001	89
	BCL2-negative vs. -positive	0.30	0.09–1.03	0.06	90
Multivariate	CD40-positive vs. -negative	0.48	0.19–1.25	0.13	89
	IPI low-risk vs. high-risk	0.20	0.07–0.55	0.002	
	BCL2-negative vs. -positive	0.33	0.10–1.14	0.08	

*10% cut-off level.
†IPI low-risk includes International Prognostic Index (IPI) score 0–2 and IPI high-risk includes IPI score 3–5.
‡One patient had a PFS of 0 months and was excluded from the analysis of PFS.
HR, hazard ratio; CI, confidence interval.

A possible mechanism for direct tumor cytotoxicity by CD40 signaling is bax-induced apoptosis, as observed in Burkitt lymphoma *in vitro* after CD40 stimulation [20]. We have previously shown the expression of CD40 and bax to be significantly correlated in patients with DLBCL [3]. Another pathway induced by CD40 stimulation is the nuclear factor (NF)-κB mediated induction of transcription factor IRF4 (MUM1), which in turn inhibits the expression of BCL6, a transcriptional repressor essential for B-cell survival [21,22]. This pathway may be disrupted by alterations in the *BCL6* gene, as shown in a subset of DLBCL [21]. In the present study, we found no significant association between the protein expressions of CD40 and BCL6, nor did we find any association between CD40 and MUM1. However, this pathway might be of interest for further studies.

In conclusion, we have shown that CD40 is a potential prognostic marker in DLBCL even after the addition of rituximab to standard chemotherapy. The robustness of CD40 as an immunohistochemical prognostic marker needs to be evaluated further in a larger independent data set before the results of this study can be considered more than preliminary, and additional studies are necessary to increase the understanding of the biological events connected to CD40 signaling. Immune activation, direct cytotoxicity, and possibly down-regulation of the *BCL6* gene are mechanisms that may contribute to the superior prognosis in CD40-expressing DLBCL. Further understanding of the genetic and molecular heterogeneity in DLBCL will allow the development of more tailored treatments and increase the possibilities to achieve cure in a larger number of patients.

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Declaration of interest: The authors report no conflicts of interest.

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Paper II

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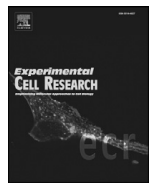

Research Article

Inhibition of geranylgeranylation mediates sensitivity to CHOP-induced cell death of DLBCL cell lines

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ABSTRACT

Prenylation is a post-translational hydrophobic modification of proteins, important for their membrane localization and biological function. The use of inhibitors of prenylation has proven to be a useful tool in the activation of apoptotic pathways in tumor cell lines. Rab geranylgeranyl transferase (Rab GGT) is responsible for the prenylation of the Rab family. Overexpression of Rab GGTbeta has been identified in CHOP refractory diffuse large B cell lymphoma (DLBCL). Using a cell line-based model for CHOP resistant DLBCL, we show that treatment with simvastatin, which inhibits protein farnesylation and geranylgeranylation, sensitizes DLBCL cells to cytotoxic treatment. Treatment with the farnesyl transferase inhibitor FTI-277 or the geranylgeranyl transferase I inhibitor GGTI-298 indicates that the reduction in cell viability was restricted to inhibition of geranylgeranylation. In addition, treatment with BMS1, a combined inhibitor of farnesyl transferase and Rab GGT, resulted in a high cytostatic effect in WSU-NHL cells, demonstrated by reduced cell viability and decreased proliferation. Co-treatment of BMS1 or GGTI-298 with CHOP showed synergistic effects with regard to markers of apoptosis. We propose that inhibition of protein geranylgeranylation together with conventional cytostatic therapy is a potential novel strategy for treating patients with CHOP refractory DLBCL.

Introduction

Diffuse large B cell lymphoma (DLBCL) is, along with chronic lymphocytic leukemia (CLL), the most common lymphoma, accounting for approximately 30–40% of all lymphoid malignancies [1]. The diversity in clinical presentation and outcome, as well as its pathological and biological heterogeneity suggests that DLBCL comprises several disease entities that may require different therapeutic approaches [2]. The conventional first-line therapy for patients with DLBCL is an antracycline-based therapy comprising cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP). The addition of the monoclonal antibody, rituximab (mAb-CD20), to the CHOP therapy (R-CHOP) demonstrates benefits in the overall survival of DLBCL patients [3–6]. Despite the success of rituximab, a considerable fraction of patients either have a primary chemotherapy refractory disease or develop, after complete remission, a recurrent chemotherapy-resistant disease and is not cured with R-CHOP-based therapy. Hence, a major challenge is to identify novel treatment strategies for patient non-responsive to conventional DLBCL therapy.

Statins are known inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-

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limiting enzyme of the mevalonate pathway [7]. Statins are widely used for the treatment of hypercholesterolemia. Several lipid isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are enzymatically generated from mevalonate. Small GTPases, including Rho, Rab, Rac, and Ras that play pivotal roles in normal and oncogenic signaling, undergo post-translational modifications by covalent attachment of FPP or GGPP, a process called prenylation [8–10]. Prenylation allows the attachment of proteins to internal cell membranes by means of the lipid isoprenoid as a lipid anchor and this is essential for proper protein localization and for their biological function [11–13]. In eukaryotic cells, prenylation is carried out by three different prenyl transferases: farnesyl transferase (FT), geranylgeranyl transferase I (GGTI) and Rab geranylgeranyl transferase (Rab GGT or GGTII) [14]. FT is responsible for prenylation of proteins such as Ras and lamins. The GGTI catalyses the geranylgeranylation of proteins in the Rho and Rac family, whereas the Rab GGT is responsible for the geranylgeranylation of the Rab protein family.

To identify genes associated with primary CHOP resistance, a gene expression analysis has previously been performed comparing the gene expression profile of DLBCL patients (at time for diagnosis) with CHOP refractory disease and patients considered cured after primary CHOP treatment [15]. Interestingly, Rab geranylgeranyl transferase beta subunit (RABGGTB) was upregulated significantly in the refractory cohort. Rab GGT functions as a heterodimer composed of an α-subunit and a β-subunit, and the mRNA of both subunits has been shown to be abundantly expressed in ovarian tumor, adenocarcinomas of the colon, large cell lung carcinomas, and melanomas. Moreover, inhibition of Rab GGT by prenyl transferase inhibitors or silencing of the alpha or beta subunit of Rab GGT by siRNA results in apoptosis in *C. elegans*, supporting a possible role during malignant transformation [16]. The Rab GTPases are important regulators of organelle biosynthesis and vesicle transport [12,17]. Interestingly, derangements of several Rab proteins are causally connected to drug resistance. For example, overexpression of Rab6 perturbs doxorubicin and vincristine resistance in breast cancer cell lines, and inhibition of Rab5 causes the intracellular deposition of the multidrug resistance p-glycoprotein [18,19].

Statins have previously been shown to have anti-proliferative and apoptotic effects on some tumor cells. These effects are mediated by the inhibition of geranylgeranylation, as addition of the substrate, GGPP but not FPP, could override the statin-induced negative effects on cell viability [20–22]. In addition, statin treatment has been shown to have chemo-sensitizing effects on several tumor cells and also in overcoming drug resistance [23,24]. Fortuny et al. reported in 2006 that the use of statins was associated with a reduced risk of lymphoma, further supporting the anti-tumor properties of statins [25]. Despite the reported effects of statins, both in vitro and in vivo, the concurrent use of statins during the treatment of patients with DLBCL has no effect on survival [26,27].

The significant role of geranylgeranylation on the survival of tumor cell lines, together with the increased expression of Rab GGTbeta in CHOP resistant DLBCL, leads us to investigate the role of farnesylation and geranylgeranylation in DLBCL. Therefore, we here demonstrate an in vitro cell line-based model for CHOP resistance in DLBCL that is used to evaluate the effect of protein prenylation for the response to CHOP therapy. Our data indicate that geranylgeranylation is important for CHOP resistance in DLBCL cells since inhibition of geranylgeranylation but not farnesylation sensitizes cell to cytotoxic treatment with CHOP, resulting in decreased cell viability, increased annexin V positivity and increased amount of cleaved caspase-3. In addition, we show that the sensitivity of DLBCL cell lines to CHOP treatment correlated to their protein expression of Rab GGTbeta.

Materials and methods

Reagents

Cyclophosphamide monohydrate (C), vincristine sulfate (O), doxorubicin monohydrate (H), prednisolone (P), simvastatin, FTI-277, GGTI-298, geranygeranyl pyrophosphate ammonium salt (GGOH) and squalene were obtained from Sigma-Aldrich (St Louis, MO). Prednisolone is the biologically active substance of prednisone. Simvastatin was chemically activated by alkaline hydrolysis. GGOH are metabolized in cells to GGPP. BMS1 (BMS-227178) was kindly provided by Bristol-Myers Squibb. Rituximab was obtained from local pharmacy.

Cells and culture conditions

The human diffuse large B cell lymphoma (DLBCL) cell lines SU-DHL-5, SU-DHL-8, Karpas-422 and WSU-NHL were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The diffuse large B cell lymphoma cell line ULA [28] was kindly provided by Dr Berglund (Uppsala University, Uppsala, Sweden). SU-DHL-5, SU-DHL-8 and Karpas-422 were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FCS) (Invitrogen). WSU-NHL was grown in RPMI 1640 supplemented with 10% FCS. ULA was grown in 45% Optimem (Invitrogen) and 45% IDEM (Invitrogen) supplemented with 10% FCS. All cell lines were cultured in a humidified atmosphere (37 °C, 5% CO2).

