Functional Analyses of Anti-B-Cell Antibodies Used to Treat Lymphoma

Dissertation

for obtaining the Doctoral degree in Natural Sciences

the Institute of Mathematics, Computer Science and Natural Sciences at the University of Hamburg

presented by

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Hamburg 2014

The experimental part of this work was performed from September 2010 until September 2014 in cooperation with Prof. Dr. Norbert Schmitz at the Department of Hematology, Oncology and Stem Cell Transplantation in Asklepios Hospital St. Georg, Hamburg, and the group of Assoc. Prof. Edzard Spillner at the Department of Chemistry, Institute for Biochemistry and Molecular Biology, University of Hamburg.

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Date of the disputation: 19.12.2014

Abstract

Rituximab, a chimeric $IgG1_K$ anti-CD20 monoclonal antibody, was first to be approved for treatment of B-cell lymphoma in 1998 after clinical studies had shown dramatic improvement in outcome of virtually all B-cell lymphoma patients when Rituximab was combined with chemotherapy. Notwithstanding the enormous success of Rituximab in treating patients with B-cell lymphoma, a substantial number of patients show no or suboptimal responses to rituximab or become refractory after initial treatment. Therefore, the search for new potentially more potent antibodies targeting malignant B cells continues.

The aim of this work was to develop general strategies how anti-B-cell antibodies can be evaluated most effectively and how the efficacy of different and new anti-B-cell antibodies (namely obinutuzumab, ofatumumab and BI 836826) can be compared with rituximab in vitro. Using healthy donor and patient NK cells isolated from peripheral blood as effector cells and various cell lines mimicking the most frequent lymphoma subtypes as target cells different functional assays based on the mechanisms of action of anti-B-cell antibodies were established: the CD 107a degranulation assay and the Europium release assay for antibody-dependent cellular cytotoxicity (ADCC), PI staining cell death assay for complement-dependent cytotoxicity (CDC), and the Annexin V / PI staining assay for programmed cell death (PCD). We also looked at immune synapse formation (ISF) and homotypic aggregation (HA) as early events when effector cells target tumor cells.

We show that performing one assay for ADCC and one assay for CDC with healthy donor cells as effector cells and cell lines SU-DHL-4 and OCY-Ly 10 as target cells give representative results allowing to gain reliable insight into the potency of old and new anti-B-cell antibodies. The use of patient effector cells as well as more cell lines or patient lymphoma cells as target cells can be restricted to antibodies which show promising results in the assays mentioned above. Immune synapse formation and homotypic aggregation showed substantial differences when various antibodies were investigated. Because no comparative

results using these methodologies so far exist, elucidating the role of these assays will need further experimentation.

Obinutuzumab, a third generation type II anti-CD 20 antibody, and BI 836826, a new Fc-engineered anti-CD 37 antibody, showed significantly stronger ADCC and PCD than established antibodies. The type I antibodies ofatumumab and rituximab performed better in CDC. BI 836826 showed moderate results in CDC and induced strong cytoskeleton polarization in ISF compared with other antibodies. The promising in vitro results with obinutuzumab were recently confirmed in clinical studies and led to licensing of this antibody for treatment of lymphoma. BI 836826 is another promising candidate anti-B-cell antibody which is being investigated in phase I clinical studies.

Comparative in vitro testing of anti-B-cell antibodies can be effectively done using a limited array of functional tests. These assays will allow to predict which antibodies can be considered promising candidates to enter the much more laborious and expensive sequence of clinical studies.

Zusammenfassung

Rituximab, ein chimärer monoklonaler $IgG1_K$ anti-CD 20 Antikörper, wurde bereits 1998 als erster Antikörper weltweit für die Behandlung maligner B-Zell Lymphome zugelassen, nachdem klinische Studien eine dramatische Verbesserung des Überlebens praktisch aller mit Rituximab (und Chemotherapie) behandelter Patienten mit B-Zell Lymphomen ergeben hatten. Trotz der enormen Behandlungserfolge mit Rituximab spricht ein nicht unbedeutender Prozentsatz von Patienten mit B-Zell Lymphomen nicht oder nur unzureichend auf die Behandlung an oder wird im Laufe der Therapie refraktär. Aus diesem Grund geht die Suche nach neuen, potentiell besseren Antikörpern weiter.

Ziel dieser Arbeit war es, allgemeine Strategien zu entwickeln wie anti-B-Zell Antikörper möglichst effektiv untersucht werden können und wie verschiedene neue anti-B-Zell Antikörper (Obinutuzumab, Ofatumumab und BI 836826) in vitro mit Rituximab verglichen werden können. NK-Zellen von gesunden Spendern oder Patienten wurden als Effektorzellen, Zelllinien oder frisch gewonnene Lymphomzellen von Patienten als Zielzellen für verschiedene Assays untersucht, die die wesentlichen Wirkungsmechanismen von anti-B-Zell Antikörpern reflektieren: der CD 107a Degranulierungs-Assay und der Europium Assay um die Antikörper-vermittelte Zytotoxizität (ADCC) zu messen, der PI staining cell death Assay um die Komplement-vermittelte Zytotoxizität und der Annexin V / PI staining Assay um den programmierten Zelltod zu untersuchen. Außerdem wurde die Ausbildung der Immunsynapse und die homotypische Aggregation als frühe Ereignisse einer zellulär vermittelten Immunantwort untersucht.

Wir zeigen, dass ein ADCC Assay und ein CDC Assay unter Verwendung von gesunden Spenderzellen als Effektorzellen und zweier Zelllinien (SU-DHL-4 und OCI-Ly 10) als Zielzellen ausreichen, um einen verläßlichen Einblick in das Potenzial verschiedener anti-B-Zell Antikörper zu bekommen. Der Einsatz von Effektorzellen, die vom Patienten stammen, kann ebenso wie die Verwendung weiterer Zelllinien oder gar von Patientenlymphomzellen als Zielzellen auf die Fälle beschränkt bleiben, in denen die ersten Tests erfolgversprechend waren. Die Ausbildung von immunologischen Synapsen und die homotypische

Aggregation zeigten sehr unterschiedliche Ergebnisse, je nachdem welcher Antikörper verwendet wurde. Da bisher keinerlei vergleichende Untersuchungen vorliegen, bleibt abzuwarten, welche Bedeutung diesen Beobachtungen beizumessen ist.

Obinutuzumab, ein Typ II Antikörper der dritten Generation, und BI 836826, ein neuer anti-CD 37 Antikörper, dessen Fc Teil zur Steigerung der Effektivität genetisch manipuliert wurde, zeigten gegenüber etablierten CD20 Antikörpern eine erheblich gesteigerte Wirksamkeit in den Tests, die ADCC und PCD messen. Die älteren Typ I Antikörper Ofatumumab und – in geringerem Ausmaß –, auch Rituximab, zeigten eine effektivere Komplement-vermittelte Zytotoxizität. BI 836826 zeigte im Vergleich zu anderen Antikörpern eine mäßige Komplement-vermittelte Zytotoxizität und eine starke Polarisation der Zellgerüststrukturen (cytoskeleton). Die vielversprechenden in vitro Ergebnisse mit Obinutuzumab fanden kürzlich ihre Bestätigung in entsprechenden klinischen Studien, die zur Zulassung von Obinutuzumab zur Therapie bestimmter Lymphome geführt haben. BI 836826 ist ein neuartiger anti-CD 37 Antikörper, dessen frühe klinische Prüfung (Phase I) aufgrund der positiven in vitro Tests derzeit läuft.

Die vergleichende experimentelle Untersuchung verschiedener anti-B-Zell Antikörper mit Hilfe weniger gut ausgewählter Standard Assays ist möglich. Diese in vitro Untersuchungen erlauben eine zuverlässige Vorhersage, welche der getesteten Antikörper erfolgversprechend erscheinen, sodass eine wesentlich komplexere und teurere klinische Prüfung gerechtfertigt erscheint.

Content

1 Introduction

1.1 Lymphoma

1.1.1 Definition

Lymphoma is a group of blood cell tumors arising from lymphoid tissues. Different lymphoma subtypes are thought to represent the malignant clonal counterpart of distinct stages of maturation normal lymphocyte progenitors regularly undergo in bone marrow, thymus, lymph nodes and other lymphatic organs (spleen and liver) before entering the circulation and tissues to cooperate with other immune cells in order to mount cellular and humoral immune responses against a plethora of infectious and toxic agents jeopardizing the integrity of the human body. Because we exclusively investigated B-cell antibodies and compare their mechanisms of action and therapeutic potential in vitro all further presentation focuses on selected aspects of B-cell development as well as the characterization of normal B-cells and B-cell lymphoma necessary to understand the background of the experimental work. The treatment of T-cell lymphoma and some rare B-cell disorders is presented in the additional sections.

1.1.2 Pathophysiology of B-cell lymphoma

Figure 1 depicts different stages of normal B-cell differentiation and their relationship to the major subgroups of B-cell neoplasms. The lower part of Figure 1 assigns the most frequent B-cell lymphoma subtypes (diffuse large B-cell lymphoma, DLBCL; follicular lymphoma, FL; and mantle cell lymphoma, MCL) to distinct stages of normal B-cell development. While MCL is thought to derive from pre-GC (germinal center) B-cells, FL and some cases of DLBCL are GC neoplasms. Other cases of DLBCL are believed to derive from cells which have already passed the GC (post-GC neoplasms). The allocation of different B-cell lymphomas to more immature or mature stages of B-cell differentiation has multiple implications ranging from diagnosis to treatment of these disorders including the use of monoclonal antibodies. We chose MCL, FL and DLBCL as

targets for our investigations because these subtypes cover more than two thirds of B-cell disorders and all stages of B-cell development.

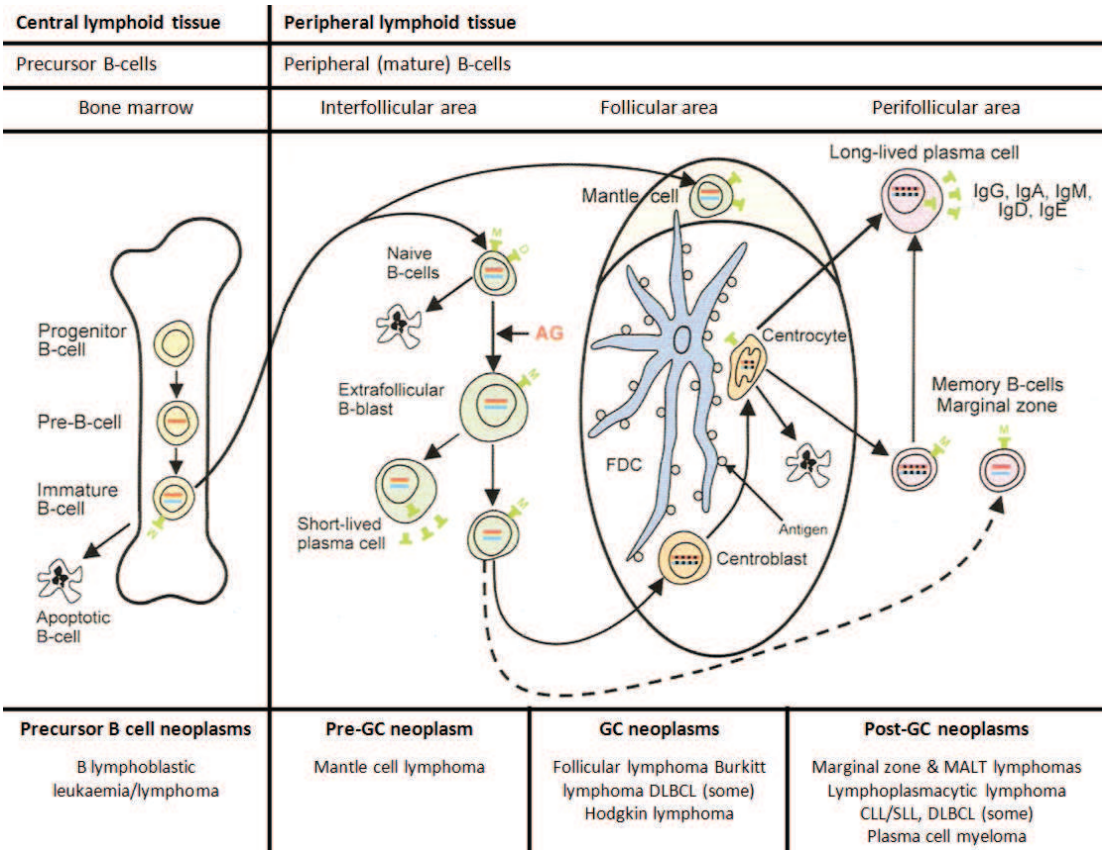

Fig. 1 B-cell differentiation and the respective lymphoma subtypes.[1]

1.1.3 Epidemiology

Non-Hodgkin Lymphoma (all lymphoma subtypes excluding Hodgkin lymphoma) is the 11th most common cancer worldwide with more than 199,000 deaths reported in 2012.[2] The annual number of new cases (incidence) in Germany is estimated at 8.5 per 100,000 persons at age-standardized rate.[2] Men are affected slightly more than women with great variation among different

subtypes. The disease can occur at any age but the incidence generally increases with age; again, important exceptions can be found for certain subtypes.

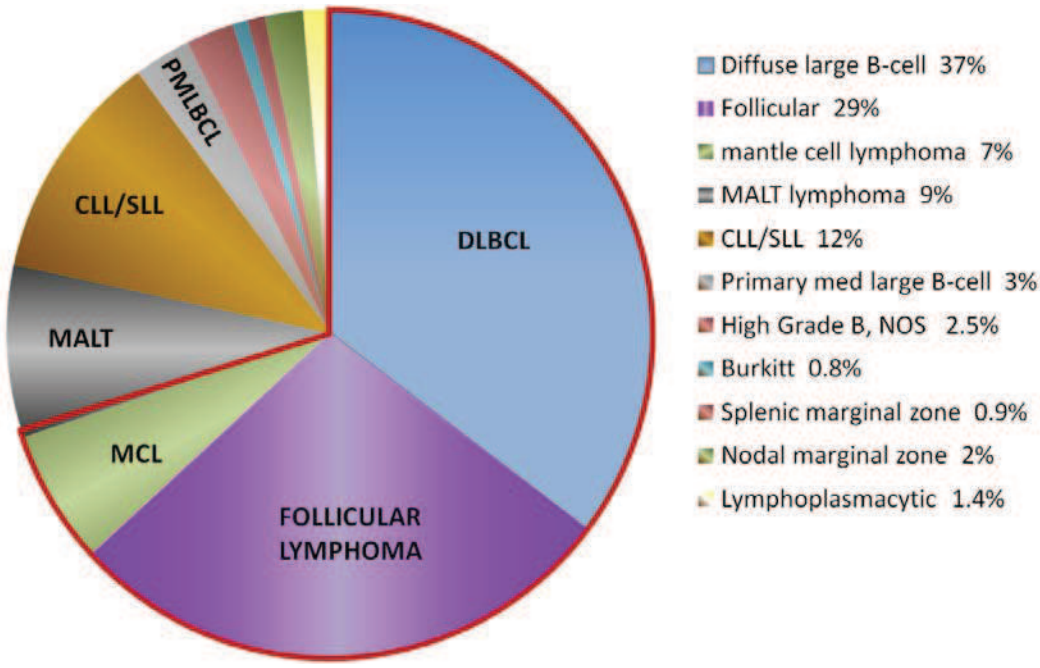

Fig. 2 Relatively frequencies of different B-cell lymphoma subtypes.[1]

1.1.4 Characterization of DLBCL, FL and MCL

1.1.4.1 Diffuse large B-cell lymphomas (DLBCL)

About one third of all B-cell lymphomas are DLBCL (Figure 2). DLBCL is a heterogeneous group of aggressive lymphomas which usually present with rapidly growing tumor masses at single or multiple nodal or extranodal sites. Microscopically, the tumor cells are large, and often are replicating.[1] Common morphologic variants include a centroblastic, immunoblastic and anaplastic

subtype. DLBCL usually express the B-cell antigens CD 19, CD 20, CD 22 and CD 79a but may also lack one or the other surface marker. Translocations of the *bcl 2* and *bcl 6* genes occur in 20 – 30 %, *MYC* rearrangement is diagnosed in about 10 % of cases.[1] Gene expression profiling (GEP) identified 2 major subgroups of DLBCL. One subgroup shows the profile of germinal center B – cells (GCB-like, 40 – 50 %) the other subtype has the profile of activated peripheral B-cells (ABC-like).[3,4] Patients with *MYC* translocations and especially patients with "double hit" lymphoma (*MYC* and *bcl 2* break) are said to have a poor prognosis[5,6] although recent data from large prospective studies suggest that the prognostic implications may have been overestimated.[7] DLBCL occur more frequently with increasing age; the median age at diagnosis is about 64 years with almost equal distribution between males and females. Nowadays, depending on the risk factor profile, sixty to more than ninety percent of patients with DLBCL can be cured. Treatment typically consists of a combination of rituximab and chemotherapy, mostly the CHOP regimen (see below). Best results are obtained in younger patients with localized disease[8] but also young high-risk patients[9] and elderly patients[10] do reasonably well with modern immunochemotherapy.

1.1.4.2 Follicular lymphoma (FL)

Follicular lymphoma accounts for approximately 20 % of all lymphomas (Figure 2) with the highest incidence in the US and Europe; it is less frequent in Asia. The median age at diagnosis is around 65 years with a male to female ratio of 1 : 1.7. It is defined as a lymphoma of follicle center B-cells (centrocytes and centroblasts) which show an at least partly follicular growth pattern in the lymph node. It is positive for the B cell markers CD 10, CD 19, CD 20, CD22 but almost always negative for CD5. The most common genetic alteration in follicular lymphoma is a balanced translocation t (14:18) (q32; q21) with deregulation of *bcl 2*. This translocation is not detected in all patients, and it is not specific for follicular lymphoma. FL occurs in any lymph node site as well as in the bone marrow. FL often does not need treatment at diagnosis (watch and wait policy). If treatment becomes necessary because of growing lymph nodes, impairment of hematopoiesis, or B-symptoms, like drenching night sweats, fever, or weight loss, a combination of rituximab and chemotherapy is used. In contrast to DLBCL, the chemotherapy is more variable and may consist of monotherapy (bendamustine)

or various forms of polychemotherapy (CHOP, CVP, FC).[11] Maintenance therapy is recommended to prolong survival.

FL still is thought to be not curable but modern treatment keeps patients alive for decades. New targeted therapies (Ibrutinib, Idelalisib)[12,13] were recently approved and will further improve long-term outcome of these patients. FL thus has changed from an acute life-threatening illness to a chronic disease which like diabetes mellitus will potentially need life-long therapy but will no longer kill the patient.

1.1.4.3 Mantle cell lymphoma (MCL)

Mantle cell lymphoma is a rarer subtype of B-cell lymphoma representing about 7% of all lymphoma subtypes (Figure 2). It preferentially occurs in patients in their sixties with a marked male preponderance (about 2 : 1 or greater). Mantle cell lymphoma is a clinically aggressive lymphoma although a rare indolent subtype also does exist. Remarkably, about 25 % of cases show extranodal involvement (bone marrow, gastrointestinal tract). Microscopically, MCL is a monomorphic lymphoid proliferation with nodular, diffuse, mantle zone or rarely follicular growth pattern. Immunohistochemically, MCL cells are surface IgM / IgD positive and express CD5, FMC-7, and CD 43; CD 23 is negative or weakly positive. MCL cells generally over-express cyclin D1 due to the translocation t(11; 14)(q13;q32).[14,15] The prognosis of mantle cell lymphoma is still poor. However, treatment including rituximab, cytosine-arabinoside and high-dose therapy followed by autologous stem cell transplantation has significantly improved the prognosis over recent years.[16] With such therapy, the progression-free survival at six years increased to 66 %. Like in FL new more specific drugs have recently become available; it is currently not possible to judge how these therapies will impact on the survival of patients with MCL.

1.1.5 Diagnosis

Lymphoma may be suspected if patients report on mostly painless enlargement of lymph nodes, so called B-symptoms (night sweats, fever, or weight loss), or other non-specific complaints like fatigue, nausea or increased susceptibility to infections. Because almost any organ of the human body may be affected by lymphoma the initial symptoms can be manifold and often misleading. In the end, any prolonged (< 4 weeks) swelling of lymph nodes which cannot be explained by an infectious disease or another underlying disorder known to cause the respective symptoms must trigger the exstirpation of an enlarged lymph node or a biopsy of any other organ involved which then should be examined by an experienced hematopathologist. Morphology, imunohistochemistry, cytogenetic and molecular methods are necessary to diagnose one of the many subtypes of lymphoma which have been described to date.[1] A precise diagnosis is of paramount importance as treatment is becoming more and more specific for certain lymphoma subgroups. In some cases, targeted therapies addressing aberrations in normal molecular pathways regulating cellular proliferation or cell death are already available. Comprehensive modern diagnostics in order to confirm the presence of a specific molecular lesion is a prerequisite before treatment with any of these drugs is initiated.