Establishment of an in vitro cell line-based model for CHOP resistance

Karpas-422, WSU-NHL, ULA, SU-DHL-5, and SU-DHL-8 were treated with increasing concentration of cyclophosphamide monohydrate (0.1–20 μM), doxorubicin hydrocloride (10–500 nM), vincristine sulfate (0.1–10 nM) and prednisolone (2–40 μg/ml) and their sensitivity to the different cytotoxic agents was noted. The degree of cytotoxicity in response to each agent varied between the cell lines utilized. Therefore, for each substance, the concentration where the most intermediately responding cell line showed an IC50 was determined, and added to the final combined CHOP regimen. The CHOP regimen used (called either CHOP or 100% CHOP) consists of 10 μM cyclophosphamide monohydrate, 20 nM doxorubicin hydrochloride, 2 nM vincristine sulfate and 20 μg/ml prednisolone. Thereafter, all cell lines were titrated with 10%, 100% and 200% CHOP to determine their CHOP sensitivity.

Cell viability

Cells were seeded in a concentration of $0.5–1 \times 10^6$/ml and treated with different substances for 48–72 h (time and concentrations are indicated in figure legends). Inhibition of prenylation was

accomplished by treating the cells with simvastatin, FTI-277, GGTI-298 and BMS1. After 48 or 72 h, cell viability was assessed by trypan blue exclusion. The effect of squalene and GGOH was investigated by treating cells with 10 μM GGTI-298 or 0.25 μM BMS1 together with either 10 μM squalene or 10 μM GGOH.

Apoptosis analysis by flow cytometry

Labeling of cells with annexin V-PE (BD Bioscience, Pharmingen, San Diego, CA) was performed according to the manufacturer's instructions. Apoptotic cells were defined as annexin V positive.

Western blot analysis

Cells (0.5×10^6/ml) were incubated for 48 h with simvastatin, FTI-277, GGTI-298, BMS1 alone or in combination with CHOP. Cells were harvested and washed once with PBS and resuspended in Laemmli sample buffer. Primary antibodies used were anti-cleaved caspase-3 (Asp175)(5A1) from Cell Signaling Technology and anti-Rab5B antibody (sc-598), anti-Rap1A antibody (sc-1482), anti-GAPDH (sc-32233), and anti-Mcl-1 (sc-12756) from Santa Cruz Biotechnology, anti-Rab5 (1/Rab5) from BD Transduction Laboratories, anti-HDJ-2 (Ab-1) from ThermoScientific and anti-Rab GGTbeta (clone 1C2) from Abnova. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, antibody binding was visualized with enhanced chemiluminescence (EZ-ECL, Biological industries, Beit, Israel) followed by detection with hyperfilm ECL (Amersham).

Cell cycle analysis

Cells ($0.5–1\times10^6$) were washed with PBS and fixed in 70% EtOH and stored at −20 °C for 1–7 days. Labeling of cells for cell cycle analysis was performed as follows. Cells were washed and stained in propidium iodide (PI)-staining solution (50 μg/ml PI, 0.05% Triton X-100, 0.1 mg/ml RNase A). Cells were incubated in the dark at room temperature for 1 h, thereafter analyzed on a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA). Markers were set to determine the percentage of hypodiploid cells (sub-G0/G1), and cells in the G0/G1, S and G2/M phase of the cell cycle.

ADCC assay

WSU-NHL cells were labeled with PKH26 red fluorescent cell linker kit for general cell membrane labeling (Sigma-Aldrich) according to the manufacturer's instructions. Heat-inactivated serum was used throughout the experiment. At day 1, the cells were plated on a round-bottom 96-well plate at a density of 10,000 cells/well. Cells were either left untreated or GGTI-298 and BMS1 were added at a concentration of 10 and 0.25 μM, respectively, followed by overnight incubation at 37 °C. At day 2, rituximab was added to the cells at concentrations of 0.01–10 μg/ml followed by 20 min incubation at 37 °C. NK cells were isolated from peripheral blood using NK cell isolation kit from MACS (Miltenyi Biotec). NK cells were used as effector cells and added at an effector to target cell ratio of 10:1. Cells were incubated overnight; thereafter, the amount of dead cells was visualized by staining with 7-AAD (BD) followed by FACS analysis. Dead target cells were identified as double positive for PKH26 and 7-AAD and were used as readout of the assay.

Statistics

Data analysis was performed with the GraphPad Prism 5.0a (GraphPad Software, Inc., La Jolla, CA) or Microsoft Excel, Version 12.2.7. Data are plotted as means ± standard error of the mean (SEM). Significant differences were evaluated using Student's unpaired *t*-test. All tests were two-sided. Effects were considered statistically significant at $P<0.05$ (*) and $P<0.01$ (**).

Results

Identification of CHOP sensitive-and insensitive DLBCL cell lines

To establish an in vitro cell line-based model for CHOP resistant DLBCL, five DLBCL cell lines, Karpas-422, WSU-NHL, ULA, SU-DHL-8 and SU-DHL-5, were used. All cell lines were treated with increasing concentrations of cyclophosphamide monohydrate (C), doxorubicin hydrochloride (H), vincristine sulfate (O) and prednisolone, (P), respectively, to establish a dose–response curve. The DLBCL cell lines showed a varying degree of sensitivity to treatment with each substance (data not shown), and the response of a particular cell line to a specific substance did not necessarily correlate to its response to another substance. Therefore, the lowest IC50 value for each substance among the five cells lines was used in the final CHOP cocktail. The final concentration of the CHOP cocktail used in all experiments was C: 10 μM, H: 20 nM, O: 2 nM, P: 20 μg/ml (these concentrations are denoted either 100% CHOP or CHOP). Ten percent CHOP means that the concentration of all substances was divided by 10, and 200% CHOP means that the concentration of all substances was multiplied by two.

To identify cell lines, resistant versus sensitive, to CHOP treatment, the cells were treated with 10%, 100%, and 200% of CHOP for 72 h and cell viability was examined by trypan blue exclusion. The cell line most resistant to CHOP was Karpas-422 followed by WSU-NHL, ULA, SU-DHL-8, and SU-DHL-5 (Fig. 1).

Simvastatin and co-treatment with simvastatin and CHOP reduces cell viability of DLBCL cell lines

Statins are inhibitors of HMG-CoA reductase, the enzyme responsible for the conversion of HMG-CoA to mevalonate [29]. Statins have been reported to induce apoptosis in other types of lymphoma cells and have been shown to sensitize cells to treatment with cytotoxic agents [23,30–32]. Therefore, we investigated if simvastatin could sensitize DLBCL cells to the cytotoxic effects of CHOP. DLBCL cell lines were treated with 10 μM simvastatin alone or in combination with CHOP for 72 h. Karpas-422, WSU-NHL and ULA cells were relatively resistant to single agent simvastatin treatment, whereas SU-DHL-8 and SU-DHL-5 showed reduction in cell viability of 40% and 30%, respectively (Fig. 1B). However, in WSU-NHL cells simvastatin treatment in combination with CHOP resulted in a potentiating effect that was demonstrated by a 40% decrease in cell viability for cells treated with both simvastatin and CHOP, as compared to 10% for CHOP alone. Also in the other cell lines, co-treatment with simvastatin and CHOP resulted in an additive negative effect on cell viability as compared to simvastatin treatment alone or CHOP treatment alone. ULA, SU-DHL-8 and SU-DHL-5 demonstrated a reduction in

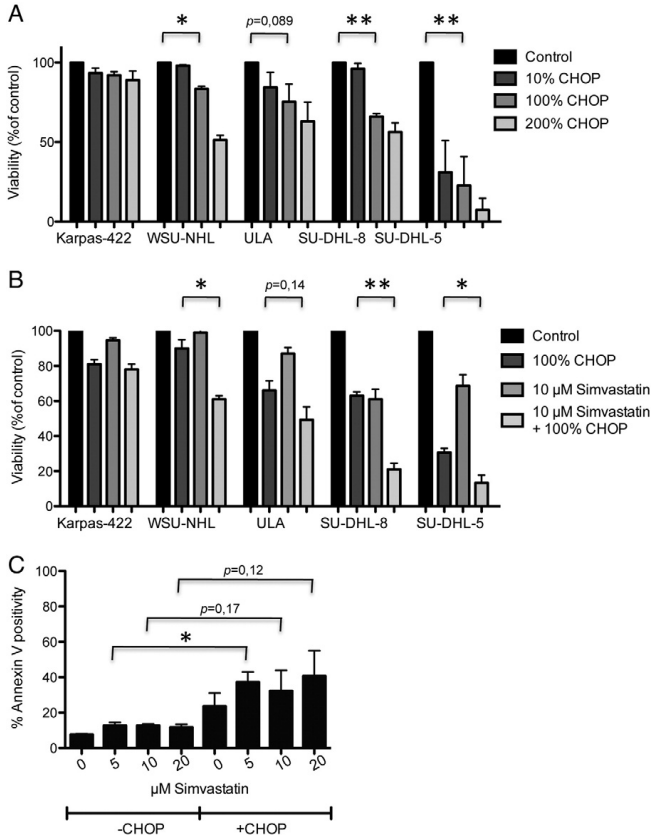

Fig. 1 – CHOP and simvastatin sensitivity of DLBCL cell lines. (A) DLBCL cell lines were treated for 72 h with three different concentrations of CHOP. The cell viability was assessed by trypan blue exclusion and normalized to untreated control cells. Data are presented as mean ± SEM, $n = 3$. (B) DLBCL cell lines were treated with 100% CHOP, 10 μM simvastatin alone or 10 μM simvastatin in combination with 100% CHOP. The cell viability was assessed by trypan blue exclusion and normalized to untreated control cells. (C) WSU-NHL cells were treated with different concentrations of simvastatin (5, 10, and 20 μM) alone or in combination with CHOP for 48 h. Cells were washed and labeled with annexin V-PE followed by FACS analysis. Data represent the percent annexin V-positive cells. Data are presented as mean ± SEM, $n = 3$.

cell viability of 50%, 80% and 90%, respectively. Karpas-422, however, did not show any cooperative effect of simvastatin and CHOP (Fig. 1B). To further investigate the sensitizing effect of simvastatin on CHOP treatment observed in the majority of the DLBCL cell lines investigated, we chose to perform further experiments on the WSU-NHL cell line, which presented apparent CHOP resistance and showed a potentiating effect of simvastatin to cytotoxic treatment. The observed cell death in WSU-NHL cells treated with simvastatin and CHOP is the result of increased apoptosis as judged by increased annexin V positivity (Fig. 1C). However, simvastatin treatment alone or in combination with CHOP did not affect the cell cycle phase distribution (data not shown).

Protein geranylgeranylation is important for DLBCL cell survival

The sensitizing effect of simvastatin to CHOP treatment indicated a possible role of a deranged mevalonate pathway on cell survival. Inhibition of the mevalonate pathway disturbs both the prenylation of farnesylated proteins and of geranylgeranylated proteins. Correct prenylation of proteins is important for the lipid attachments of a variety of signaling molecules such the small GTPase binding Ras, Rho and Ras protein families. To further investigate whether farnesylation or geranylgeranylation is involved in the increased cell death of CHOP- and simvastatin-treated cells, we used FTI-277 and GGTI-298, which are specific inhibitors of FTase

and GGTase I, respectively. Consistent with a previous report [33], treatment with the farnesylation inhibitor, FTI-277, alone had no effect on cell viability (Fig. 2A). In addition, FTI-277 treatment in combination with CHOP lacked effect on cell viability and proliferation and did not show any sensitizing effect for cytotoxic treatment (Fig. 2A and B). However, treatment of WSU-NHL cells with the geranylgeranyl transferase I inhibitor GGTI-298 alone resulted in a dose-dependent increase in cell death (Fig. 2C). In combination with CHOP, a potentiating effect of GGTI-298 was observed at low, non-toxic concentrations of GGTI-298 (5 μM, Fig. 2C). Furthermore, GGTI-298 alone reduces cell proliferation in a dose-dependent manner, an effect that is enhanced in combination with CHOP (Fig. 2D). In addition, similar results were obtained with BMS1, a farnesyl transferase and Rab geranylgeranyl transferase inhibitor [16]. BMS1 shows high cytotoxic effects in WSU-NHL cells and also demonstrates potentiating effects at lower, nontoxic concentration together with CHOP (0.05 μM, Fig. 2E). BMS1 has strong anti-proliferative effects and showed a minor additive anti-

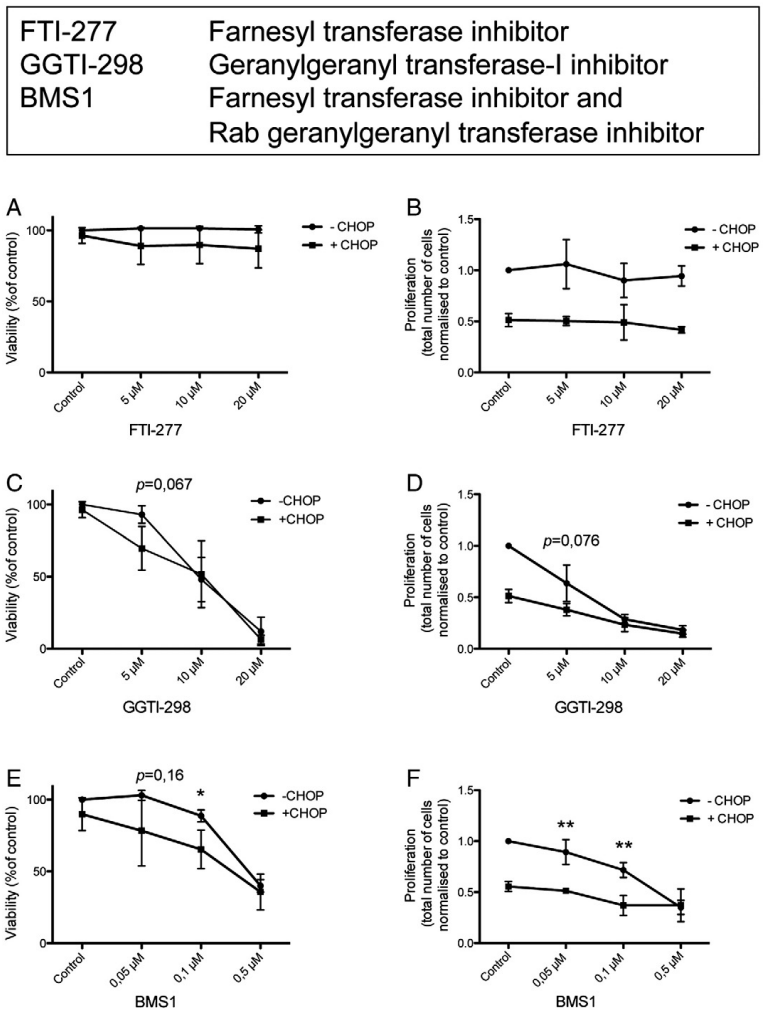

Fig. 2 – The effect of prenylation inhibitors on WSU-NHL cells. WSU-NHL cells were treated with increasing concentration of (A and B) FTI-277 (5, 10, and 20 μM) and (C and D) GGTI-298 (5, 10, and 20 μM) and (E and F) BMS1 (0.05, 0.1, 0.5 μM) alone or in combination with CHOP for 48 h. Cell viability was assessed by trypan blue exclusion. A, C, and E show cell viability and B, D, and F show total number of cells normalized to control cells. Error bars represent SEM, $n = 3$.

proliferative effect together with CHOP (Fig. 2F). The anti-apoptotic and chemo-sensitizing effect of GGTI-298 and BMS1 was verified in the DLBCL cell lines ULA, Karpas-422 and SU-DHL-8 (data not shown). Taken together, our data indicate a role for geranylgeranylation, but not farnesylation, in cell survival of WSU-NHL cells as FTI-277 failed to affect cell viability whereas treatment with GGTI-298 and BMS1, inhibitors of geranylgeranylation, resulted in reduced viability and sensitization to CHOP.

Treatment with prenylation inhibitors induces apoptosis in DLBCL cells

To establish that the increased cell death is the result of activation of apoptotic pathway, the expression of annexin V on the cell surface was investigated. Annexin V positivity is a marker for apoptotic cells that not yet have become permeable for trypan blue, a marker for late apoptosis. Treatment of WSU-NHL cells with FTI-277 for 48 h did not affect the number of trypan blue positive cells, nor alone or in combination with CHOP (Fig. 2A). However, co-treatment of FTI-277 and CHOP shows a slight increase in the number of annexin V-positive cells (Fig. 3A). Consistent with results from trypan blue exclusion, treatment of WSU-NHL cells with 5 μM GGTI-298 or 0.05 μM BMS1 alone for 48 h showed a slight increase in annexin V-positive cells, whereas co-treatment with 5 μM GGTI-298 or 0.05 μM BMS1 and CHOP resulted in 20% and 30% increase in annexin V-positive cells, respectively (Fig. 3B and C). The obvious toxicity of GGTI-298 and BMS1 at high concentrations, as judged by the low viability of cells (Fig. 2B and C), was confirmed by the increased number of annexin V-positive apoptotic cells after treatment with 10 and 20 μM GGTI-298 and 0.1 and 0.5 μM BMS1 (Fig. 3B and C). In addition, the activation of the apoptotic pathway was investigated by Western blot analysis of the expression of cleaved caspase-3 protein. CHOP treatment alone induces cleavage of caspase-3 (Fig. 3D). Treatment with FTI-277 did not affect the expression of cleaved caspase-3 nor alone or in combination with CHOP (Fig. 3D). On the other hand, GGTI-298 and BMS1 treatment alone or in combination with CHOP induces cleavage of caspase-3 in a dose-dependent manner indicating an evident apoptotic effect (Fig. 3E and F). Taken together, the increased cell death demonstrated by treatment with CHOP and GGTI-298 or BMS1 is the result of activated apoptotic pathways.

The effect of prenylation inhibitors on cell cycle distribution

To assess the effects of prenylation inhibitors with and without CHOP on cell cycle progression, WSU-NHL cells were treated with inhibitors alone or together with CHOP for 48 h. Thereafter cells were harvested and analyzed by flow cytometry. WSU-NHL cells treated with only CHOP show 10% of cells in the sub-G_0/G_1 population and demonstrated a G_0/G_1 arrest and also an accumulation of the G_2/M population probably due to the effects of doxorubicin and vincristine on the G2/M phase [34,35]. Treatment with 20 μM of FTI-277 alone or in combination with CHOP had minor effects on the cell cycle (Fig. 4A). WSU-NHL cells treated with 10 μM GGTI-298 undergo cell death as indicated by the increased presence of a sub-G_0/G_1 population from 3% in control cells to 12% in GTI-298 treated cells (Fig. 4B). In addition, GGTI-298 also induces a G2/M arrest. The sub-G_0/G_1 population increased to 20% for cells treated with both GGTI-298 and CHOP. The effect of BMS1 alone on cell cycle distribution in WSU-NHL cells resulted in approximately