1.1.6 Treatment

The cornerstones of treating lymphoma are radiotherapy, chemotherapy, monoclonal antibodies, transplantation and more recently small molecules. In many instances a combination of these modalities will be necessary to ultimately destroy the tumor cells and put patients into long-term remission and hopefully cure. Besides a correct histopathological diagnosis a number of clinical parameters (age, performance status, stage, involvement of extranodal organs), conventional blood tests, radiographic imaging in order to define the extent of disease, and molecular markers are used to characterize the disease and segregate patients into so called risk groups. In general, high-risk patients with more advanced disease will need more aggressive treatment which unfortunately also puts them at higher risks for acute and long-term side effects of therapy (e.g. secondary malignancies, organ damage). A more detailed description of treatment strategies for patients with B-cell lymphoma has been

published separately by our group.[17] It is beyond the scope of this work. Therefore, we restrict the description of therapeutic principles to patients with DLBCL, FL and MCL particularly addressing the role of anti-B-cell antibodies.

1.1.6.1 Radiotherapy

Radiotherapy at fixed doses (20-40 Gray) to lymph nodes or areas involved with disease can be very effective. Nowadays, in patients with B-cell lymphoma the role of radiotherapy is rather limited; in many countries of the Western world it is no longer recommended at all. In Germany, radiotherapy continues to be used in order to control lesions in patients with very limited spread of disease (stage I), with bulky disease (any lymphoma manifestation > 7.5 cm or 10 cm in diameter) or with extranodal disease (e.g. bone) (Figure 3).[18]

Fig. 3 Radiotherapy of involved field (left neck).

1.1.6.2 Chemotherapy

Chemotherapy still is the backbone of all therapy administered to patients with lymphoma. Chemotherapeutic agents directly interfere with patient DNA / RNA and by different and complex mechanisms lead to DNA damage which ultimately can kill the tumor cell. Because not only tumor cells but all fast growing tissues of the human body are hit it was essential to find ways how to minimize side effects of chemotherapy but at the same time increase efficacy. It was a major step forward when McKelvey et al[19] in 1976 described a combination chemotherapy regimen named CHOP. This regimen not only improved the efficacy of single chemotherapeutic agents by exploiting the different modes of action of cyclophosphamide, adriamycin, vincristine, and prednisone (CHOP) but also allowed for better tolerance of polychemotherapy by choosing a combination of drugs with non-overlapping toxicities to normal organs. For many patients with lymphoma CHOP or one of its variants which have been developed over time still form the mainstay of therapy. Alternative regimens and combinations have been developed but the principle introduced by McKelvey et al. remains valid until today.

Fig. 1 CHOP regimen.[19]

Drug	Dose	Unit	way given	Schedule	
Cyclophosphamide	750	mg/m²	i.v.	day	1
Vincristine	1,4	mg/m²	i.v.	day	1
Adriamycin	50	mg/m²	i.v.	day	1
Prednisone	100	mg abs	p.o.	day	1-- 5

6-8 courses with 2 – 3 week time intervals are administered.

1.1.6.3 Stem cell transplantation

Two different transplantation modalities are in clinical use since the early description of autologous and allogeneic transplantation in the seventies and eighties of the last century. The term autologous stem cell transplantation actually describes a procedure consisting of high-dose chemotherapy (HDT) followed by transfusion of autologous (the patient's own) hematopoietic stem cells which have been harvested from blood, frozen and kept in liquid nitrogen until thawing and re-infusion to the patient. Very high doses of chemotherapeutic agents are used to kill the tumor cells; the infusion of hematopoietic stem cells then is necessary to counteract the irreversible bone marrow aplasia induced by such high doses of cytotoxic agents which would lead to life-threatening infections and bleeding if the patient's marrow function would not be timely rescued by the infusion of hematopoietic stem cells. HDT / ASCT is regularly used in patients with relapsed lymphoma in order to improve tumor control and survival compared to conventional salvage chemotherapy.

Allogeneic stem cell transplantation also uses high doses of chemotherapeutic agents (conditioning) prior to transplantation of hematopoietic stems which in these cases, however, are donated by human leukocyte antigen (HLA)-identical family or unrelated donors. In addition to the effects of chemotherapy preceding the transplant the unique mechanism of action of allogeneic transplantation has been described as the graft-vs-tumor effect: [20] donor T-lymphocytes transplanted with the hematopoietic stem cells recognize allo-antigens on the surface of the patient's tumor cells and kill these cells by initiating a cellular immune response. Allogeneic stem cell transplantation is currently being used to treat lymphoma patients with advanced disease (relapse after conventional salvage or high-dose therapy or progression under therapy).[21]

1.1.7 Clinical outcomes

First-line therapy of DLBCL, FL or MCL is depending on patient's age and the extent of disease found to be present at diagnosis. Chemotherapy alone (e.g.

CHOP) can induce remissions in varying fractions of patients but most of these remissions are short-lived (months to few years). Results obtained with CHOP in patients with advanced DLBCL compared to other chemotherapy regimens used 20 years ago have been described by Fisher et al.[22] Overall survival after 3 years was around 50 %. For patients with MCL OS with conventional chemotherapy alone was reported to be around 75 % at 2 years,[23] for patients with FL OS was around 90 %.[24]

With the advent of rituximab, the first anti-CD 20 antibody used in man to treat patients with B-cell lymphoma PFS and OS significantly improved in all types of B-cell lymphoma. When rituximab was combined with CHOP chemotherapy the improvement seen when compared to CHOP therapy alone depended on the lymphoma subtype and prognostic subgroup. In general, improvement in OS was between 10 and 20 %. (Figure. 4)[25,8,10] This substantial survival advantage caused clinicians to integrate rituximab into all current treatment regimens for all B-cell lymphomas including DLBCL, FL and MCL.

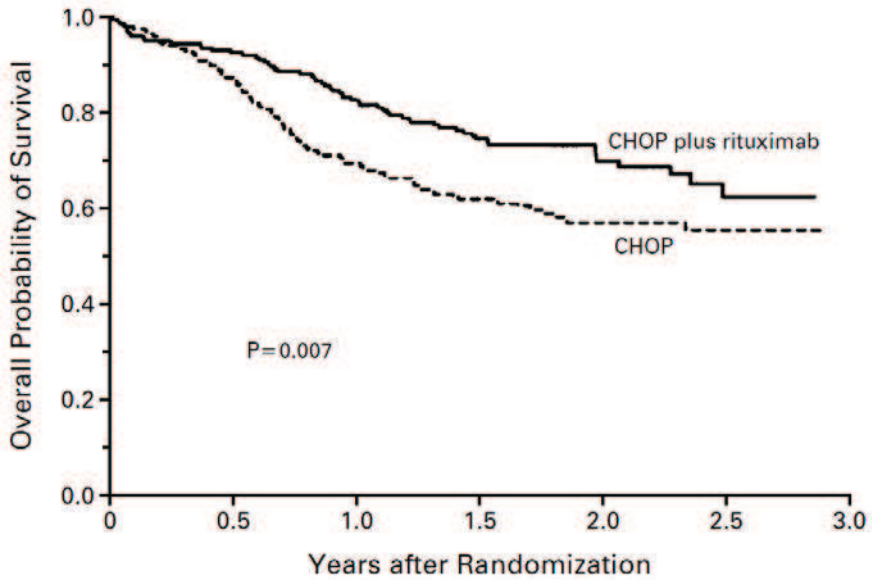

Fig. 4 Overall survival among 399 patients assigned to chemotherapy with CHOP or with R-CHOP.[25]

1.2 Monoclonal B-cell antibodies

Monoclonal antibodies (mAbs) first described by Köhler and Milstein in 1976[26] and previously used for diagnostic purposes only entered the clinical arena about 16 years ago when first reports on successful treatment of relapsed or refractory lymphoma patients with rituximab were published.[27] Following this and other reports rituximab mostly in combination with chemotherapy became the standard of care for all patients with DLBCL, FL, MCL and other B-cell malignancies.

During the last decade a number of other monoclonal antibodies mostly targeting the CD 20 antigen became clinically available. These antibodies recognize other antigens on B-cells (CD 19, CD 22, CD 37). Because these antibodies like the CD-20 antibodies, rituximab, ofatumumab and obinutuzumab, need patient effector cells and / or patient serum in order to show therapeutic efficacy, they are called ‘‘naked’’ antibodies. In contrast to naked antibodies, anti-CD 20 antibodies linked to radioisotopes (^{90}Y-labelled ibritumomab tiuxetan[28], ^{131}I-labelled tositumomab[29]) and anti-CD 22 or anti-CD 79 antibodies coupled to cytotoxic agents[30,31] use radio- or chemo- therapy to induce anti-lymphoma effects. Such antibodies direct the cytoxic agent to the tumor cell where irradiation or chemotherapeutic moieties destroy the malignant cell without the necessity of patient functional effector mechanisms of action. Because only small quantities of irradiation or the cytotoxic agent are released into the circulation, these drugs are generally better tolerated than systemic chemotherapy. Unfortunately, it is still unclear if labeled antibodies confer survival advantages to patients with B-cell malignancies as compared to unlabeled antibodies. Although a number of labeled and unlabeled antibodies targeting the CD 20 or other B-cell epitopes have been developed and some have been licensed for treatment of lymphoma, rituximab currently remains the gold standard to treat B-cell lymphoma. New antibodies are in clinical trials in order to demonstrate superior efficacy as compared to rituximab.

Table 2 shows a list of therapeutic mAbs for B- cell lymphoma as approved by the American Food and Drug Association (FDA). A number of other therapeutic antibodies are currently in early- to late-stage clinical trials. The development of candidate mAbs for the clinic involves a complex process of scientific and preclinical evaluations that include identification of the physical and chemical properties of the antibody, a detailed specificity analysis of antigen expression, the study of the immune effector functions and signaling pathway of the antibody, the analysis of *in vivo* antibody localization and distribution in transplanted or syngeneic tumor systems, and the observation of the *in vivo* therapeutic activity of the antibody in animal models.[32] Evidence from clinical trials of antibodies in cancer patients has revealed the importance of iterative approaches for the selection of antigen targets and optimal antibodies.

Interestingly, with few exceptions the combination of chemotherapy with rituximab or other monoclonal antibodies showed much better clinical results that the administration of antibody alone. This phenomenon has recently been explained by the better accessibility of target structures by antibodies after exposure to chemotherapy.[33] Although the combination of rituximab and chemotherapy dramatically improved survival rate of patients with B – cell lymphoma, there are still patients showing no response to chemoimmunotherapy or who relapse shortly thereafter. Salvage therapy of patients with relapsed or refractory B- cell lymphoma is much less successful than first-line therapy and failure to rituximab and chemotherapy is common. Therefore, the search for new and better antibodies continues.

Table 2 Monoclonal antibodies currently FDA-approved in hematological malignancies and their mechanisms of action[32,34,35]

Antibody	Target antigens	Types	FDA-approved indication
Rituximab (Mabthera; Roche)	CD20	chimeric IgG1	CD20-positive B cell NHL and CLL, and maintenance therapy for untreated follicular CD20-positive NHL
Ofatumumab (Arzerra; Genmab)	CD20	human IgG1	CLL refractory to fludarabine and alemtuzumab
Obinutuzumab (Gazyva; Roche)	CD20	humanized IgG1	in combination with chlorambucil for the treatment of patients with previously untreated chronic lymphocytic leukemia
^{90}Y-labelled ibritumomab tiuxetan (Zevalin; IDEC Pharmaceuticals)	CD20	murine IgG1	Relapsed or refractory, low-grade or follicular B cell NHL
^{131}I-labelled tositumomab (Bexxar; GlaxoSmithKline)	CD20	murine IgG2	CD20 antigen-expressing relapsed or refractory, low-grade, follicular or transformed NHL

1.2.1 B cell differentiation and clusters of differentiation (CD)

Various B cell differentiation antigens are expressed at certain stages of B cell ontogeny. Following international agreement cell surface molecules including those detected on human B-cells are classified according to clusters of differentiation (CD).[36] Five major subgroups have been defined for human B cell antigens detected by means of monoclonal antibodies. Antigens which appear before the cytoplasmic positive pre-B cell and which are lost at or just before the plasma cell stage like the CD19 and the CD20 molecule are called pan-B cell antigens.[37-39] The second group of antigens consists of antigens which are expressed on resting B cells and are lost during activation. Surface expressed IgD, CD21 and CD22 are major representatives of this group.[40-43] The third group of antigens is expressed only after activation of B cells like the CD23 antigen.[44] The fourth group of antigens appears only at terminal stages of B cell differentiation (PCA-1, and PC-1).[45,46] Finally, the fifth group includes those antigens which are expressed at several, short stages of B cell differentiation like, e.g., the cALL antigen (CD 10).[47] Figure 5 schematically demonstrates the appearance and loss of antigens on developing B-cells according to their maturation status.

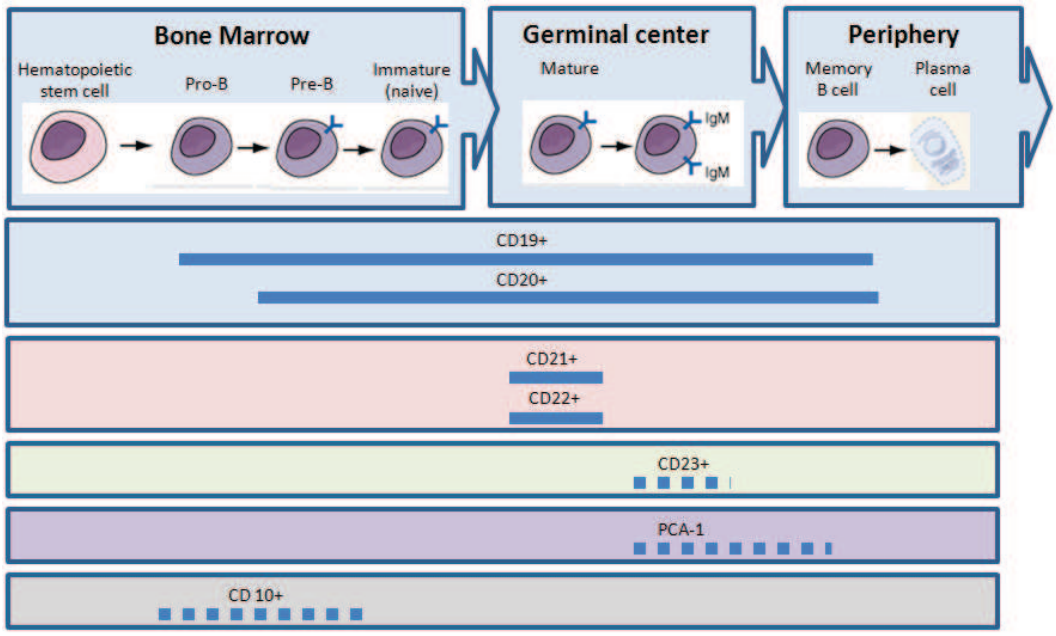

Fig. 5 Five major subgroups of human B cell antigens during B cell differentiation.[37-47]

1.2.1.1 The human CD 20 antigen

The human CD 20 molecule is expressed at early stage of B-cell development and lost upon differentiation of B cells towards plasma cells.[47-49] The CD 20 antigen is a transmembrane cellular, non-glycosylated phosphoprotein of 33 to 35kDa which in some important aspects is different from other B cell antigens.

It is an ideal target for antibody therapy because CD20 is not expressed on hematopoietic stem cells thereby avoiding the risk of irreversible marrow aplasia resulting from treatment with anti-CD 20 antibodies.[50] Moreover, CD20 is not expressed on plasma cells meaning that antibody therapy not significantly decreases immunoglobulin production. Therefore, the propensity to infections expected after treatment with rituximab and other anti-CD 20 antibodies turned out to be mild and mostly easily manageable. Other advantages of targeting CD20 are that CD20 does not circulate in the plasma,[51] is not shed from the cell surface probably because it is deeply anchored in the cell membrane with a very short extracellular segment (43 residues between 3rd and 4th transmembrane region).[52] It is also not internalized into the B-cell after antigen-antibody binding and therefore appears an ideal target for the recruitment of effector cells expressing Fc gamma receptors.[53] It means that therapeutic CD 20 antibodies can be expected to durably attach to the surface of the lymphoma cell, recruit immune effector cells and mediate sustained cell kill. Figure 6 shows the structure and topology of CD20 and the epitopes recognized by monoclonal antibodies currently FDA approved.[34]

Although CD20 is the most frequently antibody targeted antigen in general, its exact function is still unknown. It has no known ligand, and CD 20 knockout mice display an almost normal phenotype.[54,55] Thus, most of our current understanding about this antigen comes from predictions based upon the gene and protein structure. In vitro studies showed a role for CD 20 as a store-operated calcium channel following ligation of the B cell receptor. It is likely to be involved in the generation and regulation of calcium flux transduced by other receptors.[56] One of the proximal effects induced following ligation of CD20 with

type I mAb is its redistribution in the plasma membrane. It leads to the formation of lipid rafts which are recognized as signaling platforms.[56,57]

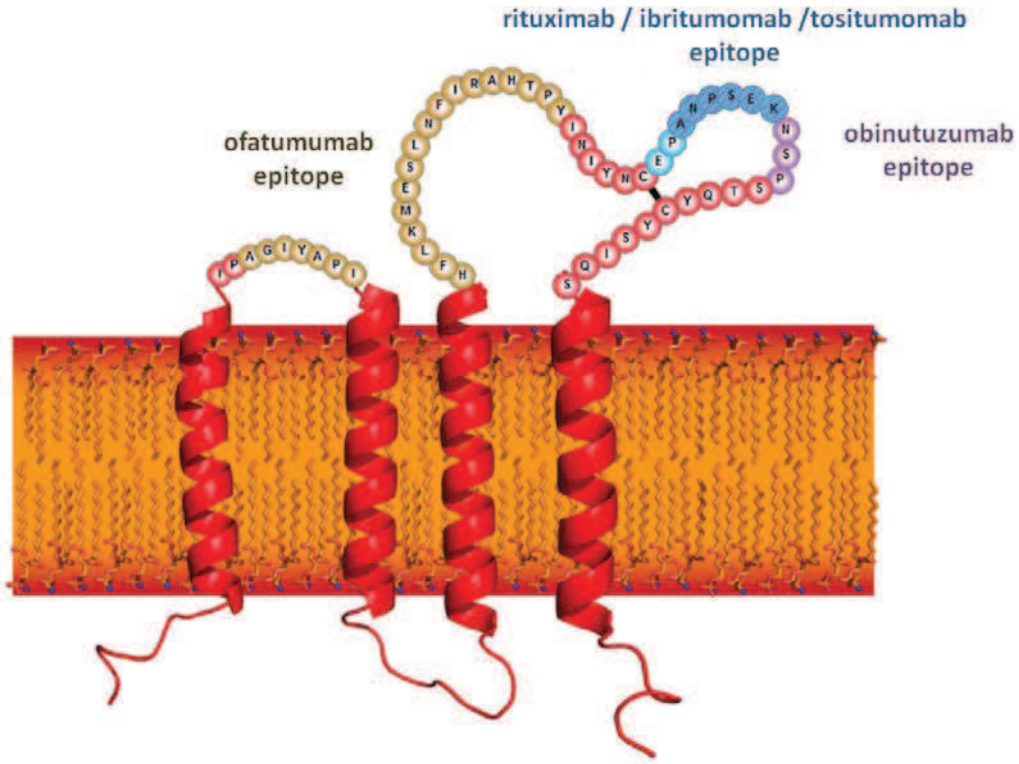

Fig. 6 The structure and topology of CD20 and the epitopes recognized by FDA approved mAb.[34]

1.2.1.2 The human CD 37 antigen

CD37 antigen is a protein which in humans is encoded by the *CD37* gene. The protein encoded is a member of the transmembrane 4 superfamily, also known as the tetraspanin family, and is highly expressed on B cells during the pre-B to peripheral mature B-cell stages. Low level expression has been reported on T-cells, granulocytes and monocytes. CD 37 is not expressed on early progenitor cells or terminally differentiated plasma cells. Strong and homogeneous CD 37

expression has been demonstrated on the surface of B-cell lymphomas and leukemias. Most members of the tetraspanin family are cell-surface proteins characterized by the presence of four hydrophobic domains (Figure 7).[58,59]

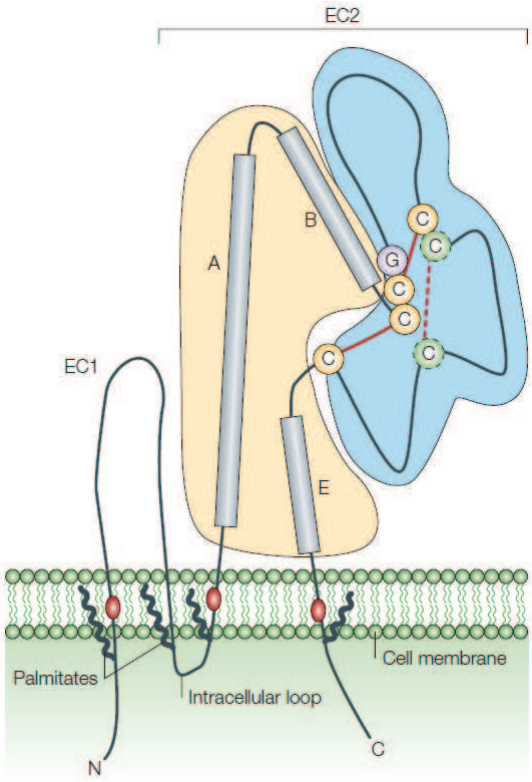

Fig. 7 Schematic structure of tetraspanins.[59]

They mediate signal transduction events playing a role in the regulation of cell development, activation, growth and motility of cells. The protein is a cell surface glycoprotein known to complex with integrins and other transmembrane 4 superfamily proteins. It may play a role in T-cell/ B-cell interactions. Alternate splicing results in multiple transcript variants encoding different isoforms. Although the exact physiological role of CD37 is unclear, it may play a role in immune cell proliferation and influences signaling via the Akt pathway.[60-62] The tetraspanin CD37 is predominantly expressed on mature B cells, with highest expression levels on peripheral blood B cells and lower levels on plasma cells. Importantly, strong and homogeneous CD 37 expression has been detected on the surface of B-cell leukemia and lymphoma cells.[58,63-66] Deckert J et al. [67]

compared the CD 37 expression with CD 20 expression in tissue biopsies from patients with various B-cell malignancies by immunohistochemistry (IHC). (Table 3) Strong positive staining for CD 37 was seen in the vast majority of DLBCL, FL, MCL and some CLL samples tested. Overall, the biopsy samples showed a similar prevalence for CD 37 and CD 20 staining. This profile suggests that CD37 represents a promising therapeutic target for B-cell malignancies.