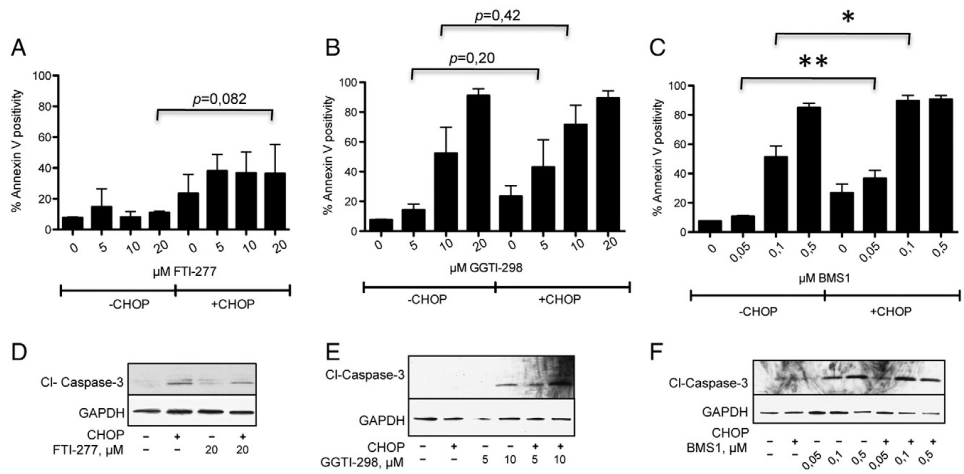

Fig. 3 – The prenylation inhibitors GGTI-298 and BMS1 induce apoptosis in WSU-NHL cells. WSU-NHL cells were treated with increasing concentration of (A) FTI-277 (5, 10, and 20 μM) and (C) GGTI-298 (5, 10, and 20 μM) and (E) BMS1 (0.05, 0.1, and 0.5 μM) alone or in combination with CHOP for 48 h. Annexin V-labeling followed by FACS analysis assessed the percentage of early apoptotic cells. Data are presented as mean ± SEM, $n = 3$. (D–F) Cells were treated with different concentration of FTI-277, GGTI-298 and BMS1. After protein isolation, cleaved caspase-3 was detected by Western blotting. Furthermore, in D, 10 μg of protein was loaded on the gel and in E and F, 5 μg of protein was loaded on the gel; thereafter, protein bands were visualized by ECL chemiluminescence.

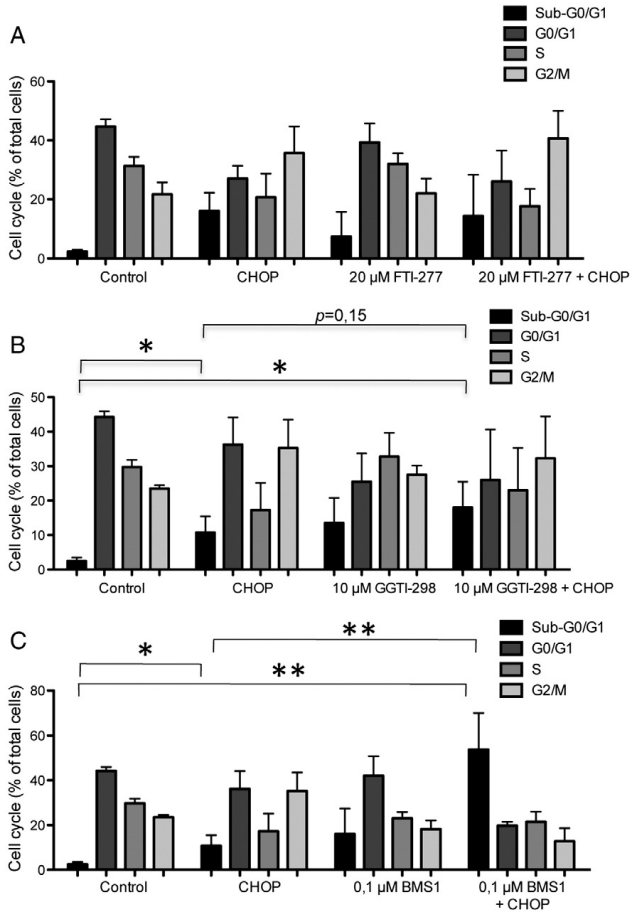

Fig. 4 – Co-treatment of CHOP and GGTI-298 or BMS1 induces increased cell death of WSU-NHL cells. WSU-NHL cells were treated with 20 μM FTI-277, 10 μM GGTI-298, or 0.1 μM BMS1 alone or in combination with CHOP for 48 h. Cells were harvested and cell cycle analysis was performed using propidium iodide labeling. Error bars represent SEM, $n = 3$.

20% of cells in sub-G_0/G_1 compared to 3% for control cells, whereas no other effect on cell cycle distribution was observed. BMS1 together with CHOP showed 50% of cells in the sub-G_0/G_1 population (Fig. 4C). In conclusion, FTI-277 did not change the cell cycle distribution, whereas GGTI-298 and BMS1 show apparent effects on the cell cycle distribution with an increased sub-G_0/G_1 population consistent with increased apoptosis, further confirming the effects of the inhibitors on proliferation and survival.

FTI-277, GGTI-298 and BMS1 are specific inhibitors as measured by the prenylation status of target proteins

The prenylation inhibitors simvastatin, FTI-277, GTI-298 and BMS1, used in this study, affect different enzymatic steps and to verify the potency and specificity of these inhibitors, we investigated the prenylation status of HDJ-2 (exclusively prenylated by farnesyl transferase), Rap 1A (exclusively prenylated by geranylgeranyl transferase I), and Rab5 (exclusively prenylated by Rab geranylgeranyl transferase). Antibodies to Rab5 and HDJ-2 that detect both the prenylated and the unprenylated forms of the proteins and an antibody to Rap1A that preferentially binds to the unprenylated form were used. As expected, treatment with 50 μM simvastatin resulted in increased expression of unfarnesylated HDJ-2 and unprenylated Rap1A (Fig. 5A). Therefore, treatment with 50 μM simvastatin was used as control of prenylation inhibition. The presence of FTI-277 showed partial inhibition of farnesylation of HDJ-2 and lack of effect on Rap1A and Rab5 that remained in their prenylated form (Fig. 5B). The prenylation status of Rab5 was not

affected by FTI-277 in accordance with the specificity of the inhibitor. As expected, treatment with GGTI-298 alone or in combination with CHOP resulted in increased amount of unprenylated Rap1A. Moreover, unprenylated HDJ-2 was also detected in the presence of GGTI-298 confirming the minor effect of GGTI-298 also on farnesylation inhibition. GGTI-298 did not affect the geranylgeranylation of Rab5B (Fig. 5C). Treatment with BMS1 alone or in combination with CHOP did not affect the prenylation status of Rap1A, consistent with reported lack of inhibitory effects on GGTI. (Fig. 5D). However, BMS1 show potent inhibitory effect on the farnysylation of HDJ-2 and the geranylgeranylation of Rab5B (Fig. 5D). To conclude, these results show that the substances used have been potent in inhibiting the expected prenylases in the cells, without unwanted effects on other prenylases.

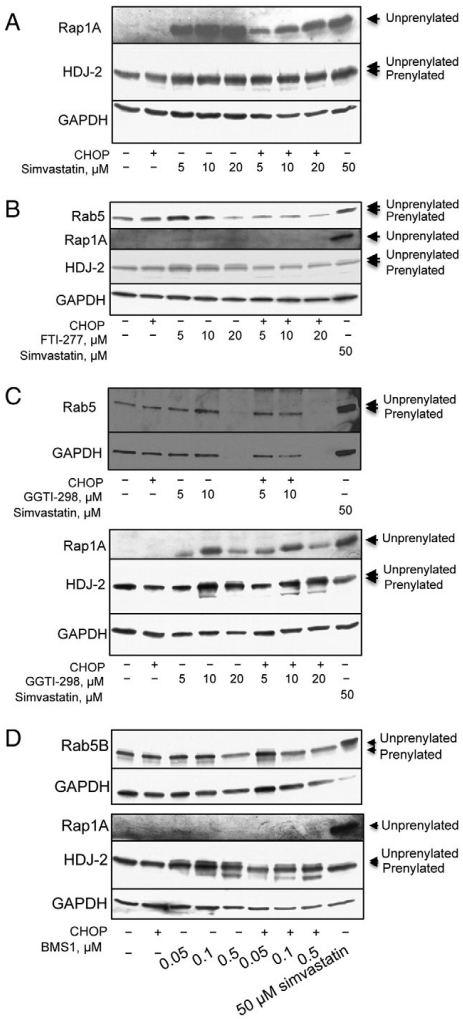

Fig. 5 – The inhibition of prenylated proteins by simvastatin, FTI-277, GGTI-298, and BMS1. WSU-NHL cells were treated for 48 h with simvastatin (5, 10, 20, and 50 μM), FTI-277 (5, 10, and 20 μM), GGTI-298 (5, 10, and 20 μM) or BMS1 (0.05, 0.1, and 0.5 μM) alone or in combination with CHOP. After protein isolation, the presence of unprenylated and prenylated HDJ-2, Rap1A, Rab5 was determined by Western blot analysis. GAPDH was used as loading control. Data are representative of at least two independent experiments.

Addition of GGOH rescues WSU-NHL cells from simvastatin-induced cell death

Statins inhibit not only the cholesterol biosynthesis, but also protein prenylation by reducing the biosynthesis of isoprenoid intermediates in the mevalonate pathway, i.e., farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). To ascertain that the sensitizing effect of simvastatin is not due negative effect on cholesterol biosynthesis, we treated the cells with squalene, a precursor for cholesterol, and monitored the effects on cell viability (Fig. 6A). The presence of squalene does not affect the sensitizing effect of simvastatin. The individual effects of the prenylation inhibitors used imply that there is a strong effect of geranylgeranylation and Rab geranylgeranylation in the increased cell death after treatment with GGTI-298 and BMS1. By adding GGOH, which metabolizes in the cells to GGPP, the intracellular pool of GGPP is restored and geranylgeranylation can be carried out also in simvastatin-treated cells. Simvastatin (10 μM) alone does not present any toxicity to WSU-NHL cells, but together with CHOP the cell viability is decreased from 90% for CHOP alone to 66% for simvastatin and CHOP. By adding 10 μM of GGOH, to simvastatin-treated cells, the viability is increased to 77%, indicating a role for geranylgeranylated proteins in the survival of WSU-NHL cells. To confirm the effect of GGOH, the prenylation status of Rab5B and Rap1A was investigated. Presence of 10 μM simvastatin alone or in combination with CHOP results in the appearance of unprenylated Rab5B and Rap1A (Fig. 6B). The addition of GGOH rescues the geranylgeranylation of both Rab5B and Rap1A in simvastatin-treated cells, which is in accordance with the expected effect. This indicates that inhibition of geranylgeranylation, by depleting the intracellular pools of GGPP, is involved in the sensitizing effect of simvastatin to CHOP treatment.

GGTI-298 and BMS1 induced cell death cannot be rescued by the addition of GGOH

To investigate the role of geranylgeranylation in the GGTI-298 and BMS1 induced cell death, WSU-NHL cells were treated with 10 μM GGTI-298 or 0.25 μM BMS1 alone or in combination with CHOP in the presence or absence of GGOH. GGOH did not reduce the cell viability of GGTI-298 or BMS1 treated cells (Fig. 6C and D). This implies that the sensitizing effect of GGTI-298 and BMS1 to cytotoxic treatment with CHOP cannot be mediated by a competitive effect of the inhibitors and GGOH.

The expression of Rab GGTbeta correlates to CHOP resistance

The finding of increased *Rab GGTbeta* mRNA in CHOP refractory DLBCL patients [15] implies a role of Rab GGTbeta in the evolution

of CHOP resistance. To study the possible connection between Rab GGTbeta and CHOP resistance in the DLBCL cell lines used in this study, we examined the expression of Rab GGTbeta protein in the DLBCL cell lines. Karpas-422 shows the highest expression of Rab GGTbeta and is also the most CHOP resistant cell line, whereas SU-DHL-5 expresses less Rab GGTbeta and is the cell line most sensitive to CHOP (Figs. 1A and 7). Consistently, the expression of Rab GGTbeta correlates to the CHOP sensitivity of the cell lines.

GGTI-298 and BMS1 do not interfere with rituximab-mediated cellular cytotoxicity

The monoclonal antibody rituximab is an important drug for patients with DLBCL as R-CHOP is considered the superior first-line treatment for DLBCL patients. To verify that the presence of GGTI-298 and BMS1 does not disturb the ability of rituximab to lyse CD20+ cells in the presence of NK cells, we performed an ADCC assay. WSU-NHL cells were pre-treated with either 10 μM GGTI-298 or 0.25 μM BMS1 for 24 h before rituximab and NK cells were added. In the absence of NK cells, rituximab alone induced 3% cell death at all rituximab concentrations as compared to 25–60% cell death in the presence of NK cells (Fig. 8A). Co-treatment of GGTI-298 and rituximab resulted in approximately 15% cell death within the absence of NK cells compared to 35–60% in the presence of NK cells (Fig. 8B). BMS1 and rituximab induced 42–53% cell death in the absence of NK cells and 50–54% cell death in the presence of NK cells (Fig. 8C). The cytotoxic effect of GGTI-298 and BMS1 is less pronounced in the ADCC setting in the absence of NK cells. This could be explained by the culture conditions probably leading to altered proliferation status thereby altered sensitivity to the substances. Taken together, GGTI-298 and BMS1 do not affect rituximab-mediated ADCC of WSU-NHL cells.

Discussion

In this study, we have established an in vitro cell line-based model for CHOP resistance in DLBCL cell lines. Using this model, we have demonstrated that specific inhibition of geranylgeranylation induces apoptosis in DLBCL cells. Furthermore, the combination of geranylgeranylation inhibitors and CHOP results in enhanced apoptotic response and this sensitizing effect of geranylgeranylation inhibitors is achieved at concentrations that are non-toxic for DLBCL cells at single agent treatments. Moreover, we have shown that the presence of the geranylgeranylation inhibitors, GGTI-298 and BMS1, does not interfere with rituximab-mediated cellular cytotoxicity but potentiates the cell death-inducing effect of rituximab.

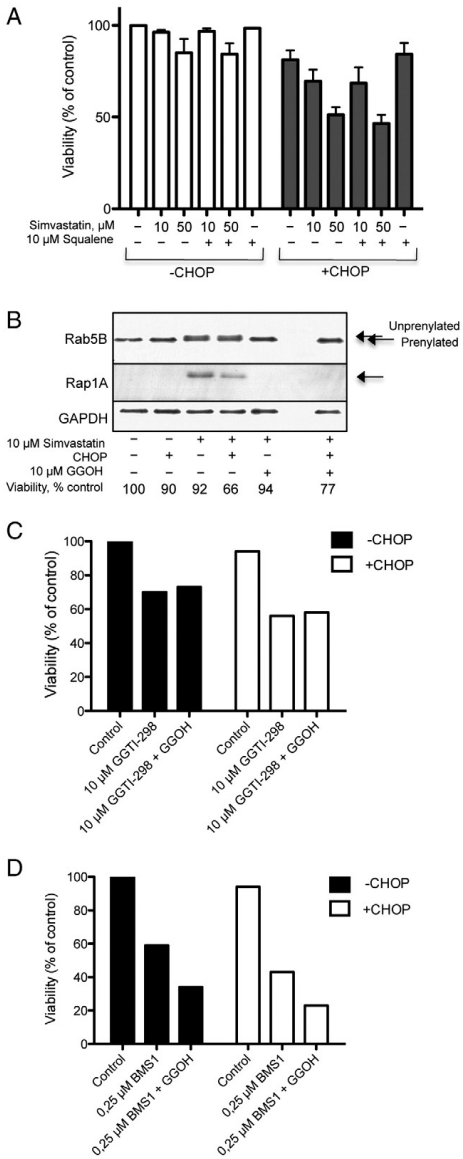

Fig. 6 – Simvastatin but not GGTI-298 or BMS1-induced cell death can be reversed by addition of GGOH. (A) WSU-NHL cells were treated for 72 h with 10 or 50 μM simvastatin alone or in combination with CHOP in the presence or absence of 10 μM squalene. Cell viability was assessed by trypan blue exclusion. Error bars represent SEM, $n = 4$. (B) WSU-NHL cells were treated for 48 h with 10 μM simvastatin alone or in combination with CHOP in the presence or absence of 10 μM GGOH. After protein isolation, presence of unprenylated and prenylated Rap1A and Rab5B was determined by Western blot analysis. Cell viability was assessed by trypan blue exclusion. The data shown are representative of two independent experiments. (C and D) WSU-NHL cells were treated for 48 h with 10 μM GGTI-298 or 0.25 μM BMS1 alone or in combination with CHOP in the presence or absence of 10 μM GGOH. Cell viability was assessed by trypan blue exclusion. The data shown are representative of two independent experiments.

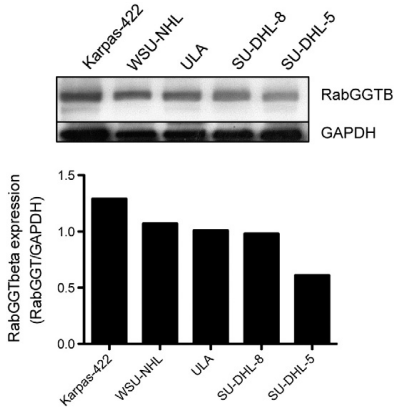

Fig. 7 – Expression of Rab GGTbeta correlates to CHOP resistance. Protein was isolated from the DLBCL cell lines Karpas-422, WSU-NHL, ULA, SU-DHL-5, and SU-DHL-8. The amount of Rab GGTbeta was determined by Western blot analysis. GAPDH was used as equal loading control. Relative amount of Rab GGTbeta was quantified by densitometry.

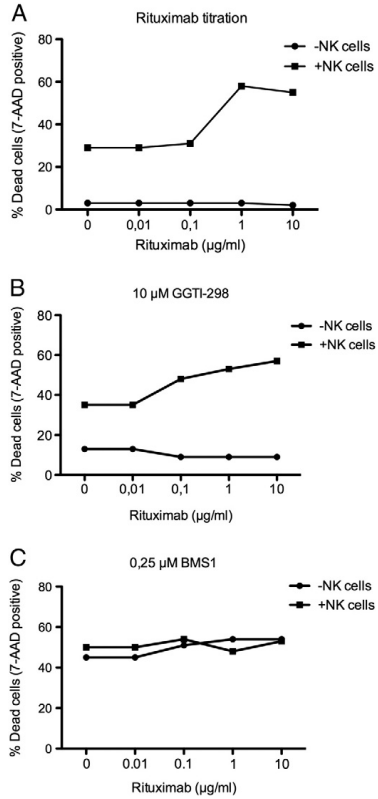

Fig. 8 – GGTI-298 and BMS1 do not interfere with rituximab-mediated cellular cytotoxicity. WSU-NHL cells were labeled with PKH26, either left untreated (A) or incubated with 10 µM GGTI-298 (B) or 0.25 µM BMS1 (C) for 24 h followed by addition of varying concentrations of rituximab. NK cells were added at an effector to target cell ratio of 10:1; thereafter, the cells were incubated for an additional 24 h. Dead target cells were identified as double positive for PKH26 and 7-AAD and used as readout of the assay. The data shown are representative of two independent experiments.