Table 3 CD 37 and CD 20 staining of paraffin-embedded lymphoma tissue cores by immunohistochemistry (IHC).[67]

		Percentage of positive cores		
Indication	**Histological subtype**	**CD37**	**CD20**	**n**
B-cell NHL	Follicular lymphoma	100	100	3
	DLBCL	93	93	14
	Mantle cell lymphoma	90	100	29
	MALT lymphoma	100	100	3
	Unspecified NHL	95	95	22
CLL		100	100	3
Multiple myeloma		0	0	10

IHC studies were conducted with an anti-CD37 antibody (clone CT1, 4 mg/mL; Novocastra) and an anti-CD20 antibody (clone L26, 0.5 µg/mL; DAKO) compared with isotype controls (Beckman Coulter). Staining results were scored by a board certified pathologist. Staining intensity scoring: 0 5 negative; 1 5 weak; 2 5 moderate; and 3 5 strong. Positive staining was defined as a score of ≥2.

1.2.2 Anti CD20 antibodies

Because of the favorable characteristics of CD20, several anti-CD 20 mAbs were developed. Rituximab, ofatumumab, and recently also obinutuzumab as well as the radiolabelled antibodies ibritumomab tiuxetan and tositumomab have been approved by FDA for certain indications. Novel anti-CD20 mAbs are currently under development with the aim of improving function and treatment results in lymphoma. The development of anti-CD 20 mAbs resulted in three generations of antibodies based on the human/mouse chimeric mAbs, like rituximab, ^{90}Y-labelled ibritumomab tiuxetan and ^{131}I-labelled tositumomab. Second generation antibodies are either humanized or fully human, like ofatumumab; third generation antibodies display further modifications of antibody structure e.g.

mutation or a-fucosylation of the Fc domain for enhanced FcR binding, like obinutuzumab (Figure 8).[68]

Anti-CD20 mAbs can also be classified as type I or type II antibodies depending on their biological activity and mechanism of action in vitro. The clinically used anti-CD20 antibodies, rituximab and ofatumumab, are categorized as type I. These antibodies are characterized by their ability to induce a translocation of CD20 into large lipid microdomains or 'lipid rafts' within the plasma membrane upon binding.[69-71] This clustering process enhances the recruitment and activation of complement, and hence Type I antibodies exert potent complement-dependent cytotoxicity (CDC)[69,72] but are weak inducers of apoptosis. Lipid rafts are sphingolipid and cholesterol enriched- microdomains of the plasma membrane which function as organizing platforms for cell signaling and receptor trafficking. The ability of anti-CD20 antibodies to induce CDC correlates with their ability to translocate CD20 into lipid rafts, whereas induction of apoptosis by anti-CD20 antibodies is independent of CD20 segregation into lipid rafts. The contribution of complement activation by anti-CD 20 antibodies to the depletion of B cells in vivo remains unclear.[73,74]

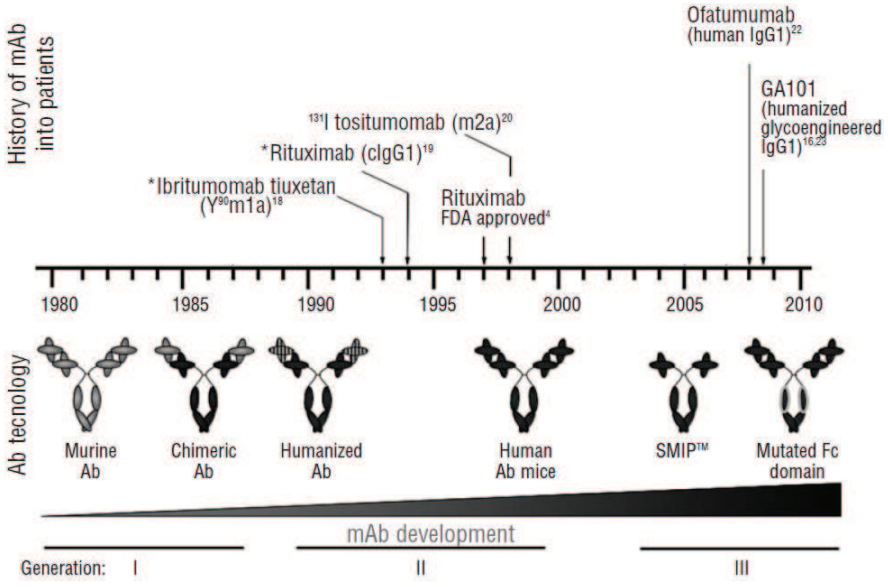

Fig. 8 The development of anti-B-cell antibodies.[68]

Another characteristic feature of type I antibodies is that B cells bind twice as many type I antibodies compared with type II antibodies[75,76] most likely due to different binding geometry. The biological significance of this phenomenon is unknown. It has been hypothesized that the 2:1 stochiometry could be explained by the binding of type I antibodies between two CD20 tetramers thereby crosslinking tetramers with two antibodies bound per tetramer whereas type II antibodies may bind within a tetramer, resulting in only one antibody bound per CD20 tetramer(Figure 9).[34,77,78]

The two clinically available type II antibodies are tositumomab and obinutuzumab (GA 101). Type II antibodies do not induce accumulation of CD20 in insoluble lipid rafts hence showing relatively little CDC activity[72,75] but more effectively inducing direct apoptosis. Type II antibodies are more potent than type I antibodies in inducing homotypic adhesion and direct cell death.[79] Although this form of cell death was initially described as apoptosis, recent studies have demonstrated that it is a non-apoptotic form of direct cell death following an actin-dependent enhancement of cell-to-cell contact, rupturing of lysosomes within the cytoplasm[80-82] and the generation of reactive oxygen species. The classical hallmarks of apoptosis such as DNA laddering or caspase dependence are not observed.[83]

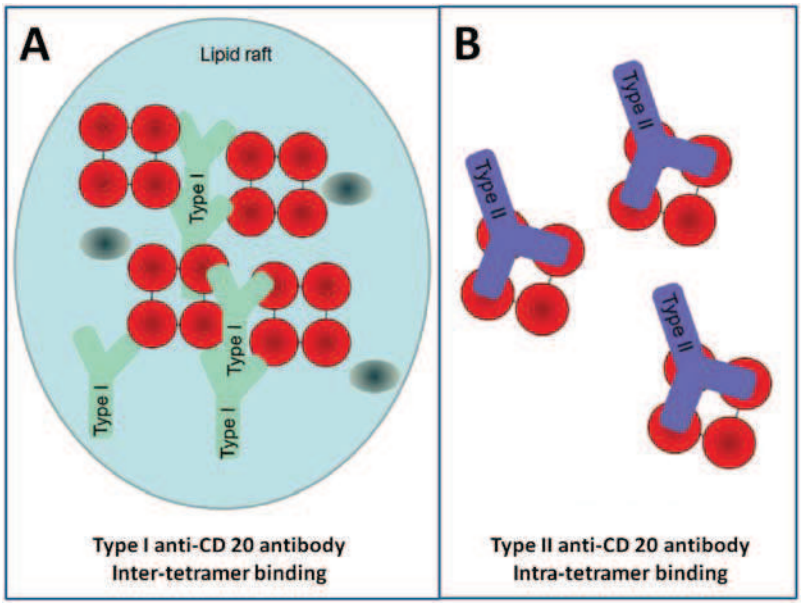

Fig. 9 Different binding ways of type I and type II anti-CD 20 antibodies.[34]

1.2.3 Mechanisms of action of CD20 antibodies

The success of rituximab has led to a general interest in the mechanism of action of anti-CD 20 mAbs. Anti-CD 20 mAbs can induce the killing of malignant B cell via various mechanisms, such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or programmed cell death (PCD). Although we nowadays have a better understanding of these mechanisms, it is still unclear which of these mechanisms is the most important for the efficacy of different anti-CD 20 mAbs action in vivo.

1.2.3.1 Antibody-dependent cellular cytotoxicity (ADCC)

Antibody-dependent cellular cytotoxicity (ADCC) is a cell-mediated innate immunity mechanism whereby an effector cell of the immune system actively lyses an opsonized target cell (malignant cell) that has been recognized by a specific antibody (Figure 10).[84,85] Effector cells that mediate ADCC include natural killer (NK) cells, monocytes, macrophages, neutrophils, eosinophils and dendritic cells. The cytotoxicity is triggered through interaction of target-bound antibodies and their Fc receptors, e.g. IgG classes to FcγRs to the effector cell. This binding leads to the release of immunostimulatory-simulants such as cytokines and chemokines as well as the release of cytotoxic granules.[86] The perforins contained in granules lead to the formation of a pore in the cell membrane of the target cell through which granzyme penetrates. Granzymes are proteases that induce apoptosis primarily in the target cells.[87] Figure 10 shows that the recognition of opsonized malignant cells by FcγRs expressed on the effector cells initiates antibody-dependent cellular cytotoxicity.

FcγRs are surface receptors for IgG and are broadly expressed on leukocytes with varying affinity to different IgG subclasses.[88] Cellular activation upon cross-linking of the receptors by IgG-IC (immune complex) is induced by the immunoreceptor tyrosin-based activation motifs (ITAM) that are located on the intracellular part of the FcRγ-chain. Immune synapse formation (ISF) may play a central role in the integration of complex receptor information.[89] (see below) Different FcγRs have different affinity to IgG subclasses. In humans, for instance, both IgG1 and IgG3 interact relatively well with all FcγRs. Most of the currently FDA approved anti-

CD 20 antibodies belong to IgG1 group. However, most of NK cells express the low affinity (Fcγ) receptors type IIIA (CD16). Therefore, Fc-engineered IgG antibodies either by introducing point mutations or by modifying the glycosylation profile are developed to enhance ADCC.

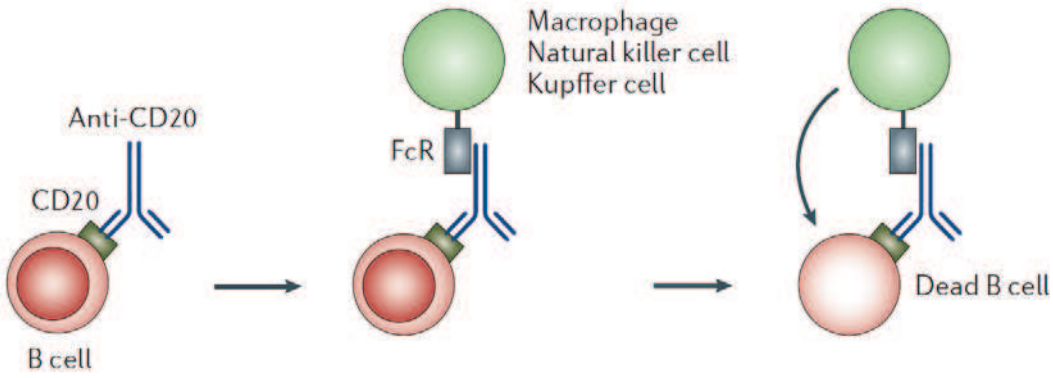

Fig. 10 Antibody-dependent cellular cytotoxicity.[85]

1.2.3.2 Complement-dependent cytotoxicity (CDC)

Another mechanism of action is complement-dependent cytotoxicity (CDC). CDC describes the activation of complement by monoclonal antibodies that initiate the complex pathway finally resulting in tumor cell lysis (Figure 11).[85] The complement system plays a key role in human health and disease due to its influence on both innate and adaptive immunity.[90]

The complement system consists of a number of small proteins synthesized by the liver circulating in the blood as a inactive precursors. When the complement cascade is triggered, proteases cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The end-result of this activation cascade is a massive amplification of the response and activation of the cell-killing membrane attack complex (MAC), which causes cell lysis.

The complement system can also be activated by an antigen-bound antibody. Early complement complexes (for example, C1 or C1q) bind to Fc fragments of the antibody (e.g. IgG or IgM) which has formed a complex with antigens. When C1q binds to IgM or IgG complexed with antigen, it activates the classical pathway of the complement system. The activation causes the release of anaphylatoxins (C3a, C4a, and C5a), and opsonins (C3b). These molecules promote phagocytosis and reinforce on the binding by complement receptors (receptors C) the activity of the effector cells. The complement cascade may lead to the formation of a MAC and thus lead directly to the lysis of the target cell.[91]

With the help of an antibody, complement is able to detect non-self pathogens or non-self cells much more specifically. For instance, type I anti-CD20 antibodies such as ofatumumab are able to induce complement activation and lyse lymphoma cells.[92,93] However, complement-dependent cytotoxicity is not always effective. Therefore, the role of CDC in vivo is still controversial.[94-96]

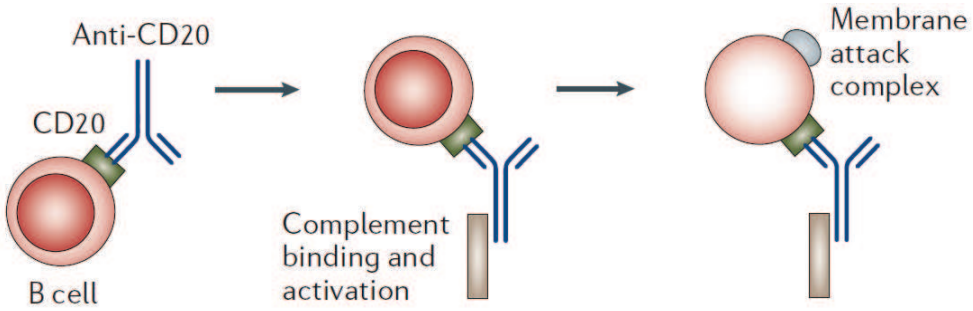

Fig. 11 Complement-dependent cellular cytotoxicity.[85]

1.2.3.3 Programmed cell death (PCD)

Binding of mAbs can induce direct effects on malignant cells, leading to transmission of intracellular signals which trigger cell cycle arrest or programmed cell death. Programmed cell death (PCD) is a physiological and highly controlled process by which unwanted or useless cells are eliminated during development and other normal biological processes (Figure 12).[85] Apoptosis and autophagy are both forms of programmed cell death. PCD also is the third important mechanism of action of anti-CD 20 antibodies. PCD can be detected in populations of cells or in individual cells. Many different methods have been devised to detect apoptosis such as the TUNEL (TdT-mediated dUTP Nick-End Labeling) analysis, ISEL (in situ end labeling), and DNA laddering analysis for the detection of fragmentation of DNA in populations of cells or in individual cells and the detection of apoptosis related proteins such p53 and Fas.

Programmed cell death induces cells to undergo numerous physiological changes including alterations in their membrane structure. Among other changes, it leads to exposure of the cells' inner phospholipids to the exterior. Apoptosis and necrosis are distinctly different processes. Fundamental differences between the two death-generating mechanisms are evident by comparing the triggers necessary to initiate these events. In contrast, necrosis is a non-physiological process that occurs as a result of infection or injury.[95] Necrosis is usually the result of an accumulation of toxic reagents within cells while apoptosis can be triggered by various environmental stimuli which lead to the activation of an endogenous endonuclease activity.[98] At the cell membrane level, disruption of internal and external membranes is a normal consequence of necrosis. Alternatively, during programmed cell death, loss of cell membrane integrity is a late event usually preceded by the destructive action of endogenous cellular enzymes.[99] The ability to exclude viable dyes such as PI is a property of cells that have an intact plasma membrane. Cells in the early phases of apoptosis fall into this category. On the other hand, necrotic cells have lost membrane integrity and therefore, easily stain with PI.

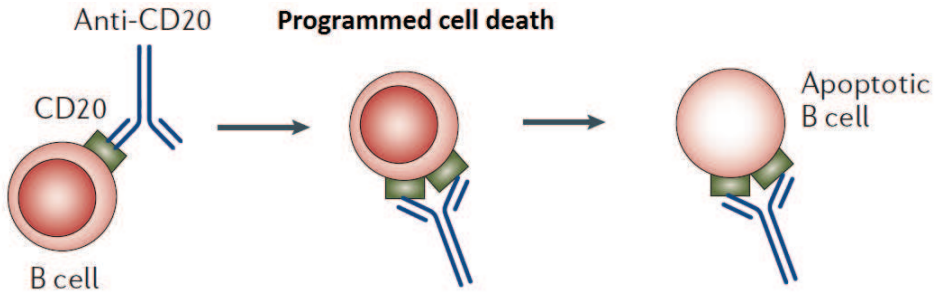

Fig. 12 Programmed cell death.[85]

One of the earlier events of PCD includes translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the cell surface. Annexin V, a Ca^{2+}-dependent phospholipid-binding protein, has high selective affinity for negatively charged phospholipids. Under defined salt and calcium concentrations, Annexin V preferably binds to PS as compared to other phospholipid species and can therefore be used for the detection of PS on cell surfaces using flow cytometry. The binding of Annexin V to phospholipids is very rapid, extremely dependent on the presence of Ca^{2+}. Combining the staining properties of Annexin V and propidium iodide (PI) can be used to distinguish early stages of apoptosis from later stages of apoptosis or necrosis. Cells staining positive for Annexin V but negative for PI indicate an early stage of apoptosis. In contrast, cells undergoing necrosis or late stage apoptosis stain with both Annexin V and with PI.[99]

1.2.3.4 Immune Synapse Formation (ISF)

The immune synapse (IS) was originally defined in the late 1990s as an adaptive immune response which is initiated by the interaction of T-cell antigen receptors with major histocompatibility complex (MHC) molecule-peptide complexes in the nanometer scale gap between a T cell and an antigen-presenting cell (APC).[89,100,101]

Clustering in the synapse is a dynamic process: IS formation depends on ligand binding, proximal signaling by kinase, calcium flux, cytoskeletal reorganization and costimulation. Imaging studies of the IS have revealed a remarkable organization that may help account for the longevity and specificity of signaling. The mature IS has been defined by a bull's eye arrangement of supramolecular activation clusters (SMACs) that form within a few minutes of T-cell-APC contact. SMACs can be detected by fluorescence microscopy and appear as increased densities of specific molecules. Actin filaments are crucial for microdomain formation and centripetal transport. Therefore, actin filaments can transport certain microdomain (especially TCR and MHC microdomain) toward the actin-sparse cell center and form the cSMAC (central SMAC) or bull's eye. The center of the bull's eye or cSMAC is enriched for TCR and MHC complexes which can trigger TCR signaling and further release cytotoxin to lyse the APC.[102] The ring of the bull's eye or pSMAC (peripheral SMAC) contains the integrin lymphocyte function-associated antigen-1 (LFA-1) and its major counterreceptor intercellular adhesion molecular-1 (ICAM-1). The formation of IS requires an intact T cell cytoskeleton and begins as an inverted structure with a central adhesion cluster surrounded by a ring of engaged TCR.[103] Figure 13 shows a model for the segregation of TCR and adhesion molecules during the immune synapse formation.[104]

Subsequent studies extended the observation of ISF and identified immune synapse formation between different types of immune cells. Natural killer cells are the cytolytic effector cells of the innate immune system and also can form immune synapses like initially shown for helper T cells. On the surface of NK cells,

CD16 behaves as a non-MHC specific activating receptor. When it recognizes antibody-coated targets, they can be killed by ADCC.