The sensitivity to CHOP treatment differed between the DLBCL cell lines used, perhaps illustrating the heterogeneity of this aggressive lymphoma type. Interestingly, treatment with single agent simvastatin resulted in increased cell death of the cell lines that was also most sensitive to CHOP treatment. Surprisingly, in combination with CHOP, simvastatin treatment resulted in a sensitizing effect to CHOP treatment in the CHOP resistant WSU-NHL cell line and showed an additive effect with CHOP in the simvastatin sensitive cell lines ULA, SU-DHL-5, and SU-DHL8. However, Karpas-422 was resistant to both CHOP and simvastatin, both as single agent treatment and in combination with CHOP. Statin-induced apoptosis has been described in several tumor cell lines such as melanoma [36], thyroid cancer [37,38], colon cancer [39], multiple myeloma [40], breast cancer [41], malignant lymphoma [21,22] and acute myeloid leukemia [42]. In addition, simvastatin has a chemo-sensitizing effect both in vivo [43] and in vitro [23,31,36,44,45]. Hitherto, the molecular mechanism behind the effect of simvastatin on apoptosis induction in tumor cells is not yet defined but involves the inhibition of prenylation of proteins important for cell cycle progression and cell signaling. Simvastatin treatment results in inhibition of the mevalonate pathway resulting in depletion of the intracellular pools of the isoprenoid substrates FPP and GGPP and as a result inhibits farnesylation and geranylgeranylation of proteins. However, these effects have not been shown to correlate to clinical effects for lymphoma patients as concomitant treatment with statins and R-CHOP does not affect outcome [26,27]. This calls for more specific inhibitors of the mevalonate pathway. Thus, to investigate which prenylation pathway might be involved in simvastatin-induced apoptosis, we used FTI-277 and GGTI-298, two reported inhibitors of farnesyl transferase and geranylgeranyl transferase I, respectively. In addition, we also used a combined Rab geranylgeranyl transferase and farnesyl transferase inhibitor, BMS1, kindly provided by Bristol-Myers Squibb. We demonstrated that GGTI-298 and BMS1, but not FTI-277, mimicked the effect of simvastatin, strongly indicating that geranylgeranylation but not farnesylation plays an important role in the regulation of growth and cell survival of WSU-NHL cells. Moreover, the role of geranylgeranylation in the simvastatin-induced apoptosis and chemo-sensitization of DLBCL cell lines was examined by the addition of the isoprenoid substrate, GGOH, which is converted to GGPP in the cell, and serves as substrate for the geranylgeranylation of proteins such as Rap1A and Rab5. The presence of GGOH during simvastatin treatment showed a positive effect on cell viability, further strengthening the importance of geranylgeranylated proteins in the sensitizing effect

of simvastatin. On the contrary, the addition of GGOH to the treatment of WSU-NHL cells with GGTI-298 did not rescue the cells to that extent that would be expected if a geranylgeranyl transferase I targeted protein was involved in the GGTI-298-induced apoptosis as GGTI-298 is considered a competitive inhibitor in regard to GGOH. Moreover, the addition of GGOH did not rescue the viability of BMS1 treated WSU-NHL cells, suggesting that BMS1 is an uncompetitive inhibitor with respect to GGOH. Taken together, inhibition of geranylgeranylation by GGTI-298 and BMS1 could not be prevented by GGOH. This is consistent with specific inhibition of the enzyme Rab GGTase, which catalyses the final step in the mevalonate pathway downstream from GGOH, that leads to the prenylation of the Rab family of proteins [46]. Previous studies have shown that inhibition of Rab GGT cannot be reversed by the addition of GGOH [47]. GGTI and Rab GGT share similar active site structures and both enzymes have a core structure that consists of α- and β-subunits with significant homology. The discrepancy regarding the apoptosis-rescuing effect of GGOH on simvastatin, GGTI-298 and BMS1 treated cells could be explained by the mevalonate pathway specific site of action of the inhibitors as well as the specific mechanism of action of the inhibitors. The possible inhibitory mechanism of GGTI-298 on Rab GGTase has to be further characterized. It cannot be excluded that GGTI-298 and BMS1 have effects unrelated to prenylation that also can affect DLBCL cell viability. In addition, these effects could potentially be involved in the CHOP sensitization induced by these inhibitors. As the level of prenylation inhibition by GGTI-298 and BMS1 does not seem to correspond to the CHOP sensitization, we speculate that the effects on important cellular signaling pathway by affecting the membrane localization of prenylated proteins renders cells more prone to respond to cytotoxic agents. It is possible that GGTI-298 and BMS1 affect a specific prenylated protein in DLBCL cells that has this significant effect on CHOP sensitization. Although no direct evidence for the causal link between inhibition of Rab geranylgeranylation and sensitization of CHOP-induced apoptosis could be provided in this study, we propose that the chemo-sensitizing effect of GGTI-298 and BMS1 in DLBCL cells is due to the loss of geranylgeranylated proteins and/or accumulation of ungeranylgeranylated proteins resulting in dysregulated cell cycle progression and signal transduction, mechanisms affecting cellular growth and survival. We speculate that the balance between farnesylation and geranylation of cellular proteins is essential for correct signal transduction, in which these proteins are involved, and which is crucial for cellular survival.

Consistent with a role of Rab proteins in CHOP resistance is the abundant expression of both Rab GGTalfa and Rab GGTbeta subunits in several different tumors [16]. Additionally, the elimination of either Rab GGTalfa or Rab GGTbeta by siRNA results in induction of apoptosis of cancer cell lines, further illustrating the important role of Rab proteins in cell survival [16]. Our data demonstrate an association between Rab GGTbeta protein expression and resistance to cytotoxic treatment as the expression of Rab GGTbeta protein in our DLBCL cell lines correspond to their CHOP sensitivity. This association is further supported by the finding of upregulated Rab GGTbeta in patients with refractory DLBCL disease [15].

A possible implementation of geranylgeranylation inhibitors to the conventional R-CHOP therapy is dependent on the sustained effect of the monoclonal antibody, rituximab, also in the presence of these inhibitors. The therapeutic efficacy of rituximab includes several mechanisms such as direct cell death-inducing effects, CDC and ADCC [48]. We here demonstrate an unaffected rituximab-mediated cellular cytotoxicity in the presence of GGTI-298 and BMS1, further supporting a future role for geranylgeranyl transferase inhibitors in the treatment of DLBCL patients.

In this study, we have established an in vitro cell line-based model for CHOP resistant DLBCL. We demonstrate a chemo-sensitizing effect of the geranylgeranylation inhibitors GGTI-298 and BMS1, suggesting that interference with geranylgeranylation could be a plausible way to sensitize DLBCL cells to CHOP treatment. Prenylation inhibitors, especially FTIs, have been used for several years as cancer drugs and it has recently been discovered that certain FTIs can potently inhibit also Rab GGTase. It is not impossible that their cytotoxic effects are applicable to inhibition of Rab GGT. Future studies will determine a potential correlation of these effects to the levels of Rab GGT. We propose that inhibition of protein geranylgeranylation together with conventional cytotoxic therapy is a potential novel strategy for treating patients with CHOP refractory DLBCL.

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Paper III

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ORIGINAL ARTICLE: RESEARCH

Gene expression profiling indicates that immunohistochemical expression of CD40 is a marker of an inflammatory reaction in the tumor stroma of diffuse large B-cell lymphoma

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Abstract

Immunohistochemical expression of CD40 is seen in 60–70% of diffuse large B-cell lymphoma (DLBCL) and is associated with a superior prognosis. By using gene expression profiling we aimed to further explore the underlying mechanisms for this effect. Ninety-eight immunohistochemically defined CD40 positive or negative DLBCL tumors, 63 and 35 respectively, were examined using spotted 55K oligonucleotide arrays. CD40 expressing tumors were characterized by up-regulated expression of genes encoding proteins involved in cell–matrix interactions: collagens, integrin αV, proteoglycans and proteolytic enzymes, and antigen presentation. Immunohistochemistry confirmed that CD40 positive tumors co-express the proinflammatory proteoglycan biglycan ($p = 0.005$), which in turn correlates with the amount of infiltrating macrophages and CD4 and CD8 positive T-cells. We postulate that immunohistochemical expression of CD40 mainly reflects the inflammatory status in tumors. A high intratumoral inflammatory reaction may correlate with an increased autologous tumor response, and thereby a better prognosis.

Keywords: Diffuse large B-cell lymphoma, CD40, gene expression profiling, tumor stroma

Introduction

In the search for new prognostic and predictive markers in diffuse large B-cell lymphoma (DLBCL), in many cases a curable disease [1], our group has previously identified and confirmed that immunohistochemical expression of CD40 is associated with superior overall survival (OS) after treatment with anthracycline-based chemotherapy [2,3]. We have also shown that CD40 has a potential favorable prognostic impact in DLBCL even after the addition of rituximab to chemotherapy [4].

In order to explore possible mechanisms and relationships of the positive CD40 effect, we have in previous studies tried to correlate the immunohistochemical expression of CD40 with other established or potential prognostic markers such as BCL-6, germinal center profile according to Hans *et al.* [5] and tumor infiltrating T-cells, but have not been able to find any correlation [2–4].

The aim of the present study was to use whole genome oligonucleotide arrays to identify differences in gene expression profiles between immunohistochemically CD40 positive and CD40 negative tumors, in order to further elucidate the mechanisms behind the impact of CD40 on prognosis.

Materials and methods

Patients

From a cohort of 98 patients with *de novo* DLBCL, stage I–IV (patients with mediastinal large B-cell lymphoma, transformed lymphoma or central nervous system [CNS] involvement were excluded), two sets of patients were selected, one with patients whose tumors expressed the CD40 antigen immunohistochemically, and the other where the CD40 antigen was lacking. Seventeen patients presented with stage I disease and all received stage-adequate treatment, i.e. at least three courses of anthracycline-based chemotherapy followed by local irradiation. Of the 81 patients with stage II–IV, 68 received adequate treatment, i.e. at least six courses of anthracycline-based chemotherapy. Tumor material from the remaining 13 patients with stage II–IV who had not received adequate chemotherapy was used in the gene expression analysis, but these patients were not included in the survival analyses. Twenty-nine of the 98 patients were previously included in a gene expression study comparing cured ($n = 17$) versus primary refractory ($n = 12$) disease [6]. In the cured cohort, 13 of 17 (77%) tumors were CD40 positive and in the primary refractory cohort six out of 12 (50%). The local ethics committee approved the study.

RNA isolation, labeling, hybridization and image analysis

RNA extraction, labeling and hybridization followed standard procedures using 55K oligonucleotide arrays produced

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at the Swegene Resource Center, Department of Oncology, Lund University Hospital. The image analysis was performed in GenePix Pro 4.1. The procedure is described in detail in an earlier publication [6].

Data extraction

The data were uploaded to the BioArray Software Environment (BASE; http://base.onk.lu.se/int/) [7], and spots flagged as missing in image processing, spots with a non-positive intensity, and spots with 10% or more saturated pixels, in either channel, were removed. Also, reporters annotated to be non-specific, and reporters without known gene symbol, were removed. Normalization was done with the lowest algorithm [8]. The 2-logarithm of the intensity ratio was used as a measurement of expression, and all measurements were assigned an uncertainty based on the signal to background variation in both channels [9].

All reporters were printed in duplicate on the assays, and these duplicates were merged in a weighted fashion, taking each spot uncertainty into account [10]. Similarly, reporters representing the same gene symbol, according to UniGene 181, were combined in a weighted merge.

The 98 assays were subject to an error model reducing the importance of low quality spots [9]. Unsupervised hierarchical clustering revealed that one of the two available duplicate assays clustered tightly together, whereas the other duplicate pair were separated.

After this quality confirmation, the data prior to the error model were revisited, replicate assays were subject to the weighted merge, and the error model was applied on the merged data. Reporters with more than 10% missing values among the 98 assays were removed, as were reporters where the standard deviation of expression was less than 0.4.

The (absolute value of the) Fisher score was used to rank reporters by differential expression, and the false discovery rate (FDR) [11] was estimated with a permutation test. Given the mean m and the variance V of expression within each sample group 1 and 2, the Fisher score F is given by: $F = (m_1 - m_2)/(V_1 + V_2)^{½}$.

Gene biology

Gene ontology classification was performed using the Functional Annotation Clustering program at DAVID Bioinformatic Resources (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/home.jsp)

Immunohistochemistry

The two cohorts were initially selected according to their immunohistochemical CD40 status, positive or negative, using a 10% cut-off level [2]. A specimen with more than 10% positive cells of the total number of tumor cells was regarded as positive. Paraffin blocks of the tumor tissue were previously made applying the tissue microarray (TMA) technique using standard procedures previously described [6]. CD4, CD8, CD68 and urokinase-type plasminogen activator receptor (uPAR) were already analyzed in 84, 84, 85 and 83 cases, respectively, using a 5% cut-off level [2,6]. A specimen with more than 5% positive cells of the total number of cells was regarded as positive.

In the present study immunohistochemistry was used to confirm, on the protein level, some of the major results obtained from gene expression profiling (GEP). Eighty-six cases of 98 were gathered in a TMA, but due to the earlier usage of the material only 62 (biglycan) and 69 (integrin αV) cases, respectively, were available for proper evaluation in the present study. The immunostaining was performed using the Tech-Mate instrument (Dako) and EnVision method (Dako) according to the manufacturer's instructions. Commercially available antibodies against biglycan, lumican, versican and, as a marker of integrin αV, CD51, were used. However, the results for lumican and versican were impossible to evaluate for technical reasons, and the data could not be further used (Table I). The assessment of immunostaining was performed by visual estimation by one hematopathologist (P.J.). To be consistent with our previous analyses of the markers correlating with or representing tumor infiltrating cells [2,6], the cut-off level for biglycan was set to 5% of the total number of cells. Likewise, to be consistent with previous analysis of CD40 [2], a marker expressed on tumor cells, the cut-off level for CD51 was set to 10%, since this is a marker also expressed on tumor cells. No other cut-off values were tested.

Statistical analysis

Associations between categorical and/or categorized patient characteristics were evaluated using the χ^2 test. The trend version of the test was used for variables with more than two ordered categories. The two-sample Wilcoxon rank-sum (Mann–Whitney) test was used to compare the age distribution. The log-rank test was used to test for differences in OS. All tests were two-sided and the significance level was set to 0.05. Stata 12.0 (StataCorp LP) and SPSS for Windows 17.0 (SPSS Inc.) were used for statistical analysis.

Results

Gene expression analysis

Among the 3211 reporters with acceptable missing value rate and overall variation, 28 reporters were found at a FDR of ≤ 10%, and are listed in Supplementary Table I to be found online at http://www.informahealthcare.com/lal/doi/10.3109/10428194.2012.666541. The CD40 gene itself was ranked number 36 of 3211 reporters.

Gene biology

Function analysis of the top 28 genes revealed that 21 were relatively up-regulated in the CD40 positive cohort and encoded proteins involved in cell–matrix interactions and antigen presentation, e.g. collagens type VI α1 and α2, integrin αV, proteoglycans (biglycan, lumican, versican)

Table I. Antibodies used for immunohistochemistry.

Antibody	Article no.	Source	Dilution
BGN*	HPA003157	Atlas Antibodies/Sigma	1:400
CD51/ITGAV†	HPA004856	Atlas Antibodies/Sigma	1:100

*Biglycan.
†Integrin αV.

Table II. Genes up-regulated in CD40 positive DLBCL tumors and associated with cell–matrix interactions and antigen presentation.

Biological function	Up-regulated genes
Structure of ECM and cell communication	Collagen type VI α1/2
	Integrin αV (CD51)
	Biglycan
	Lumican
	Versican
Proteolysis	Urokinase-type plasminogen activator receptor (*uPAR*)
	Matrix metallopeptidase 2 (*MMP-2*)
Antigen presentation	Proteasome β type 5 (*PSMB5*)

DLBCL, diffuse large B-cell lymphoma; ECM, extracellular matrix.

and proteolytic enzymes (uPAR, matrix metallopeptidase 2 [MMP-2] and proteasome β type 5 [PSMB5]) (Table II).

Immunohistochemistry

In order to confirm some of the gene expression data at the protein level, immunohistochemistry was used. However, there was a shortage of commercially available antibodies and others could not be evaluated due to non-specific staining. Expression of biglycan was seen on stromal cells and macrophages, the latter identified by having an abundant cytoplasm and a smaller nucleus without prominent nucleoli. The cut-off level of 5% for biglycan was congruent with the visual impression under the microscope. For most positive cases, a minority of the total number of cells were positive in the range 5–20% [Figure 1(A)], and for the majority of negative cases, no positive reactive cells could be detected [Figure 1(B)]. CD51 was seen on tumor cells. Expression of uPAR was mainly seen in macrophages [6], and to a lesser extent in other reactive cells such as follicular dendritic cells and lymphocytes. The correlation between immunohistochemical expression of biglycan, uPAR and CD51 with CD40 status was investigated, and only biglycan correlated significantly ($p = 0.005$) with CD40 expression. The results are summarized in Table III.

Due to the GEP findings indicating an increased inflammatory activity in the CD40 positive cohort, the association of expression of CD40 with tumor infiltrating macrophages and T-cells was evaluated, but no correlation was found. Furthermore, the relationship between all possible inflammatory parameters, CD40, biglycan, uPAR, macrophages and T-cells, was investigated. Expression of biglycan was found to correlate significantly with the expression of uPAR and with an increased infiltration of CD68 positive cells (macrophages) and CD4 and CD8 positive T-cells, respectively. The results are summarized in Table IV. Moreover, the expression of CD68 correlated significantly with the amount of CD4 ($p = 0.004$) and CD8 ($p = 0.000$) positive T-cells. The correlation between expressions of CD68 and uPAR did not reach statistical significance ($p = 0.062$).

Survival analysis

The major clinical characteristics of the patients are shown in Table V. When all adequately treated patients, irrespective of stage, were included in the analysis ($n = 85$), expression of CD40 was associated with a prolonged OS: median survival 62 months in patients with CD40 positive tumors (66%) versus 20 months in patients with CD40 negative tumors. The difference did not reach statistical significance ($p = 0.055$). When the analysis was restricted to patients in stage II–IV ($n = 68$), expression of CD40 was associated with a significantly prolonged OS: median survival 53 months in patients with CD40 positive tumors (65%) versus 19 months in patients with CD40 negative tumors ($p = 0.015$).

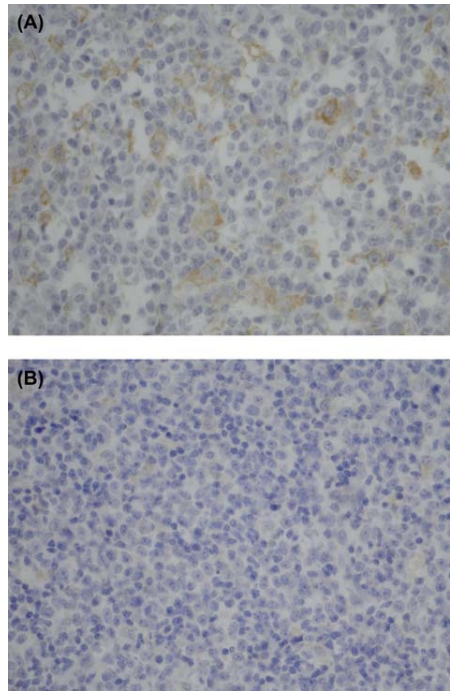

Figure 1. Immunohistochemical expression of biglycan, positive versus negative (A, B) in DLBCL. Positivity of the epitope is seen on stromal cells and macrophages.