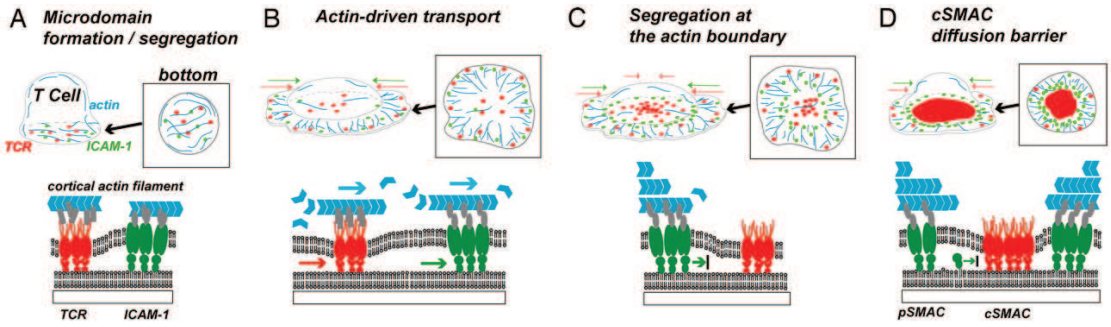

Fig. 13 Model for the segregation of TCR and adhesion molecules during the immune synapse formation.[104]

1.2.4 Antibodies used in the study

1.2.4.1 Rituximab

Rituximab, a chimeric $IgG1_K$ anti-CD 20 monoclonal antibody, is the standard anti-CD 20 antibody licensed for treatment of B-cell lymphoma. It contains the complement-determining regions of the murine anti-CD 20 antibody 2B8 in conjuction with human kappa and IgG1 heavy-chain constant region sequences. The vector was cloned into Chinese hamster ovarian cells as the production source of immunoglobulin. Rituximab is composed of two heavy chains of 451 amino acid and two light chain of 213 amino acids with a molecular weight of 145KDa. It binds to the large extracellular loop at residues 170-173 and has a binding affinity for the CD 20 antigen of approximately 8.0 nM, which is similar to the parent murine antibody, 2B8. Rituximab is classified as type I antibody and belongs to the first generation of anti-CD 20 mAbs.[34,105]

1.2.4.2 Ofatumumab

Ofatumumab, a human $IgG1_K$ anti-CD 20 monoclonal antibody, is approved for the treatment of chronic lymphocytic leukemia (CLL). It was generated in transgenic mice. The heavy and light chain genes from a human anti-CD 20 cell line, 2F2, were transfected into a murine myeloma cell line (NS/O) for the production of ofatumumab. It recognizes an epitope different from that of rituximab. Peptide scanning and mutagenesis studies have revealed that ofatumumab recognizes a completely novel epitope located N-terminally of the motif (the other side of large extracellular loop), also including the small extracellular loop of CD 20 (see Fig. 6). Ofatumumab dissociates more slowly from the cell surface than rituximab. Ofatumumab is also classified as type I antibody and belongs to the second generation of anti-CD 20 mAbs.[34,105-107]

1.2.4.3 Obinutuzumab

Obinutuzumab, a glycoengineered, humanized $IgG1_K$ anti-CD 20 monoclonal antibody, was recently approved for the treatment of patients with previously untreated chronic lymphocytic leukemia (CLL). It is a glycoengineered CD 20 antibody derived from the murine Bly-1 antibody. It has been glyco-engineered to decrease fucosylation of carbonhydrate attached to Asn-297 glycosylation site of Fc region which substantially enhances the affinity of this antibody for different variants of FcγRIIIa. The epitopes of obinutuzumab and rituximab overlap. The obinutuzumab epitope is shifted toward the C-terminus of CD 20 antigen. Unlike ofatumumab, obinutuzumab does not appear to directly interact with the small extracellular loop of CD 20 or the region preceding the larger loop. Obinutuzumab is classified as type II antibody and categorized as the third generation of anti-CD 20 mAbs.[34,105,108]

1.2.4.4 BI 836826

BI 836826, a chimeric IgG1 anti-CD 37 monoclonal antibody, is currently in phase I clinical studies in order to evaluate the safety profile and the optimal dose of this antibody in man. BI 836826 was expressed in DHFR-deficient Chinese hamster ovary (CHO) DG44 suspension cells. This antibody is constructed by a

chimerized high-affinity mouse antibody to CD 37 which has been engineered at the CH2 domain to improve binding to human Fc gamma receptors.[66] Anti-CD 37 antibodies unlike the CD 20 antibodies are not classified into different types or generations.

1.3 Experimental questions

Although rituximab is clinically successful in treating B-cell lymphoma, varying percentages of patients, especially those with poor prognostic factor, show no response or a poor response to rituximab. Therefore, the development of new antibodies targeting malignant B cells is urgently needed. In this work, we established different functional assays to evaluate different anti-B-cell antibodies used to treat B-cell malignancies.

a. We compared the efficacy of various anti-CD 20 antibodies by using different cell lines, representing GCB-type and ABC-type of diffuse large B-cell lymphoma, and primary lymphoma cells (focused on FL, MCL, and DLBCL).

b. We compared the efficacy of various anti-CD 20 antibodies with different mechanism of action.

c. We compared the newly developed anti-CD 37 antibody with the commercially available anti-CD 20 antibodies.

1.4 Additional information on treatment of T- and B-cell lymphoma

1.4.1 Allogeneic Transplantation in T-Cell Lymphomas[109]

Mature T- and NK-cell neoplasms represent an extremely heterogeneous group of diseases with distinct epidemiologic, pathophysiological, immunophenotypic, molecular and clinical features. First-line therapy may differ depending on the T-cell subtype but in many instances consists of CHOP or CHOP plus etoposide (CHOEP). Unfortunately, treatment results are often not satisfactory and especially patients with advanced disease and a high International Prognostic Index (IPI), progress during therapy or relapse frequently. Survival of patients with relapsed T-cell lymphoma who do not receive an autologous or allogeneic transplant is dismal; therefore, number of alloSCT in T-cell lymphoma have steadily increased during recent years. Most papers on alloSCT focus on the three most frequent sub-entities: ALCL, anaplastic lymphoma kinase (ALK)-negative, angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). With few exceptions ALK-positive ALCL has been excluded from recent reports on alloSCT because prognosis of these patients is relatively good with conventional chemotherapy and the results of high-dose therapy and autologous stem cell transplantation (ASCT) seem reasonable in patients with relapsed ALCL. Other sub-entities like adult T-cell leukemia/ lymphoma (ATLL), NK/ T-cell lymphoma, hepatosplenic T-cell lymphoma or cutaneous T-cell lymphoproliferative disorders are so rare and/ or patient numbers reported have been so low, that reports focusing on these entities do not exist until today or suffer from very low patient numbers, making it difficult to judge the real impact of alloSCT. On the other hand, most publications on alloSCT for the more frequent T-cell lymphoma entities (ALK-negative ALCL, AITL and PTCL) are "contaminated" by cases of these very rare subentities. It is also important to note, that studies on alloSCT exclusively deal with patients transplanted for very advanced, often refractory disease.

1.4.2 Hematopoietic Stem Cell Transplantation in Patients with Lymphomatoid Granulomatosis[110]

Lymphomatoid granulomatosis (LG) is a very rare, Epstein-Barr virus (EBV)-driven B cell lymphoproliferative disorder first described as a distinct clinicopathological entity by Liebow in 1972. At diagnosis, the majority of patients are between 40 and 60 years of age with a definite male preponderance. Immunocompromised patients are more likely to develop LG, probably because cellular immunodeficiency compromises EBV elimination. Three histological grades have been described, depending on the number of EBV-positive, CD-20 positive, large atypical B cells in relation to reactive tissue. Whereas grade 1 lesions contain very few and grade 2 lesions contain only scattered EBV-positive cells, grade 3 lesions are characterized by sheets of large atypical cells showing a high propensity to transform into diffuse large B cell lymphoma. Because of lung involvement in >90% of cases, first symptoms are often cough and shortness of breath in combination with B symptoms, such as fever, night sweats, and weight loss. The skin, kidneys, liver, and the central nervous system are affected in about one third of the patients, although involvement of lymph nodes or spleen is rare. In older series, median survival of patients with LG was under 2 years. Patients with grade 1 or 2 lesions may achieve durable remissions with corticosteroids and interferon-alpha. Dunleavy et al. investigated the use of interferon-alpha in 28 patients with grade 1/2 disease and achieved a 5-year progression-free survival of 56%. In 24 patients with grade 3 disease, progression-free survival was 40% with dose-adjusted etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone ± Rituximab (DA-EPOCH±R) with a median follow up of 28 months. Despite a number of case reports, there are no reliable data available if aggressive first-line therapy, including combination chemotherapy with or without rituximab, can improve outcome of patients with advanced disease as recently reviewed. Patients with relapsed disease often demonstrate higher histologic grades and carry a very bad prognosis with no curative approach having been described. Because case reports of high-dose therapy followed by autologous stem cell transplantation (HDT/ASCT) or allogeneic stem cell transplantation (alloSCT) indicated that patients with LG might benefit from these procedures, we aimed to collect all cases of autologous and allogeneic transplantation reported to the EBMT to gain better insight into the therapeutic impact of transplantation in this rare disease.

1.4.3 Risk of CNS Recurrence and Role of Prophylaxis in Diffuse Large B-Cell Lymphoma[111]

Current practice how to estimate an individual patient`s risk of CNS disease (defined as progression during or recurrence after first-line treatment in the cerebrospinal fluid or the brain parenchyma) is largely based on early reports from single institutions or cooperative groups. These series do no longer reflect diagnostic and therapeutic standards and therefore are of limited value today. In the last decade, a number of investigators did post hoc analyses of CNS recurrences occurring in patients with DLBCL who participated in prospective randomized studies or performed analyses on other larger, more or less well-defined cohorts of patients with aggressive B-cell lymphoma, mostly DLBCL. The latter studies mostly include patients treated with state-of-the-art chemoimmunotherapy (R-CHOP or variants) or compare the incidence and type of CNS disease in patients treated with R-CHOP or CHOP. Before summarizing and discussing the major findings of these studies it is important to note that all studies featured varying definitions of patients considered high risk for CNS disease, stipulated different imaging technology and methods how to diagnose involvement of the cerebrospinal fluid (CSF), and gave differing recommendations for prophylaxis of CNS disease. While some of the recently published studies did not ask for any diagnostic and prophylactic measures or left the decision at the discretion of the individual investigator other protocols precisely describe which patients should receive which type of prophylaxis. These major inter-study variations will have influenced the percentages of patients diagnosed with CNS relapse, the identification of risk factors for CNS disease, and the answer to the question if intrathecal (i.th.) prophylaxis is effective or not.

This review is restricted to risk factor analyses and prophylactic strategies reported for patients with DLBCL treated with R-CHOP and its variants. Patients with rare subtypes of aggressive B-cell lymphoma or patients who are HIV-positive may have higher incidences of CNS relapse. Because data are even more scarce than in classical DLBCL these cases are not further discussed.

2 Materials and Methods

2.1 Materials and preparation

2.1.1 Cell lines

Human lymphoma cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C in medium (Table 4) supplemented with 2 mM glutamine and 1% penicillin-streptomycin (Sigma-Aldrich GmbH, Munich, Germany)

Table 4 Cell lines used in study

Cell line	Cell type	Description	Medium
SU-DHL-4	Human aggressive B cell lymphoma*	GCB-like subtype of diffuse large B-cell lymphoma	RPMI + 10% FCS
Ramos	Burkitt lymphoma*	-	RPMI + 10% FCS
Karpas 422	Human aggressive B cell lymphoma *	GCB-like subtype of diffuse large B-cell lymphoma	RPMI + 10% FCS
OCI-Ly-3	Human diffuse large B-Cell lymphoma**	ABC-like subtype of diffuse large B-cell lymphoma	IMDM + 20% HS + 55µM ß-mercaptoethanol
OCI-Ly-10	Human diffuse large B-Cell lymphoma**	ABC-like subtype of diffuse large B-cell lymphoma	IMDM + 20% HS + 55µM ß-mercaptoethanol

GCB-like: germinal center B-cell like lymphoma subtype; ABC-like: activated B-cell like lymphoma subtype; FCS: fetal calf serum; HS: human serum; IMDM: Iscove's modified Dulbecco's medium; RPMI: Roswell Park Memorial Institute medium
*gift from G. Wulf, University Medicine Göttingen, Göttingen, Germany
**gift from A. Rosenwald, University of Würzburg, Würzburg, Germany

2.1.2 Cell Preparation from Lymphoid Tissues

Lymphoid tissues were obtained from the *Department of Hematology, Oncology, and Stem Cell Transplantation at the Asklepios Hospital St. Georg, Hamburg*. Diagnoses were rendered by the *Institute of Hematopathology, Hamburg*, the *Institute of Pathology, University of Würzburg* or the *Institute of Pathology, Section of Hematopathology, University Hospital of Kiel*. Lymph node material

was obtained from patients with FL, MCL and DLBCL at the time of diagnosis or at relapse. A single cell suspension was obtained by mechanical disruption of lymphoid tissue. Cells were collected in culture media and passed through a cell strainer to eliminate debris and clumps. Cell suspensions were collected in a 50 mL conical centrifuge tube and washed with culture media. Normal and malignant B cells were positively selected using mouse CD19 microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach Germany). The purity of the isolated B cells (higher than 90%) was determined by flow cytometry using the anti-CD 20 antibody-PE (BD Biosciences, Heidelberg, Germany). Cells were cryopreserved at -80°C.

2.1.3 Isolation of lymphocytes and granulocytes from peripheral blood

Peripheral blood from healthy volunteers and patients with lymphoma was obtained from the *Department of Hematology, Oncology, and Stem Cell Transplantation in Asklepios Hospital St. Georg, Hamburg*, and collected into heparinized tubes. All patients gave written informed consent to draw additional blood sample at the time they had routine blood collections. Patients' or healthy donors' mononuclear cells were separated by Ficoll-Paque plus (GE healthcare Europe GmbH, Freiburg, Germany) density gradient centrifugation. CD56+ cells were positively selected using the CD56 MultiSort Kit and MACS separation columns (Miltenyi Biotec, GmbH, Bergisch Gladbach Germany). The purity of the isolated CD56+ cells (higher than 95%) was determined with flow cytometry (BD FACS ARIA III; BD, New Jersey, USA).

2.1.4 Serum and plasma preparation

Serum and plasma were separately collected into S-Monovette® tubes or heparinized tubes and centrifuged (3,000 rpm and 1,100 rpm, respectively) at room temperature for 10 minutes (Sarstedt AG & Co. Nümbrecht, Germany). Serum and plasma were cryopreserved at -20°C and -80°C until next measurements.

2.1.5 Antibodies and reagents

2.1.5.1 Monoclonal antibodies

2.1.5.1.1 Rituximab

Rituximab (MabThera®, Roche Pharma AG) was purchased from the hospital pharmacy at a concentration of 10 mg/mL.

2.1.5.1.2 Obinutuzumab

Obinutuzumab (Gazyva®, Roche Pharma AG) was provided by Roche Pharma AG at a concentration of 10.5 mg/mL.

2.1.5.1.3 Ofatumumab

Ofatumumab (Arzerra®, GlaxoSmithKline GmbH & Co. KG) was purchased from the hospital pharmacy at a concentration of 20 mg/mL.

2.1.5.1.4 BI 836826

BI 836826 was provided by Boehringer Ingelheim GmbH. The antibody was used at a concentration of 10 mg/mL.

2.1.5.2 Antibodies used for in vitro labeling of cells in FACS analysis

Antibody	Clone	Format	Company
CD3	UCHT1	PE-Cy7	BD Biosciences
CD16	3G8	FITC	BD Biosciences
CD20	2H7	PE	BD Biosciences
CD56	B195	PE	BD Biosciences
CD107a	H4A3	APC	BD Biosciences
Annexin		FITC	BD Biosciences

2.2 Methods

2.2.1 Immune synapse formation (ISF)

2.2.1.1 Immunofluorescence sample preparation

B cells (target cells) were labeled by CellTracker™ Blue CMAC (Life Technologies GmbH, Darmstadt, Germany). Equal amounts of target cells (at a concentration of $1x10^6$ cells/mL) and CD56+ PBMCs (effector cells) were incubated together with different monoclonal antibodies at 10 µg/mL for 15 minutes. After fixation, coverslips were permeabilized in 0.3% Triton-X-100 for 5 minutes. Blocking and washing was performed in PBS / 0.05% saponin / 0.25% fish skin gelatin. F-actin was stained with rhodamine phalloidin (Life Technologies GmbH, Darmstadt, Germany). After labeling, specimens were mounted on Prolong Gold anti-fade reagent (Life Technologies GmbH, Darmstadt, Germany). PBMCs stimulated by IL-2 were used as positive control.

2.2.1.2 Confocal microscopy image acquisition

2.2.1.2.1 General settings

Analyses and medial optical section images were acquired on a confocal laser scanning microscope (Leica TCS SP5 II, Wetzlar, Germany) by means of a 63x NA 1.4 oil objective lens. Dual color fluorescence (CellTracker™ Blue CMAC and rhodamine phalloidin) was excited by solid state lasers at 405 nm and 561 nm, respectively. Fluorescence was captured with novel hybrid detectors HyD (for more information please go to http://www.leica-microsystems.com/products /confocal-microscopes/leica-tcs-sp8-configurable confocal/details/product/leica-hyd/) with emission gates set to 417-542 nm and 569-702 nm, respectively.

2.2.1.2.2 Quantification acquisition settings

3D image stacks were acquired using high resolution (63X NA=1.4 objective lens) confocal laser scan microscopy to visualize and quantify actin aggregation and relocation. The area was scanned with a resonant scanner at 8000Hz, 6 to 8-fold frame averaging, pinhole set to 0.88 airy unit, 1024x1024 pixels, and 1.7-fold

zoom, resulting in a pixel size of ca. 230 nm. Multi-point z-stack acquisition was performed with an automated XY-stage and piezo-driven focus drive with z-spacing set to 0.3 µm for 30-60 slices depending on cellular structure after aggregation e.g. single or multi-layer; the distance between two adjacent images was kept at 300 nm. Z stacks were used to collect the entire synapse binding volume in order to have a precise and less user-biased evaluation of bond strength, size, and morphology. The detectors gain was kept at the same value for all measurements. For the red channel, all acquisitions were kept under the saturation level to allow quantitative analyses. The laser excitation was kept constant in all experiments; exceptions were made for few datasets to keep the signal under saturation. The slightly different excitation values were recorded to rescale quantification accordingly.

2.2.1.2.3 Higher quality image acquisition settings

A galvo-scanner was used at 400 Hz and 3-fold frame averaging. Multi frame acquisition (tiled scanning) was employed in order to produce images on areas bigger than the field of view. Pinhole was set to 0.88 airy unit, 512x512 pixels, and 1.25-fold zoom, resulting in a pixel size of ca. 440 nm and all the confocal images were having 8 bit pixel depth (255 gray scales). For each tile z-stack acquisition was performed with an automated piezo-driven focus drive with z-spacing set to 0.3 µm for 30-60 slices, depending on the sample thickness. Fluorescence was acquired sequentially to prevent passage of fluorescence from other channels. Detectors were set to detect an optimal signal below the saturation limits. Image stacks were visualized as maximum intensity projections with the supplied Bitplane Imaris 6 software (Zürich, Switzerland).