Discussion

Gene expression profiling indicates that immunohistochemical expression of CD40 reflects an inflammatory reaction in the tumor stroma of DLBCL, most likely favorable for prognosis.

CD40 is a member of the tumor necrosis factor receptor family expressed on several immune cells including normal B-cells, as well as on non-immune cells and on tumor cells such as the majority of B-cell malignancies [12,13]. The

Table III. Correlation between immunohistochemical expression of CD40 and biglycan, uPAR and CD51.

Epitope	CD40 positive, *n* (%)	CD40 negative, *n* (%)	*p*-Value*
Biglycan	21/39 (54)	4/23 (17)	0.005
uPAR	22/54 (41)	7/29 (24)	0.101
CD51	27/43 (63)	16/26 (62)	0.558

uPAR, urokinase-type plasminogen activator receptor.
*χ^2 test.

Table IV. Correlation between immunohistochemical expression of biglycan and uPAR, tumor infiltrating macrophages and T-cells.

Epitope	Biglycan positive, *n* (%)	Biglycan negative, *n* (%)	*p*-Value*
uPAR	16/24 (67)	7/37 (19)	0.000
Macrophages	14/24 (58)	10/37 (27)	0.014
T-cells, CD4	23/24 (96)	28/37 (76)	0.038
T-cells, CD8	19/24 (79)	18/37 (49)	0.017

uPAR, urokinase-type plasminogen activator receptor.
*χ^2 test.

natural ligand CD154 is primarily expressed on activated T-helper cells [12]. CD40 is considered a critical regulator of humoral and cellular immunity, since CD40 signaling stimulates proliferation and differentiation in normal B-cells and is involved in the activation of cytotoxic T-cell response, mostly mediated through enhanced antigen presentation on dendritic cells after their CD40 interaction with T-helper cells [12,14]. To date, the mechanisms behind the prognostic impact of remaining CD40 expression on the malignant cells of DLBCL have not been fully understood. *In vitro* experiments have shown that CD40 signaling can increase antigen presentation from malignant B-cells, evoking an autologous immune response [15,16]. In order to investigate whether CD40 expression on the tumor cells of DLBCL is associated with an increased T-cell infiltration, we have in a previous study examined the amount of tumor infiltrating CD4 and CD8 positive T-cells in CD40 negative and positive tumors, respectively. By using this simplified method of measuring an autologous tumor response, no correlation was found [2]. However, in the present study we found an up-regulated gene expression of *PSMB5* in the CD40 positive cohort, which strengthens the hypothesis that there might be a difference in autologous immune response between CD40 positive and negative tumors. The proteasome β type 5 (PSMB5) is a part of the intracellular antigen processing machinery necessary for tumor antigen presentation via major histocompatibility complex type 1 (MHC-1), which can eventually evoke a T-cell response against the tumor cell. Components of the antigen processing machinery, including PSMB5, have been shown to increase in mRNA expression after CD40 and interleukin-4 stimulation in acute lymphoblastic leukemia cells [17], and defects in the antigen processing machinery have been correlated with tumor growth in solid tumors and AML [18,19].

Table V. Patient characteristics.

Characteristic	CD40 negative, *n* (%)	CD40 positive, *n* (%)	*p*-Value
Patients ($n = 98$)	35 (100)	63 (100)	
Sex			
Male	18 (51)	37 (59)	0.485*
Female	17 (49)	26 (41)	
Age			
< 60	12 (34)	25 (40)	0.514†
≥ 60	23 (66)	38 (60)	
IPI			
0	6 (17)	12 (19)	0.293‡
1	9 (26)	16 (26)	
2	7 (20)	21 (34)	
3	7 (20)	8 (13)	
4	5 (14)	3 (5)	
5	1 (3)	2 (3)	
Missing	—	1§	

IPI, International Prognostic Index.
*χ^2 test.
†*p*-Value corresponds to two-sample Wilcoxon rank-sum (Mann–Whitney) test.
‡*p*-Value corresponds to χ^2 test for linear trend.
§Missing value is listed in table but not included in calculation of percent or *p*-value.

In addition to possible differences in antigen presentation between CD40 positive and negative tumors, a prominent finding in the present study was a difference in gene expression regarding genes encoding proteins involved in cell–matrix interactions. In the CD40 positive cohort there was a relative up-regulation of genes encoding collagen type VI α1/2, integrin αV, proteoglycans (biglycan, lumican and versican) and proteolytic enzymes (uPAR and MMP-2). The intention was to use immunohistochemistry to confirm the results on the protein level. However, many of the pertinent antibodies could not be used for proper evaluation, and only biglycan, uPAR, and CD51 (integrin αV) were used for further assessment. Biglycan, a small leucine-rich proteoglycan and a component of the extracellular matrix, correlated significantly with CD40 expression. The biological function of biglycan is not fully understood, although it is associated with inflammation and macrophage activity [20]. During tissue injury, biglycan can be released from activated macrophages [21]. High gene expression levels of biglycan have been associated with superior prognosis in DLBCL [22]. Biglycan expression has also been associated with the transcription regulator, hypoxia-inducible factor-1α, which in turn predicts superior survival in DLBCL [23]. In the present study, immunohistochemical expression of biglycan was found to be a common denominator for CD40, uPAR and the degree of tumor infiltrating T-cells and macrophages. The presence of tumor infiltrating macrophages, as defined by more than 5% CD68 positive cells, was also associated with superior OS (Linderoth, unpublished data). The correlation between protein expression of uPAR and tumor infiltrating macrophages did not reach statistical significance, which might be explained by the fact that uPAR is found not only in macrophages but also in other reactive cells [6], and that the identification of macrophages is difficult without double staining with CD68.

The present gene expression findings support earlier reports regarding the importance of the tumor microenvironment for outcome in DLBCL [6,22,24]. Our group has previously investigated the differences in gene expression profiles of cured versus primary progressive DLBCL tumors and found up-regulation of genes and corresponding proteins involved in inflammation and proteolysis in the cured cohort [6]. In a large GEP study analyzing DLBCL tumors, a prognostically favorable "stroma-1 signature" was identified, characterized by extracellular matrix deposition and histiocytic infiltration [22]. The present study indicates that factors in the tumor microenvironment, which in solid cancers are associated with local aggressiveness and increased metastatic potential, e.g. collagen VI [25], integrin αV [26], biglycan [27], lumican [28,29], versican [30,31], uPAR [32] and MMP-2 [33], in the context of DLBCL might have a beneficial function, probably due to an adequate autologous anti-tumor response.

In conclusion, CD40 is a critical regulator of immunity expressed on a variety of cells. The mechanisms behind the superior prognostic effect of CD40 expression on the malignant cells of DLBCL have to date not been understood. The present study indicates that immunohistochemical

expression of CD40 to a great extent is a marker of an inflammatory reaction in the tumor stroma of DLBCL. This stromal inflammatory process might explain the better prognosis in CD40-expressing DLBCL. Further studies regarding the complex stromal activity of DLBCL are important in order to investigate other prognostic factors and new therapeutic targets.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Supplementary Table I showing genes expressed in CD40 positive and negative DLBCL

Supplemental Table I. Genes differently expressed in CD40 positive versus CD40 negative DLBCL tumors.

Rank	Entrez Gene ID	Fischer score	Sign*	Gene symbol	Gene name
1	55103	0.692812892950724	-	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2
2	51104	0.611738991312038	-	C9orf77	Chromosome 9 open reading frame 77
3	56904	0.625872222515538	-	SH3GLB2	SH3-domain GRB2-like endophilin B2
4	1462	0.650952084627139	+	CSPG2	Chondroitin sulfate proteoglycan 2 (versican)
5	4060	0.623526056912165	+	LUM	Lumican
6	11117	0.62421165704717	+	EMILIN1	Elastin microfibril interfacer 1
7	489	0.601363311504891	-	ATP2A3	ATPase, Ca++ transporting, ubiquitous
8	26608	0.581287599819649	-	TBL2	Transducin (beta)-like 2
9	4837	0.58322735358876	+	NNMT	Nicotinamide N-methyltransferase
10	4313	0.604910815795919	+	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
11	79368	0.55513854846212	-	SPAP1	SH2 domain containing phosphatase anchor protein 1
12	54453	0.611242126756406	+	RIN2	Ras and Rab interactor 2
13	1292	0.594092068172947	+	COL6A2	Collagen, type VI, alpha 2
14	5209	0.5691170493806	+	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
15	23176	0.562772768857112	+	SEPT8	Septin 8
16	8895	0.571450590567395	+	CPNE3	Copine III
17	4741	0.554320426836385	+	NEF3	Neurofilament 3 (150kDa medium)
18	10630	0.564085063734485	+	T1A-2	Lung type-I cell membrane-associated glycoprotein
19	5693	0.537029846938961	+	PSMB5	Proteasome (prosome, macropain) subunit, beta type, 5

20	10159	0.552121966476898	+	ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2
21	152137	0.516238992821558	-	C3orf6	Chromosome 3 open reading frame 6
22	3685	0.527834808941328	+	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
23	1291	0.542121114385934	+	COL6A1	Collagen, type VI, alpha 1
24	5329	0.537900418179173	+	PLAUR	Plasminogen activator, urokinase receptor
25	217	0.519829743299222	+	ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)
26	633	0.53153121859838	+	BGN	Biglycan
27	4792	0.534521321092526	+	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
28	1293	0.530987956309618	+	COL6A3	Collagen, type VI, alpha 3
⋮					
36	958	0.493752009405273	+	CD40	CD40 antigen (TNF receptor superfamily member 5)

* +, up-regulated in CD40 positive tumors, -, up-regulated in CD40 negative tumors.