2.2.1.3 Quantitative image analysis of conjugate formation and F-actin polymerization

Conjugation intensity was scored by visual counting under 3D image stacks acquired by a confocal microscope with random selection of at least 30 contacts of target and effector cells in one area. The background was determined from the image histogram and subtracted from the images. In areas of cell to cell

contact a region of interest was selected and the automated segmentation algorithm applied. The setting of the segmentation was kept constant to ensure that there was no user bias. From such segmented volumes, the sum of the total intensity and the volume parameters were extracted for further analysis and plotting. Polarization of proteins at the effector cell contact site was scored independently. Those conjugates showing a distinct polymerized protein band at the effector cell contact site were considered polarized. Conjugates lacking protein polymerization or showing weak protein polymerization were also included in this analysis. Statistical significance was calculated using the Student's *t*-test (two-tailed). From segmented volumes, the sum of the total intensity and the volume parameters were extracted for further analysis and plotting. These findings were verified using Bitplane Imaris 6 software (Zürich, Switzerland) to calculate the mean fluorescence intensity (MFI) at synapse of proteins to the T cell-B cell contact site.

2.2.2 Antibody-dependent cellular cytotoxicity (ADCC)

Cell lines or lymphoma cells from patients were used as target cells. Follicular lymphoma cells (FL), mantle cell lymphoma cells (MCL) and diffuse large B-cell lymphoma cells (DLBCL) were isolated from lymph nodes obtained from patients with FL, MCL, DLBCL at the time of diagnosis or at relapse. Effector cells were freshly isolated from healthy donor blood or from the blood of patients donating lymphoma cells (autologous effector cells) before chemotherapy started. Effector cells were positively selected from peripheral blood of healthy donors or patients using the CD56 MultiSort Kit and MACS separation columns. Frozen lymphoma target cells were thawed shortly before the experiment.

2.2.2.1 CD 107a degranulation assay

Antibody-dependent cellular cytotoxicity was determined by CD 107a degranulation assay. Target cells were collected, washed and re-suspended in complete RPMI 1640 medium containing 10% fetal bovine serum and 1% Penicillin/Streptomycin. Five µM CellTracker™ blue CMAC (7-amino-4-chloromethylcoumarin) (Life Technologies GmbH, Darmstadt, Germany) was

added and samples were incubated for 30 minutes at 37°C in a 5% CO_2 humidified atmosphere. The cells were then incubated with 50 µg/mL mAb (rituximab, obinutuzumab, BI 836826 or ofatumumab) for 25 minutes at 37°C. CD56 positive cells were re-suspended at a concentration of 10^6 cells/mL in complete RPMI 1640 medium. 100 µL of cell suspension were seeded into 96-well round-bottom plates. An equal volume of labeled target cells re-suspended in complete RPMI 1640 at a concentration of 10^5 cells/ml was added to the wells (effector: target ratio = 10:1). Effector cells incubated with 100 U IL-2 were used as positive controls. 2 µL of Anti-CD107a mAb was directly added to the culture wells and incubated at 37°C and 5% CO_2 humidified atmosphere. Following incubation for 1 hour, Golgi-Stop (2 µM) was added and the cells were incubated for an additional 5 hours at 37°C and in 5% CO_2 humidified atmosphere. Samples were then washed and stained with 20 µL Anti CD16-FITC, 20 µL Anti CD56-PE and 5 µL Anti CD3-PEcy7 for 20 minutes in the dark at 4°C. $2x10^4$ effector cells were used in the assay. Data acquisition and subsequent analyses were performed on BD FACSAria III using BD FACSDiva software (BD, New Jersey, USA).

2.2.2.2 Europium release assay

In order to monitor the final stage of cytoxicity induced by different antibodies, the radioactive chromium (^{51}Cr)-release assay has been used as a 'gold standard' to measure cell-mediated cytotoxicity since 1968. The major disadvantage of the ^{51}Chromium-release assay (CRA) is the necessity to use radioactivity which can be hazardous to health and is not practical and costly due to the short half-life of ^{51}Cr and requirements for radiation safety training and licensing. Therefore, we chose to use an alternative method named Europium-release assay (time-resolved fluorescence-based cytotoxicity assay) to replace the conventional ^{51}Chromium-release assay.

This assay is based on loading target cells with an acetoxymethyl ester of a fluorescence enhancing ligand (BATDA; bis (acetoxymethyl) 2,2':6',2''-terpyridine- 6,6''- dicarboxylate). After the hydrophobic ligand has penetrated

the cell membrane the ester bonds are hydrolyzed within the cell to form a hydrophilic ligand (TDA, i.e. 2,2':6',2" -terpyridine-6,6" -dicarboxylic acid), which no longer passes the membrane. After cytolysis the released ligand is introduced to a DELFIA™ europium solution to form a fluorescent chelate (EuTDA). The measured signal correlates directly with the number of lysed cells.

Target cells were labeled with 5 µL DELFIA® enhancing ligand [BATDA, bis (acetoxymethyl) 2,2':6',2''- terpyridine- 6,6''- dicarboxylate)] and free BATDA was removed by washing with PBS. The target cells were seeded in 96-well round bottom microtiter plates at a concentration of 10^4 in triplicates. Effector cells were added in a ratio of 10:1 and co-cultured for 4 hours at 37°C in a 5% CO_2 humidified atmosphere. After 4 hours the supernatants were harvested. The released Eu-TDA fluorescence was measured in a time-resolved fluorometer.

(Note: DELFIA is a registered trademark of PerkinElmer, Inc.)

Definitions of background, spontaneous release, and maximum release

Background (= media without cells):

Loaded target cells were centrifuged immediately after dilution in culture medium. 100 µL of the supernatant was pipetted into the wells and 100 µL of the cell culture medium was added.

Spontaneous release (= target cells without effector cells):

Incubate the target cells (100 µL) with 100 µL of cell culture medium instead of effector cells during the assay.

Maximum release (= lysed target cells):

Incubate the target cells (100 µL) with 100 µL of cell culture medium supplemented with 10 µL of lysis buffer.

Formulas for calculating

$$\text{Spontaneous release (\%)} = \frac{\text{Spontaneous release (counts) - background (counts)}}{\text{Maximum release (counts) - background (counts)}} \times 100$$

$$\text{Specific lysis (\%)} = \frac{\text{Experimental release (counts) - Spontaneous release (counts)}}{\text{Maximum release (counts) - Spontaneous release (counts)}} \times 100$$

Note: Probenecid is an inhibitor of the multidrug resistance transporter (MDR); it was needed for some patients' lymphoma cells to prevent spontaneous release. Probenecid was freshly prepared every day at a stock concentration of 250 mM, and used at a working concentration of 2.5 mM.

2.2.3 Complement-dependent cytotoxicity (CDC)

Complement-dependent cytotoxicity was determined by PI assay using flow cytometry. Target cells were pipetted in complete RPMI 1640 at a concentration of $2x10^5$ cells/well into round-bottom 96-well plates. 10µg/ml mAb (rituximab, obinutuzumab, BI 836826 or ofatumumab) was added. Healthy human serum or patient serum at a volume of 0.1 µL, 1 µL, 2 µL, 5 µL or 10 µL was added to the wells. After incubating the plates for 4 hours at 37°C, cells were washed with cold PBS and cells were re-suspended in 100µl 1x Annexin binding buffer. 5µL propidium iodide was added to the samples after incubation for 15 minutes at room temperature. Cell viability was detected by flow cytometry (BD FACS ARIA III; BD, New Jersey, USA).

2.2.4 Programmed cell death (PCD)

The percentage of cells undergoing apoptosis was determined by PI / Annexin V assay. Target cells were re-suspended in complete RPMI 1640 medium at a concentration of $1x10^6$ cells/mL treated with 10 µg/mL mAb (rituximab, obinutuzumab, BI 836826 or ofatumumab) for 4 hour at 37°C. After incubation

cells were washed with cold PBS and stained with 5 µL propidium iodide (PI) as well as 5 µL Annexin-V-FITC in 100 µL Annexin-V-binding buffer. The cell viability was examined by flow cytometry (BD FACS ARIA III; BD, New Jersey, USA).

2.2.5 Microscopic analysis of homotypic aggregation

Homotypic cell adhesion (HA) plays an important role in many cell-cell interactions of normal and malignant cells. Activation of lymphoma cell lines and lymphoma cells by e.g. antibodies is accompanied by the formation of autologous cell aggregates called HA.

2.2.5.1 Sample preparation

Lymphoma cell lines were allowed to settle in 8-well flat-bottomed glass µ-Slides (ibidi GmbH, Planegg-Martinsried, Germany) overnight in a humidified atmosphere with 5% CO_2 at 37 °C. A Zeiss Axiovert 200M inverted wide field microscope was used for optical live cell imaging experiments. Wide field live phase contrast imaging of cells, employing a low magnification lens i.e. 20x 0.4 NA, was used for investigating the dynamics of homotypic aggregation in the first 24 hours after the addition of different anti-CD20 mAbs (10 µg/mL). The measurement was done in a humidified atmosphere with 5% CO_2 at 37 °C. The imaging technique used for all live cell imaging experiments was phase contrast microscopy.

Image acquisition was employed to allow for a larger field of view during live acquisition. The images were captured every 5 minutes and were acquired using a CCD AxioCam HRm 1.4MP monochrome camera. The images were exported by using Zeiss Axiovision 4.8 software (ZEISS Deutschland, Oberkochen, Germany), videos were generated using Bitplane Imaris 6 software (Zürich, Switzerland).

2.2.6 CD 20 and CD37 expression levels

CD20 expression of cell lines and lymphoma cells can vary with culture conditions and also spontaneously. Whenever possible, the relative numbers of CD 20 and CD37 molecules were measured on human lymphoma cell lines and the cells from human lymphoma tissues by using PE-labeled anti-20 antibody and FITC-labeled anti-37 antibody and analyzed with flow cytometry (BD FACS ARIA III; BD, New Jersey, USA).

2.2.7 Statistical methods

Values were expressed as means ± standard deviation from at least 3 independent experiments. Statistical significance was calculated using the Student's *t*-test (two-tailed). Differences were considered significant when a $p<0.05$ was obtained.

3 Results

3.1 Immunological synapse formation (ISF)

The interface between an antigen-presenting cell or target cell and an effector T- or NK- cell is called the immunological synapse (IS). Formation of the IS requires an intact T- or NK- cell cytoskeleton and begins as an inverted structure with a central adhesion cluster surrounded by a ring of engaged receptors on T- or NK-cells. In order to study IS formation after binding of various monoclonal antibodies to NK- or T-cells dual color 3D image stacks acquired by using high resolution confocal laser scan microscopy were used.

3.1.1 Quantification of immunological synapse formation (ISF)

Immunological synapse formation occurs at the contact surface between a target cell and a T- or NK- cell. In order to observe the segregated protein clusters and to compare the intensity of ISF resulting from the use of different antibodies, a three-dimension quantification method was used. The confocal 3D sections have a thickness ranging between 10 and 30 μm depending on cellular structure after aggregation, e.g. single or multi-layer; the distance between two adjacent images was kept to be 300 nm. Fig. 14 (A) shows the F-actin re-organization which is highly correlated with the formation of the ring of the bull's eye or pSMAC (peripheral supramolecular activation cluster) in three dimension. F-actin in both target and effector cells was stained with rhodamine dye in red, targets were separately labeled with Celltracker™ blue CMAC. F-actin accumulation was shown in green and quantified by Bitplane Imaris 6 software (Zürich, Switzerland).

3.1.2

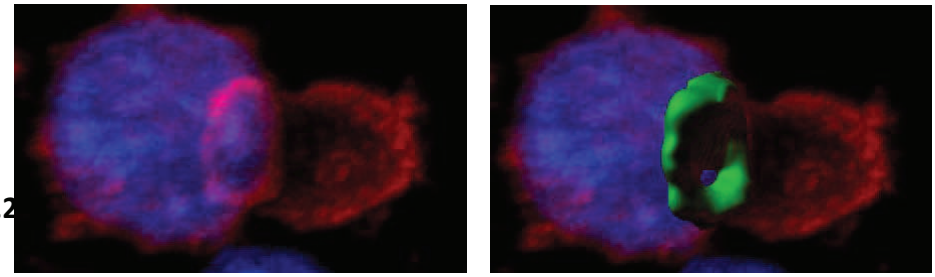

Fig. 14 Quantification of ISF (A) ring of the bull's eye or pSMAC (B) quantification area of ISF.

To compare ISF induced by different antibodies in vitro, a GCB-type lymphoma cell line (SU-DHL-4) and an ABC-type lymphoma cell line (OCI-Ly 10) were used as targets. Effector cells were incubated with rituximab-, obinutuzumab-, ofatumumab- or BI 836826- (10 μg/ml each) labeled target cells at a 1 to 1 ratio (at a concentration of $1x10^6$ cells/mL) over 15 minutes. Representative data from different cell lines are shown in Fig. 15 (A) and (B).

Su-DHL-4 cells labeled with BI 836826 showed a similar intensity of F-actin accumulation compared to targets labeled with rituximab. These two antibodies showed significantly stronger intensity of ISF than obinutuzumab, ofatumumab or no antibody ($p<0.05$). The intensity of ISF with SU-DHL-4 can be summarized as follows: BI 836826 or rituximab >> ofatumumab, obinutuzumab or no antibody.

For OCI-Ly 10, target cells labeled with BI 836826 showed a significantly stronger intensity of F-actin accumulation than target cells labeled with rituximab. Targets labeled with rituximab showed stronger intensity than obinutuzumab or ofatumumab. The intensity of ISF with OCI-Ly 10 can be summarized as follows: BI 836826 >> rituximab >> ofatumumab, obinutuzumab or no antibody.

3.1.3 ISF with patient lymphoma cells

Target cells obtained from patients with FL, MCL, or DLBCL labeled with different antibodies showed significantly different intensity of F-actin accumulation although antibodies tested showed similar patterns of intensity regardless of the lymphoma subtype. Figure 16 (A) to Figure 18 (A) give an overview on ISF in 2 dimensions when FL, MCL and DLBCL labeled with different antibodies were incubated with autologous effector cells. To capture these images, contrast was adjusted. Real images may differ due to underestimated strength of signals. The comparison among different antibodies was based on the intensity data calculated by the automated segmentation algorithm programmed by Imaris 6 software. The intensity of ISF with lymphoma cells from patients with FL, MCL

and DLBCL can be summarized as follows: BI 836826 > rituximab >> ofatumumab, obinutuzumab or no antibody.

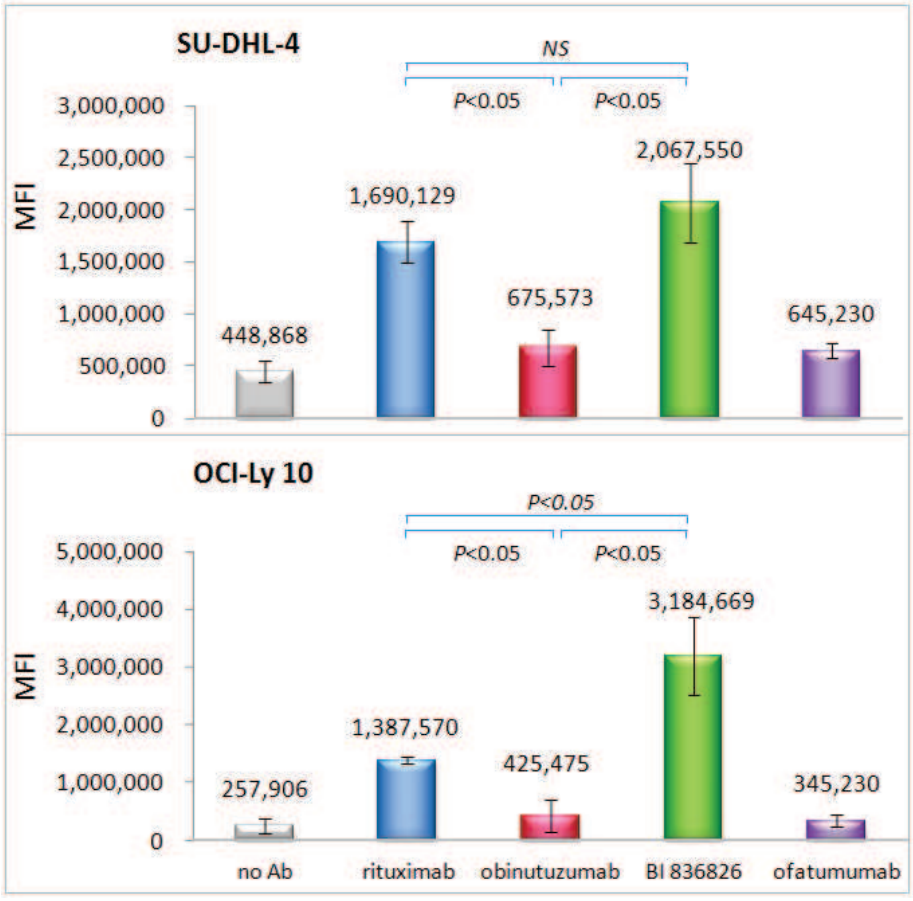

Fig. 15 Quantification of ISF using various antibodies with SU-DHL-4 (A) and OCI-Ly 10 (B) cell lines.

Fig.16 ISF using p

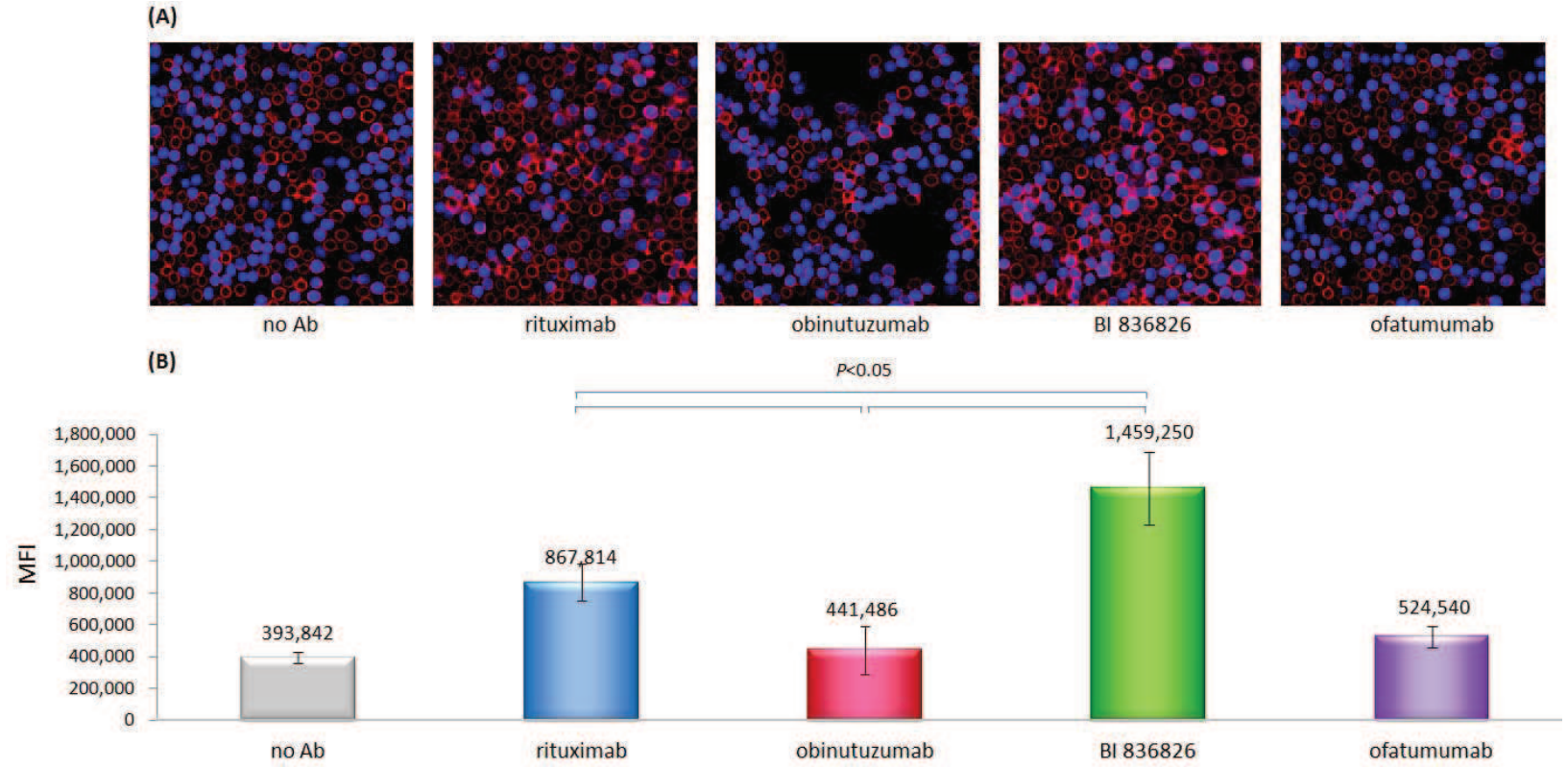

Fig. 17 ISF using patient effector cells and patient MCL cells as target labeled with various antibodies. (A) overview (B) quantification result.

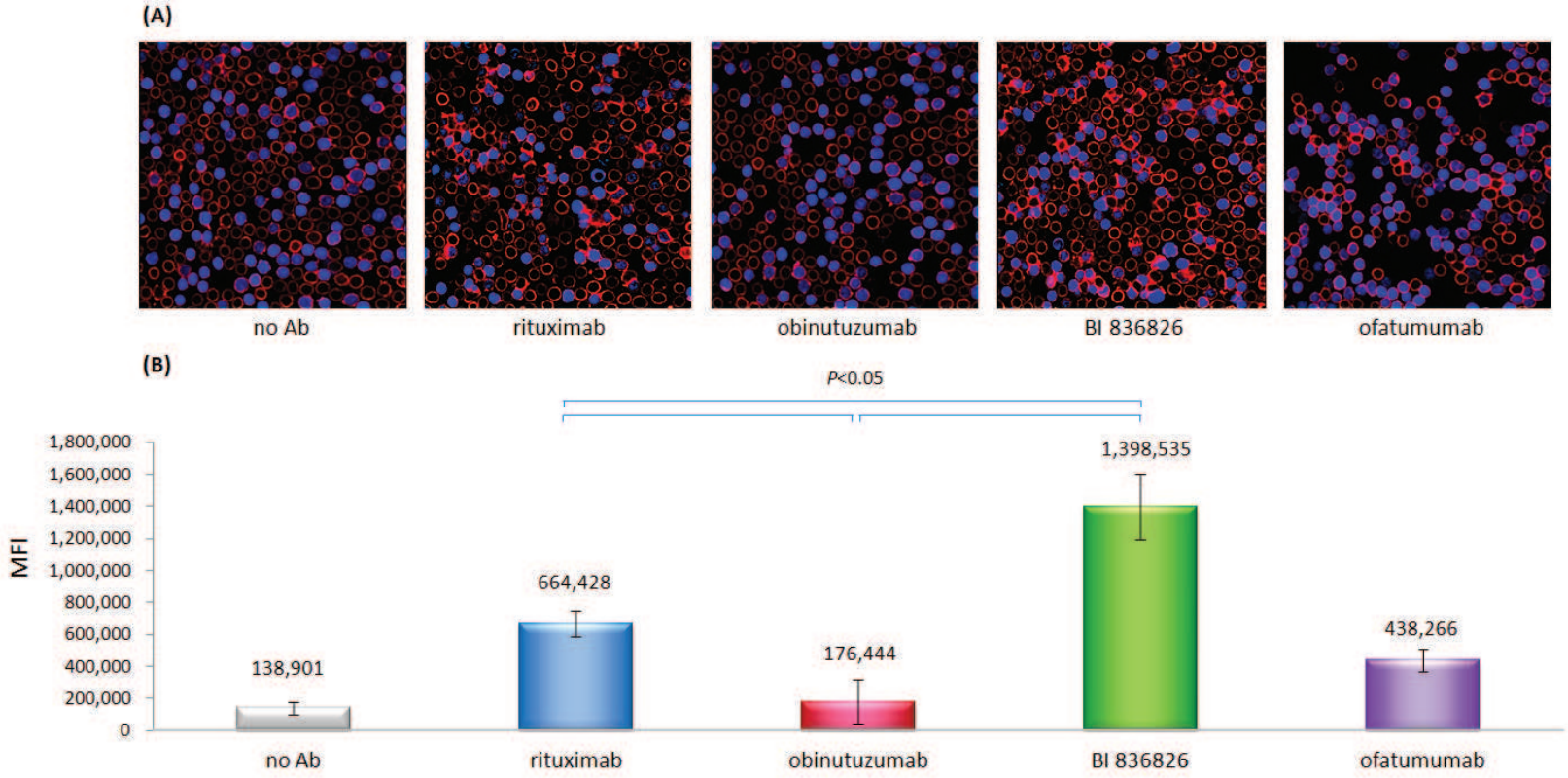

Fig. 18 ISF using patient effector cells and patient DLBCL cells as target labeled with various antibodies. (A) overview (B) quantification result.

3.2 Antibody-dependent cellular cytotoxicity (ADCC)

Antibody-dependent cellular cytotoxicity (ADCC) is thought to be one of the most important mechanisms of action of anti-B-cell antibodies in vivo. To evaluate and compare ADCC induced by different monoclonal antibodies, a multiparametric flow cytometry-based cytotoxicity assay (CD 107a degranulation assay) and a time-resolved fluorescence-based cytotoxicity assay (Europium-release assay) were used.

3.2.1 CD 107a degranulation assay

3.2.1.1 Establishment of the CD 107a degranulation assay

The CD 107a degranulation assay measures the CD 107a expression on the surface of effector cells when the effector cells recognize a target cell and start to release lytic proteins such as perforin and granzymes. This process indicates the initial phase of cytotoxic activity of natural killer cells or cytokine induced killer cells.[112,113]

3.2.1.2 Gating strategies for the flow-cytometry-based assay

Due to the limited amount of fresh cells obtained from peripheral blood or lymph nodes, an adequate gating strategy must be applied to effectively discriminate CD 107a expression on different subsets of effector or target cells. Target cells were labeled by CellTracker™ Blue CMAC while effector cells were freshly isolated from peripheral blood and positively selected by using the CD56 MultiSort Kit and MACS separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Following a 6-hour incubation period in the presence of monensin and CD107a antibody, cells were stained for CD3, CD16 and CD56 in order to identify T-cells, NK/T-cells and NK- cells.

Figure 19 shows the first step to differentiate effector from target cells labeled by CellTracker™ Blue CMAC. Using an effector / target cells ratio of 10 to 1, around 10 % of total cells being CellTracker™ Blue positive can be identified as target cells and excluded from further analysis.

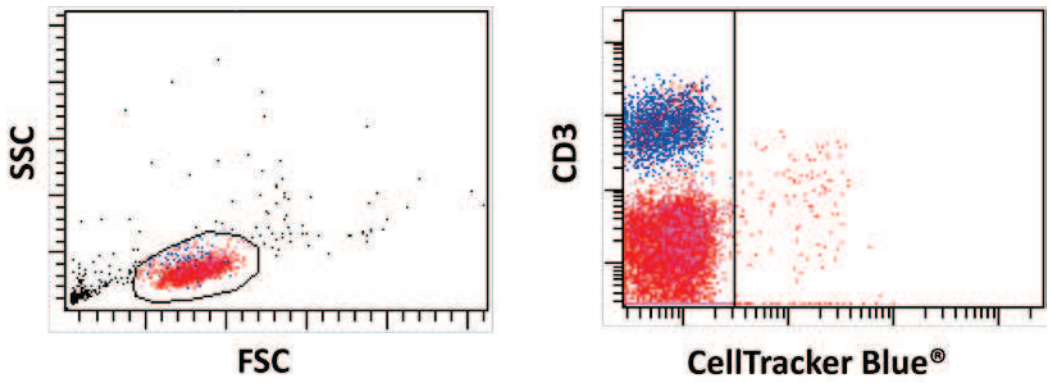

Fig. 19 Gating strategy to exclude target cells from measuring CD 107a expression of effector cells in CD 107a degranulation assay.

After excluding interference from target cells, CD56+ cells were further subdivided. NK cells (CD3-/CD16+) are discriminated from other lymphocytes, e.g. NK/ T cells or cytokine-induced killer (CIK) cells, by specific gating strategies as shown in Figure 20 A-C. Within the lymphocyte population we gated on CD 107a+/CD3- and CD107a+/CD3+ populations. Within these populations, and particularly in the CD107a+/CD3- population, we investigated the expression of CD107a for each sample following stimulation with different targets.

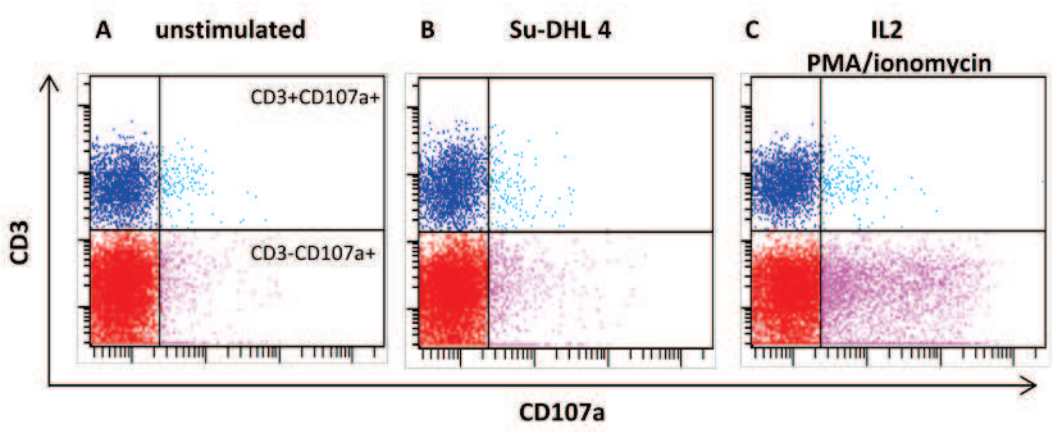

Fig. 20 CD 107a is expressed at high levels on the surface of NK cells after stimulation. Flow cytometry shows the percentage of CD3-/CD56+ cells expressing CD107a without stimulation(A), stimulation with SU-DHL-4 cells (B) or with PMA/ionomycin and interleukin-2 (C). Data from a single representative experiment are shown.

Surface expression of CD 107a was low on unstimulated NK cells (0.8±0.3%, n=10) and other effector cells (<0.1%) (Figure 20A). Following stimulation with target cells, CD 107a expression on the surface of CD3-/CD56+ NK cells increased; for example stimulation with SU-DHL 4 cells resulted in 6.1±1.5 % of NK cells expressing CD 107a (7.6- fold increase over non-stimulated cells) while CD 107a expression on the surface of other effector cells (CD3+/CD56+) increased 5.8-fold, resulting in 0.58±0.1% of effector cells expressing CD 107a (Figure 20B). Following maximal stimulation of effector cells with PMA (10 ng/mL) / ionomycin (0.25 µM) and 100 U/mL interleukin-2, CD 107a expression on the surface of NK cells was up-regulated 45.25-fold, reaching a percentage of 36.2±6.8% of NK cells and 4.1±1.1% of other effector cells (Figure 20C) (p= 0.02, Student's t test, compared to baseline CD 107a expression). The CD 3-positive cells remaining in the culture system did not significantly influence test results (either due to the low expression of CD 107a or the low absolute number of cells) and were ignored in all following measurements. Results of CD 107a expression on effector cells from 10 individuals are reported.

Taken together, data demonstrate that activation of NK cells by different target cell populations causes CD 107a degranulation which can be reliably measured by the assay implemented.

3.2.1.3 ADCC measured by the CD 107a degranulation assay with lymphoma cell lines

To compare the cytotoxic effects of different antibodies in vitro, GCB-type lymphoma cell lines (SU-DHL-4, Ramos, and Karpas 422) and ABC-type lymphoma cell lines (OCI-Ly 10 and OCI-Ly3) were used as target cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10µg/ml each) labeled target cells at a 10 to 1 ratio over 6 hours. Data obtained with different lymphoma cell lines are shown in Figures 21 and 22. Results of the CD 107a degranulation assay using different lymphoma cell lines are from 10 experiments using healthy donor effector cells.

With GCB-type lymphoma cell lines, CD 107a expression on the surface of CD3-/CD56+ NK cells incubated with target cells labeled with rituximab was significantly higher than with unlabeled target cells (Figure 21). In contrast to rituximab, target cells labeled with ofatumumab did not elicit CD 107a expression of NK cells different from baseline. Target cells labeled with obinutuzumab increased CD 107a expression by at least 2-fold [4.85- (25.7±2.7% vs. 5.3±1.5%), 2.57- (20.3±1.9% vs. 8.9±1.2%), and 8.58-fold (20.6±1.8% vs. 2.4±0.9%), respectively] on CD56+/CD3- NK cells compared to unlabeled target cells. Using BI 836826 as labeling antibody gave results comparable to those obtained with obinutuzumab [3.87- (20.5±2.8% vs. 5.3±1.5%), 2.51- (22.3±3.1% vs. 8.9±1.2%), and 5.28-fold (13.2±2.1% vs. 2.4±0.9%), respectively] when SU-DHL 4, Ramos or Karpas 422 cell lines were targeted.

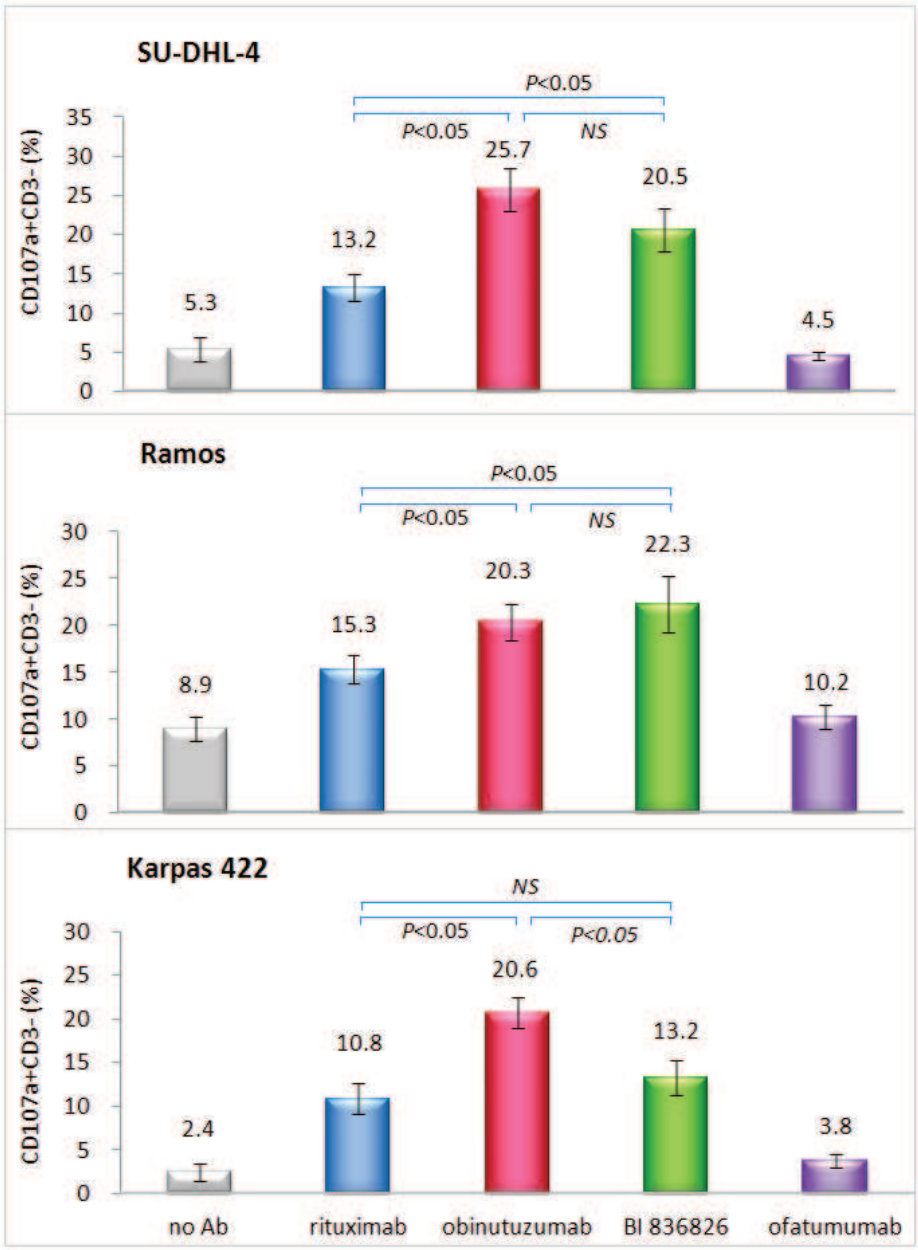

Fig.21 Results of ADCC as measured by CD107a degranulation assay using GCB-type lymphoma cell lines as target and healthy donor CD56+ cells as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 6 hours.

For ABC-type lymphoma cell lines, target cells labeled with ofatumumab showed no difference in CD 107a expression compared to stimulation without mAb. OCI-Ly 10 labeled with rituximab, obinutuzumab, and BI 836826 resulted in at least 2-fold [2.18- (8.3±1.5% vs. 3.8±1.3%), 5.32- (20.2±1.4% vs. 3.8±1.3%), and 4.16-fold (15.8±0.8% vs. 3.8±1.3%), respectively] increase of CD 107a expression on the surface of CD56+/CD3- NK cells compared to stimulation without mAb. OCI-Ly 10 labeled with obinutuzumab or BI 836826 elicited significantly higher killing efficacy as compared to rituximab (20.2±1.4% and 15.8±0.8% vs. 8.3±1.5%, p=0.018, n=10). With OCI-Ly 3 only BI 836826 resulted in a significantly higher killing efficacy compared to rituximab (13.6±0.6% vs. 8.9±0.8%, p=0.03, n=10) (Figure 22).

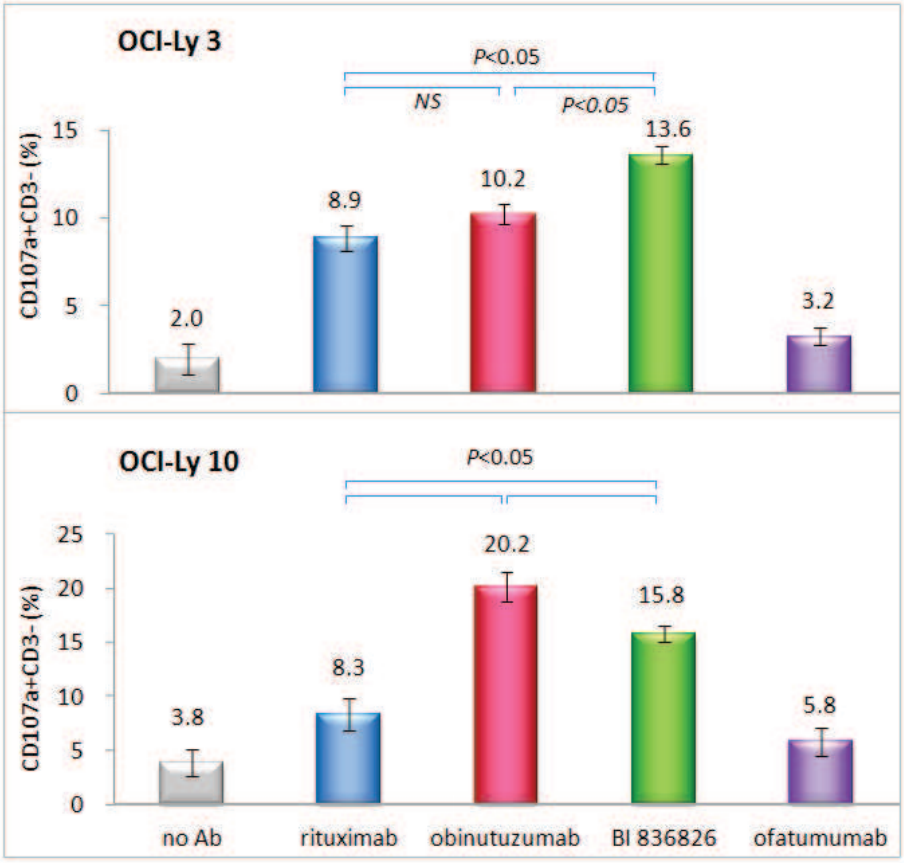

Fig. 22 Results of ADCC as measured by CD107a degranulation assay using ABC-type lymphoma cell lines as target and healthy donor CD56+ cells as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 6 hours.

3.2.1.4 ADCC with patient cells by CD 107a degranulation assay

3.2.1.4.1 Obinutuzumab and BI 836826 enhance degranulation of NK cells obtained from healthy donors or patients with follicular lymphoma

Fresh CD56+ NK cells were isolated from peripheral blood of healthy donors. CD 107a expression of healthy donor NK cells incubated with follicular lymphoma cells and labeled with rituximab was significantly higher than CD 107a expression of NK cells not labeled with rituximab (13.8±1.7% vs. 6.7±0.7%, $p<0.01$, n=3).

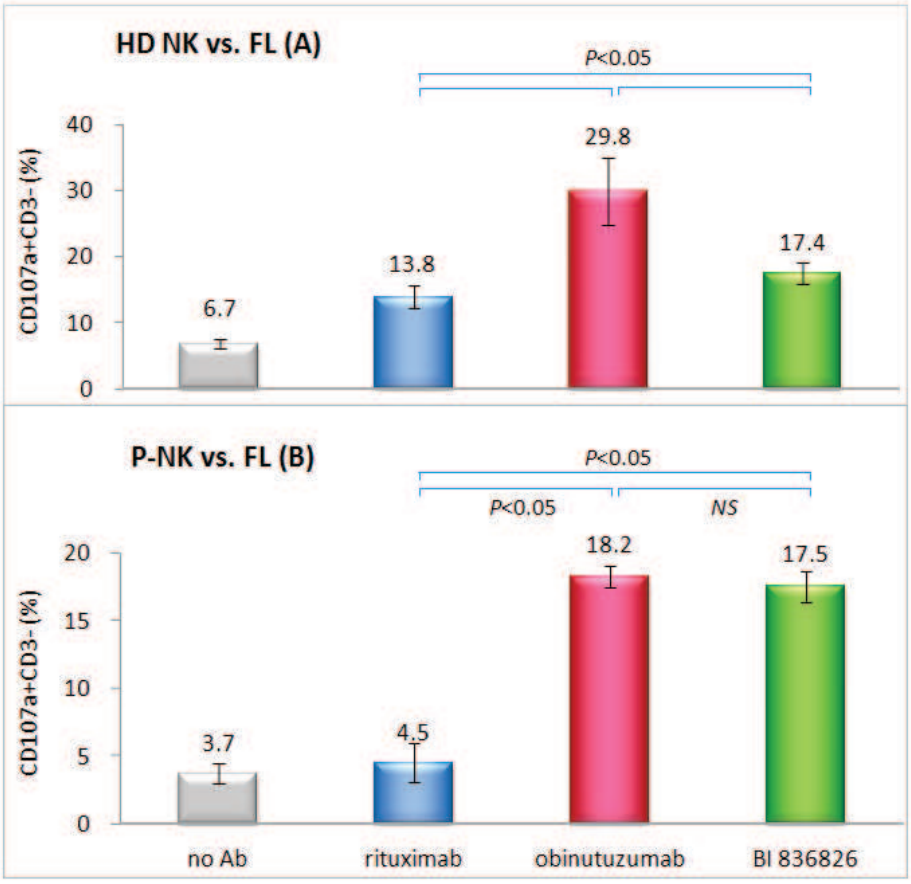

Fig. 23 Results of ADCC as measured by CD107a degranulation assay using patient follicular lymphoma cells as target cells and healthy donor CD 56+ NK cells (A) or patient CD56+ NK cells (B) as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, or BI 836826- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 6 hours.

When follicular lymphoma cells were labeled with obinutuzumab and BI 836826, CD 107a expression of healthy donor CD3-/CD56+ NK cells increased at least 2-fold [4.45- (29.8±5.2% vs. 6.7±0.7%) and 2.59- (17.4±1.6% vs. 6.7±0.7%), respectively] compared to stimulation without antibody. Obinutuzumab showed significantly better cytotoxicity compared to rituximab and BI 836826 (Figure 23A). (29.8±5.2% vs. 13.8±1.7% and 17.35±1.6%, p=0.04, n=3)

CD 107a expression on the surface of autologous NK cells incubated with FL cells and labeled with rituximab showed no significant difference to CD 107a expression of NK cells incubated with unlabeled target cells (4.5±1.43% vs. 3.7±0.8%, p>0.05, n=3). In contrast to rituximab, follicular lymphoma cells labeled with obinutuzumab and BI 836826 increased CD 107a expression at least 4-fold [4.92- (18.2±0.8% vs. 3.7±0.8%) and 4.73- (17.5±1.1% vs. 3.7±0.8%), respectively] compared to stimulation without antibody (Figure 23B).

3.2.1.4.2 Only BI 836826 enhances degranulation of patient NK cells in the presence of cells from patients with mantle cell lymphoma

Following stimulation by mantle lymphoma cells labeled with rituximab, obinutuzumab or BI 836826, both healthy donor and autologous NK cells showed low expression of CD 107a when compared to NK cells stimulated by labeled lymphoma cell lines tested. As shown in Figure 24(A), mantle cell lymphoma cells labeled with obinutuzumab showed 2.78-fold (3.2±0.2% vs. 1.2±0.3%, p=0.02, n=4) killing efficacy compared to rituximab; target cells labeled with BI 836826 elicited significantly superior expression of CD 107a on healthy donor NK cells compared to target cells labeled with rituximab or obinutuzumab (5.4±0.3% vs. 3.2±0.2% vs. 1.2±0.3%, p<0.01, n=4).

In contrast to the results with healthy donor NK cells, patient NK cells significantly increased killing of MCL only when BI 836826 was used as labeling antibody (3.7±0.6%, n=4). Rituximab, obinutuzumab like no antibody had no significant effect on killing efficacy (1.0±0.1%, 0.5±0.1% and 0.1±0.1%, p>0.05, n=4)(Figure 24B).

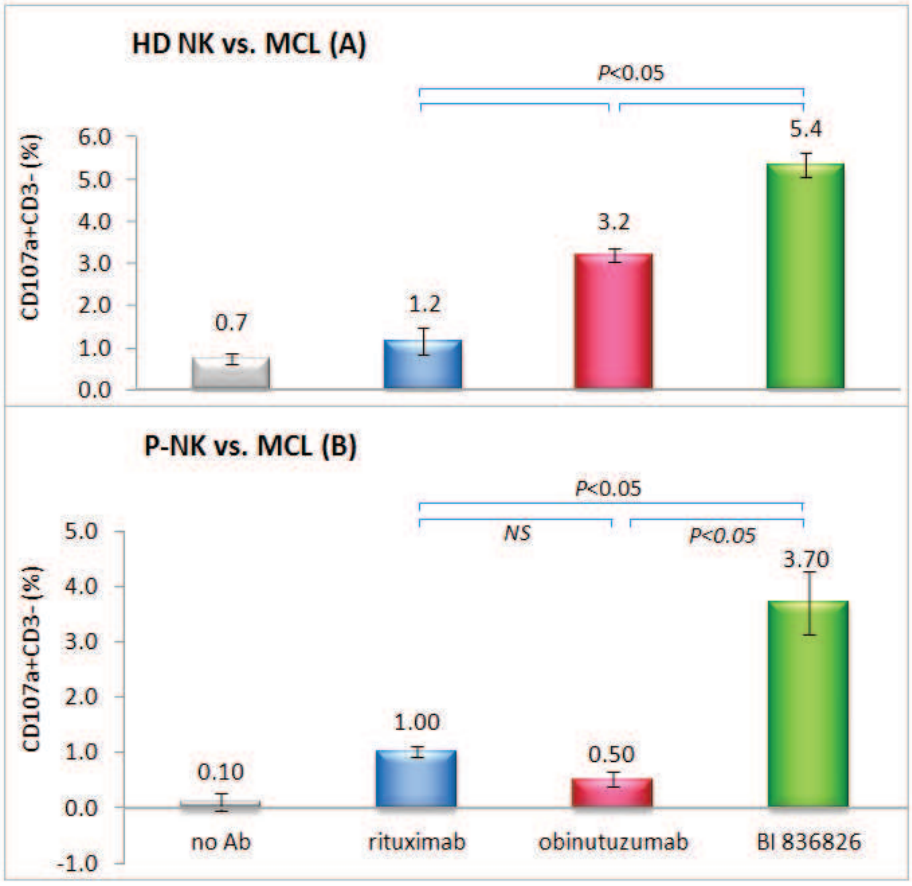

Fig. 24 Results of ADCC as measured by CD107a degranulation assay using patient mantle cells as target cells and healthy donor CD 56+ cell (A) or patient CD56+ cells (B) as effector cells. Effector cells were incubated with rituximab, obinutuzumab, or BI 836826 (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 6 hours.

3.2.1.4.3 Diffuse large B-cell lymphoma cells labeled with obinutuzumab and BI 836826 enhance degranulation of healthy donor and patient NK cells

Following stimulation with diffuse large B-cell lymphoma cells labeled with rituximab, obinutuzumab or BI 836826, CD107a expression of healthy donor NK cells showed a higher killing efficacy compared to stimulation without antibody, with 5.7±0.6%, 13.7±2.7%, and 12.5±1.3% of NK cells being CD 107a positive, respectively (Figure 25A).

CD 107a expression on the surface of autologous NK cells incubated with diffuse large B-cell lymphoma cells labeled with rituximab showed no difference to CD 107a expression when target cells were unlabeled. In contrast to rituximab, diffuse large B-cell lymphoma cells labeled with obinutuzumab and BI 836826 increased CD 107a expression on the surface of CD3-/CD56+ NK cells more than 9-fold (9.27- and 9.79-fold respectively) [9.27- (8.9±1.9% vs. 0.9±0.8%) and 9.79- (9.4±3.1% vs. 0.9±0.8%), respectively] compared to stimulation without antibody (Figure 25B).

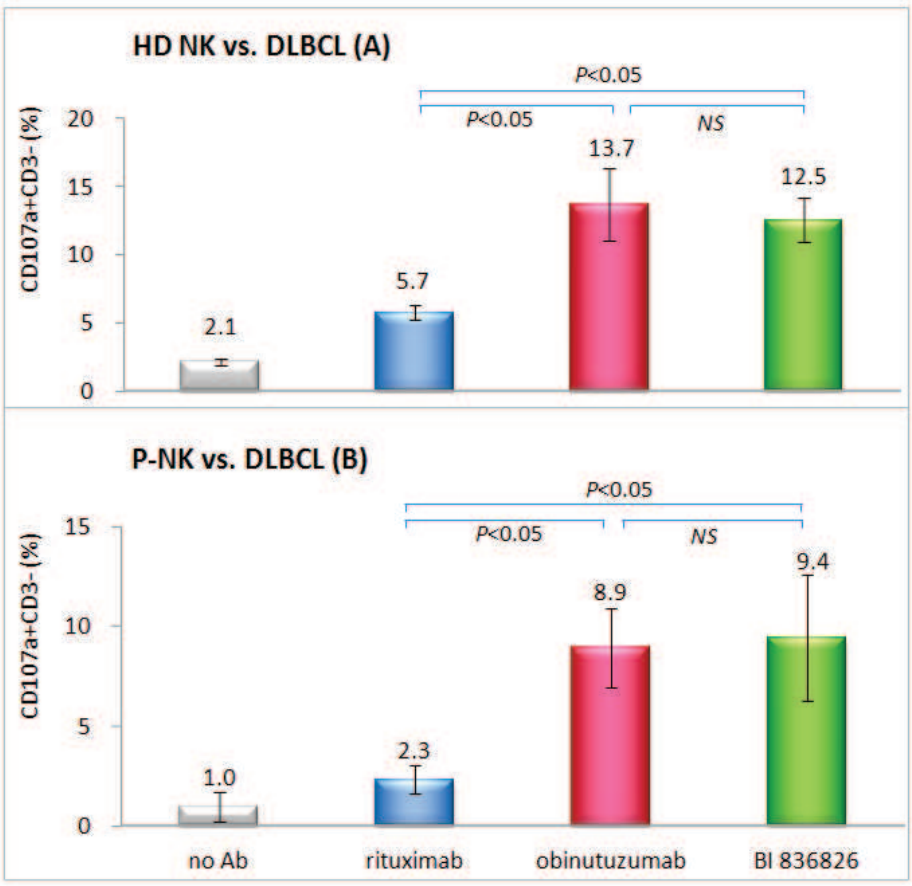

Fig. 25 Results of ADCC as measured by CD107a degranulation assay using DLBCL cells as target cells and healthy donor CD 56+ NK cell (A) or patient CD56+ NK cells (B) as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, or BI 836826- (10µg/mL each) labeled target cells at a 10 to 1 ratio over 6 hours.

3.2.2 Europium release assay

3.2.2.1 Europium release assay with various lymphoma cell lines

To compare the cytotoxic effects of different antibodies in vitro, GCB-type lymphoma cell lines (SU-DHL-4, Ramos, and Karpas 422) and ABC-type lymphoma cell lines (OCI-Ly 10 and OCI-Ly 3) were used as target cells. Effector cells were incubated separately with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10µg/mL each 10^6cells) labeled target cells at a 10 to 1 ratio over 4 hours. Representative data using different lymphoma cell lines are shown in Figures 26 and 27. All lymphoma cell lines showed spontaneous release in the range of 12.94±3.91% to 15.33±4.79%, respectively (n=12) (Table 5) except for Ramos and OCI-Ly 3, both of which showed a higher spontaneous release (21.53±4.51% and 21.06±4.59%, respectively).

Table 5 Spontaneous release of Eu^{3+} from GCB-type or ABC-type lymphoma cell lines in a 4-h cytotoxicity assay[a].

Cell lines		% release of Eu^{3+}
GCB- type	SU-DHL-4	14.14 ± 1.75%
	Ramos	21.53 ± 4.51%
	Karpas 422	12.94 ± 3.91%
ABC-type	OCI- Ly 10	15.33 ± 4.79%
	OCI-Ly 3	21.06 ± 4.59%

[a] Target cells were labeled with Eu3+, as described in Materials and Methods. The data are mean percentages ± SD of spontaneous release. Data obtained from 12 cytotoxicity assays performed with normal controls.

For GCB-type lymphoma cell lines, target cells labeled with rituximab showed higher specific lysis than after labeling with ofatumumab or no antibody. Obinutuzumab and BI 836826 showed superior killing efficacy compared to rituximab, reaching specific lysis of 82.38±7.16% and 83.03±13.46% with SU-DHL-4, 59.46±21.30% and 52.81±21.20% with Ramos, and 72.78±7.38% and 87.12±9.12% with Karpas 422 (n=12).

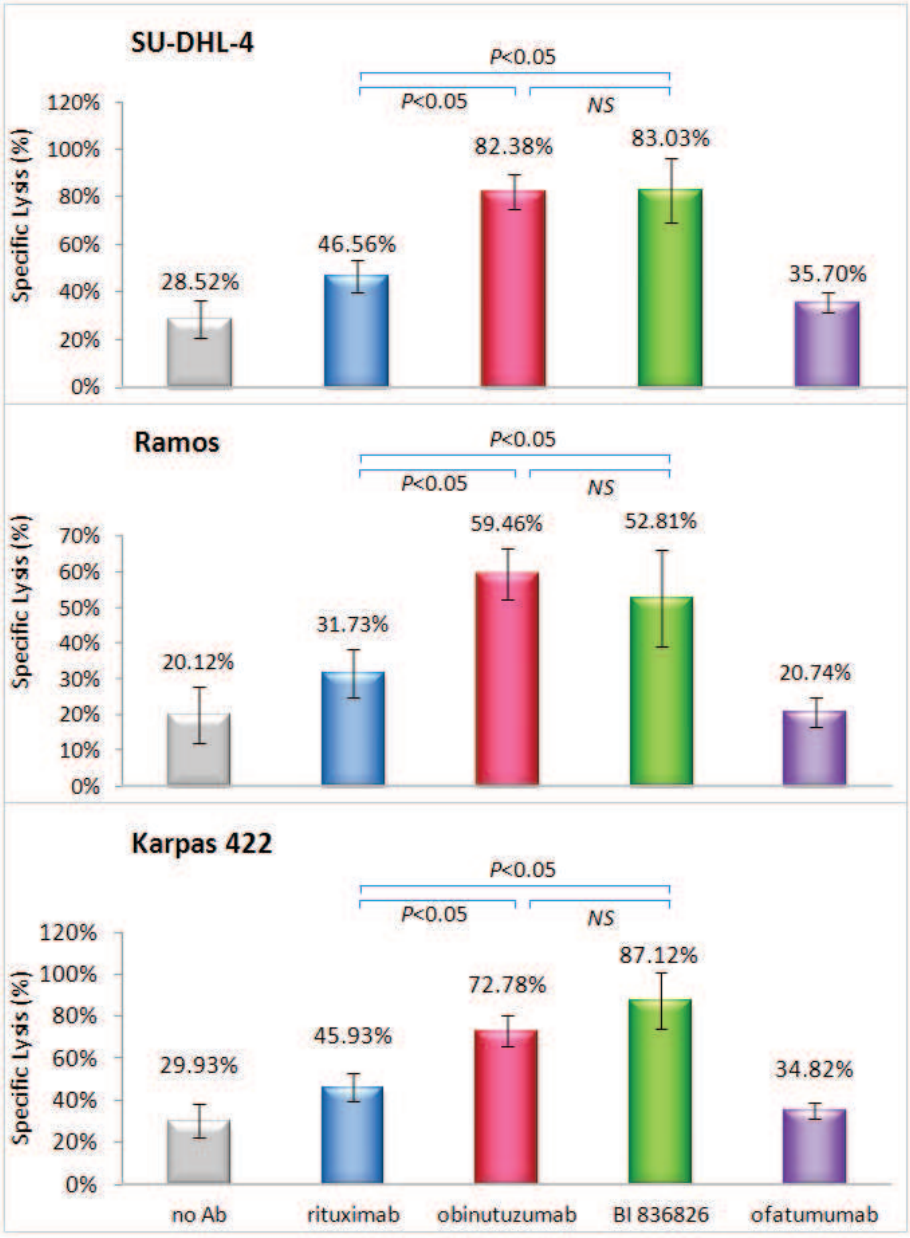

Fig. 26 Results of ADCC as measured by Europium release assay using GCB-type lymphoma cell lines as target cells and healthy donor lymphocytes as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 4 hours.

Comparing the killing of different ABC-type lymphoma cell lines, OCI-Ly 10 labeled with obinutuzumab or BI 836826 induced specific lysis of more than 80% (88.54±12.81% vs. 82.10±14.04%) compared to rituximab where specific lysis was only 58.36±10.35%. All antibodies except ofatumumab elicited higher cytotoxicity than stimulation without antibody. With OCI-Ly 3, lysis induced by any of the tested antibodies was in the range of 52.49±17.89% (ofatumumab) to 67.30±19.66% (rituximab) with no significant difference between antibodies (n=12).

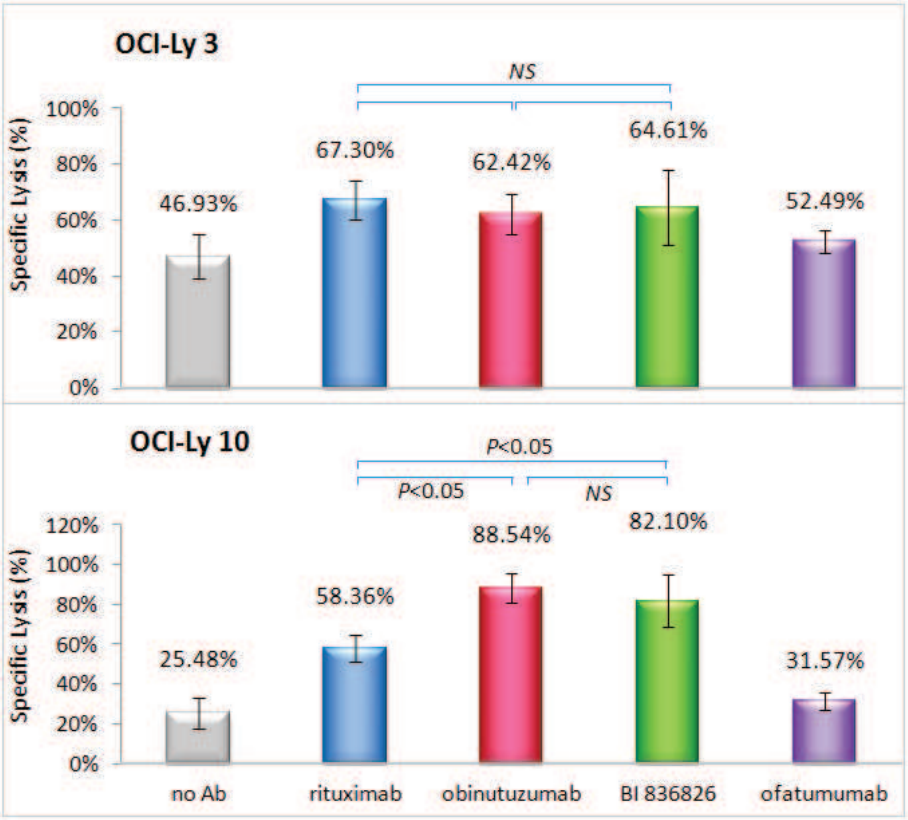

Fig. 27 Results of ADCC as measured by Europium release assay using ABC-type lymphoma cell lines as target cells and healthy donor lymphocytes as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 4 hours.

3.2.2.2 ADCC with patient cells by Europium release assay

Spontaneous release of primary lymphoma cells was measured 2 hours after thawing. Primary lymphoma cells showed relatively higher spontaneous release compared with lymphoma cell lines (in the range of 24.35±3.67% to 29.23±3.91% vs. 12.94±3.91% to 15.33±4.79%,) (Table 6).

Table 6 Spontaneous release of Eu^{3+} from follicular lymphoma cells (FL), mantle cell lymphoma cells (MCL) and diffuse large B-cell lymphoma cells (DLBCL) in 4-h cytotoxicity assay[a].

Lymphoma cells	% release of Eu^{3+}
FL	24.35 ± 3.67
MCL	27.64 ± 3.07
DLBCL	29.23 ± 3.19

[a] Target cells were labeled with Eu3+, as described in Materials and Methods. The data are mean percentages ± SD of spontaneous release. Data obtained from at least 6 independent experiments performed with normal controls.

3.2.2.2.1 Obinutuzumab and BI 836826 enhance specific lysis of FL cells by NK cells obtained from healthy donors or patients

Overall, follicular lymphoma cells showed higher spontaneous release (24.35±3.67%, n=3) (Table 6) than GCB- or ABC-type lymphoma cell lines, except for Ramos and OCI-Ly 3. Follicular lymphoma cells labeled with rituximab showed significantly higher specific lysis in the presence of healthy donor NK cells (53.66±3.01% vs. 31.06±5.03%, p=0.01, n=3) or autologous NK cells (33.89±6.05% vs. 20.58±2.11%, p<0.05, n=3) compared to stimulation without antibody. In contrast to rituximab, labeling with ofatumumab showed no difference compared to stimulation without antibody. Both, obinutuzumab and BI 836826, showed superior specific lysis in the presence of healthy donor NK cells (93.46±7.03%, and 89.12±8.06%, vs. 53.66±3.01%, p=0.01, n=3) or autologous NK cells (73.89±9.02% and 68.36±8.17% vs. 33.89±6.05%, p=0.01, n=3) compared to follicular lymphoma cells labeled with rituximab.

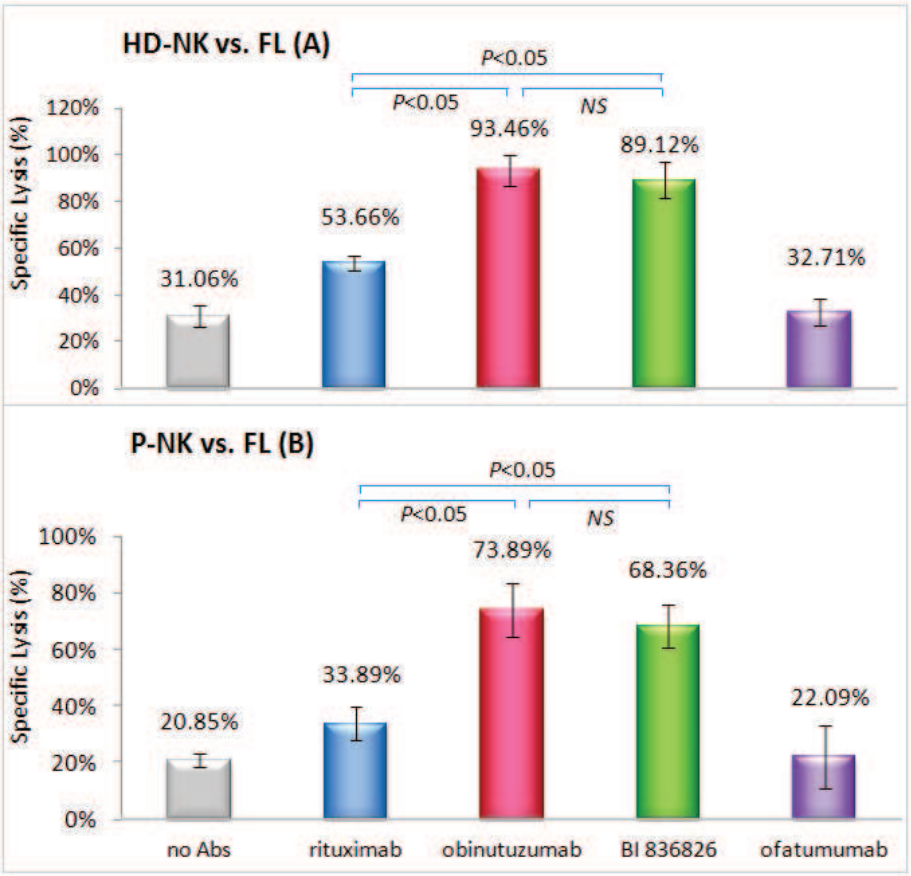

Fig. 28 Results of ADCC as measured by Europium release assay using follicular lymphoma cells as target cells and healthy donor CD 56+ cells (A) or patient CD56+ cells (B) as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 4 hours.

3.2.2.2.2 Obinutuzumab and BI 836826 enhance specific lysis of patient MCL by NK cells obtained from healthy donors or patients

Mantle cell lymphoma cells from patients showed higher spontaneous release than GCB-type or ABC-type lymphoma cell lines reaching a mean percentage of 27.64±3.07%. Mantle cell lymphoma cells labeled with rituximab showed significant higher specific lysis in presence of healthy donor NK cells

(49.44±9.12%, vs. 28.67±4.01%, *p*=0.03, n=4) or autologous NK cells (5.79±0.09% vs. 1.13±1.97%, *p*=0.01, n=4) compared to stimulation without antibody. In contrast to rituximab, ofatumumab made no difference compared to stimulation without antibody.

Both, obinutuzumab and BI 836826, showed superior specific lysis in the presence of healthy donor NK cells (80.05±9.89% and 81.77±20.01% vs. 49.44±8.86%, *p*<0.02, n=4) or autologous NK cells (11.22±2.79% and 12.94±3.15% vs. 5.79±0.93%, *p*=0.02, n=4) compared to mantle cell lymphoma cells labeled with rituximab.

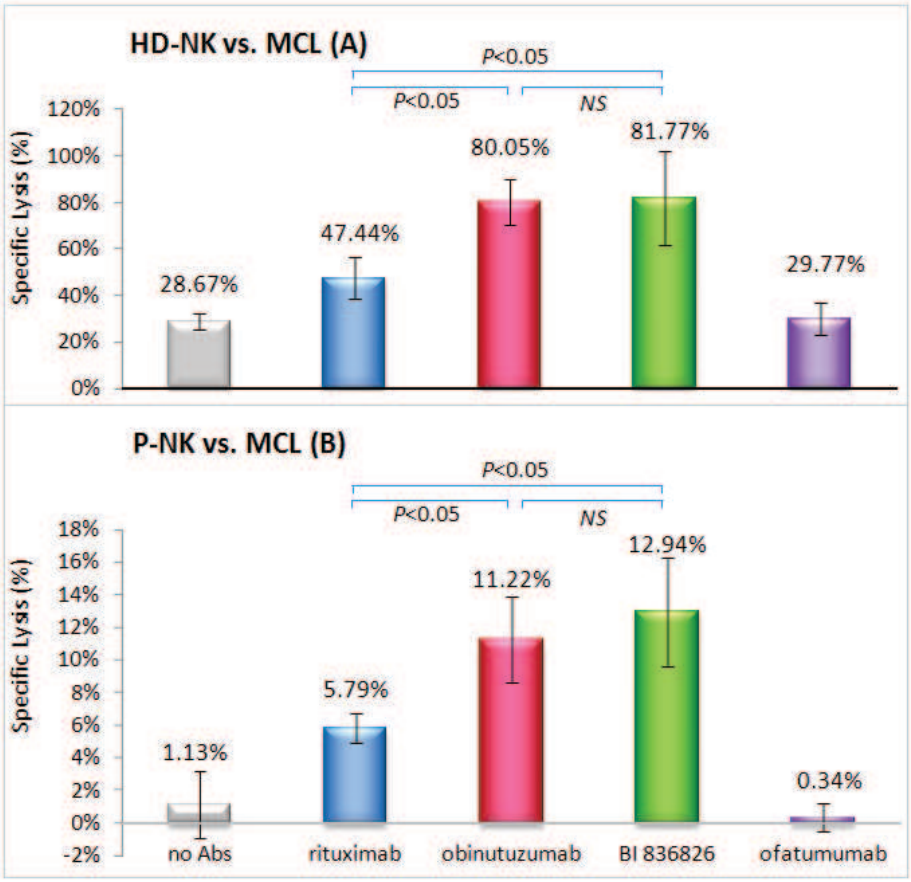

Fig. 29 Results of ADCC as measured by Europium release assay using mantle cell lymphoma cells as target cells and healthy donor CD 56+ cells (A) or patient CD56+ cells (B) as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 4 hours.

3.2.2.2.3 Obinutuzumab and BI 836826 enhance specific lysis of patient DLBCL by NK cells from healthy donors or patients

Diffuse large B-cell lymphoma cells from patients showed higher spontaneous release than GCB-type or ABC-type lymphoma cell lines with a mean percentage of 29.23±3.19%.

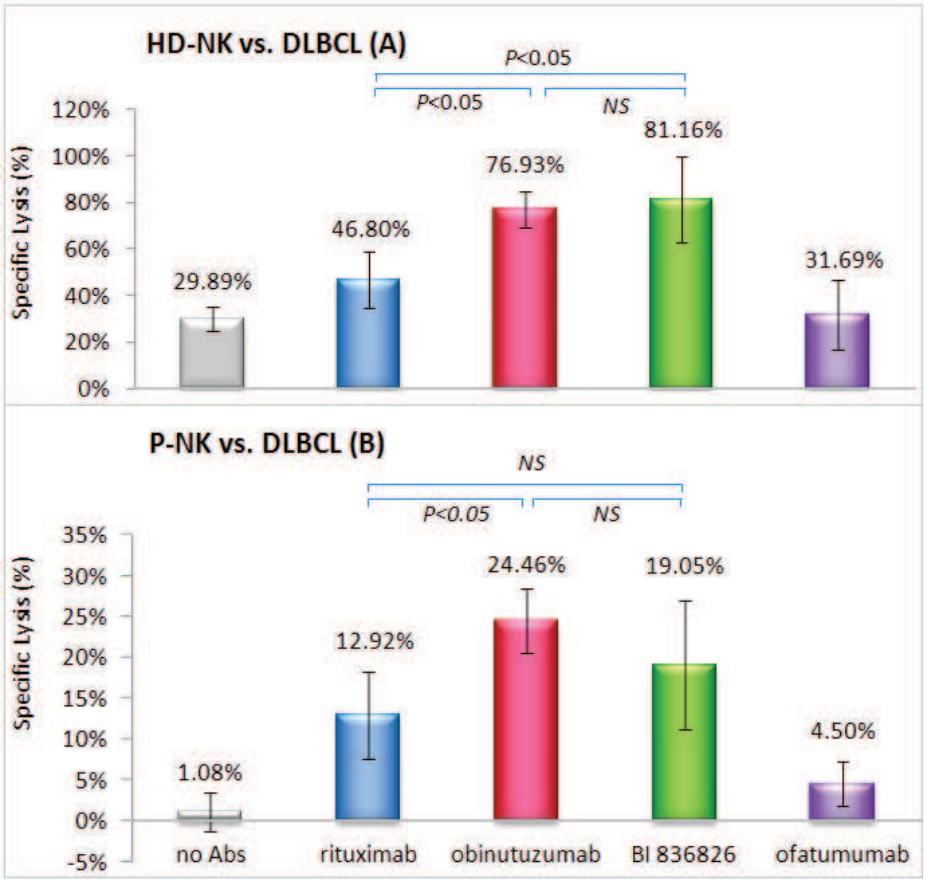

Fig. 30 Results of ADCC as measured by Europium release assay using diffuse large B-cell lymphoma cells as target cells and healthy donor CD 56+ cells (A) or patient CD56+ cells (B) as effector cells. Effector cells were incubated with rituximab-, obinutuzumab-, BI 836826- or ofatumumab- (10 µg/mL each) labeled target cells at a 10 to 1 ratio over 4 hours.

Diffuse large B-cell lymphoma cells labeled with rituximab showed significant higher specific lysis in the presence of healthy donor NK cells (46.80±12.10% vs. 29.89±4.99%, $p<0.05$, n=5) or autologous NK cells (12.92±5.05% vs. 1.08±2.07%, $p=0.02$, n=5) compared to stimulation without antibody. In contrast to rituximab, ofatumumab made no difference compared to stimulation without antibody.

Both, obinutuzumab and BI 836826 showed superior specific lysis in the presence of healthy donor NK cells (76.93±8.23% and 81.16±19.17% vs. 46.80±12.10%, $p=0.01$ and $p=0.03$, respectively, n=5) compared to diffuse large B-cell lymphoma cells labeled with rituximab. With autologous NK cells, only obinutuzumab showed superior specific lysis (24.46±3.89% vs. 12.92±5.05%, $p=0.02$, n=5) compared to DLBCL labeled with rituximab.

3.3 Complement-dependent cytotoxicity (CDC)

Complement-dependent cytotoxicity (CDC) is thought to be another important mechanism of action of the anti-CD 20 antibodies. In order to evaluate CDC, measurement of cell viability is necessary. Several methods to measure cell viability have been described: the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay determines mitochondrial activity, the lactate dehydrogenase assay evaluates the extent to which live cells are damaged. Assays using fluorescent dyes like 7-AAD (7-aminoactinomycin D) or PI (Propidium Iodide) use the property of living cells to actively excrete these dyes while dead or damaged cells are penetrated and labeled. Fluorescence-based assays are not only compatible with a wide range of cytological labeling techniques but also more sensitive and convenient. To evaluate CDC induced by various monoclonal antibodies, a flow cytometry-based cytotoxicity assay, namely the PI staining cell death assay, was used.

3.3.1 Establishment of the PI staining cell death assay

PI (propidium iodide) is a fluorescent DNA-intercalating agent that can be used to stain living cells. PI binds to nucleic acids by intercalating DNA bases with little or no sequence preference and with a stoichiometry of one molecule per 4-5 DNA base pairs.[114] To investigate complement-dependent cytotoxicity evoked by different antibodies, we applied the PI staining cell death assay to various lymphoma cell lines and patient cells varying the concentration of healthy donor or patient serum.

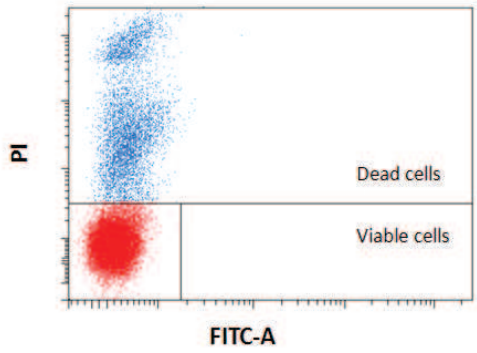

Fig. 31 Gating strategy to exclude viable target cells during PI staining cell death assay.

3.3.2 CDC with lymphoma cell lines

We found that target cells labeled with different mAbs induce CDC to different extents (Figure 32). The viability of lymphoma cell lines used in these experiments was in the range of 80.5±2.2% to 84.8±3.1%. For OCI-Ly 3 cells, the viability was 74.5±3.3% after 4 hours incubation.

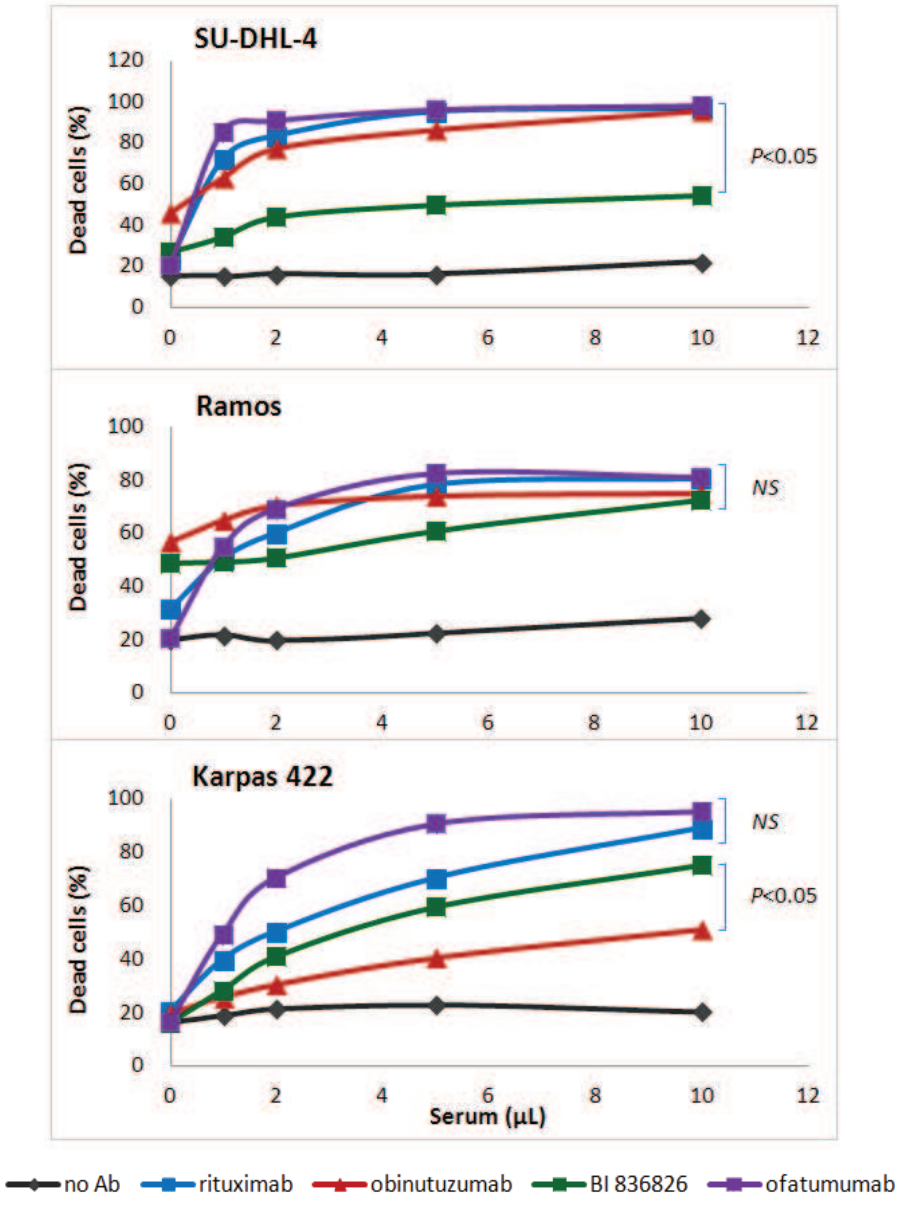

Fig. 32 Results of CDC as measured by PI assay using GCB type lymphoma cell lines incubated with healthy donor serum and rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

By increasing the volume of serum from healthy donors, all mAbs used showed increasing CDC although with different intensity. In general, ofatumumab showed the highest CDC in all GCB-type lymphoma cell lines while obinutuzumab and BI 836826 showed low or no cytotoxicity. The induction of CDC in Su-DHL-4 was as follows: ofatumumab or rituximab >> obinutuzumab > BI 836826. With Ramos cells, obinutuzumab and BI 836826 induced very low CDC compared to ofatumumab and rituximab.

For ABC-type lymphoma cell lines, ofatumumab again showed higher CDC than rituximab and obinutuzumab but no significant difference between ofatumumab and BI 836826 was detected. Induction of CDC with OCI-Ly 3 cells was as follows: ofatumumab or BI 836826 > rituximab >> obinutuzumab. When OCI-Ly 10 was labeled with ofatumumab, 2ul serum of a healthy donor could kill 70% of target cells. Lymphoma cells labeled with rituximab and BI 836826 showed the same degree of CDC while obinutuzumab showed almost no CDC (Figure 33).

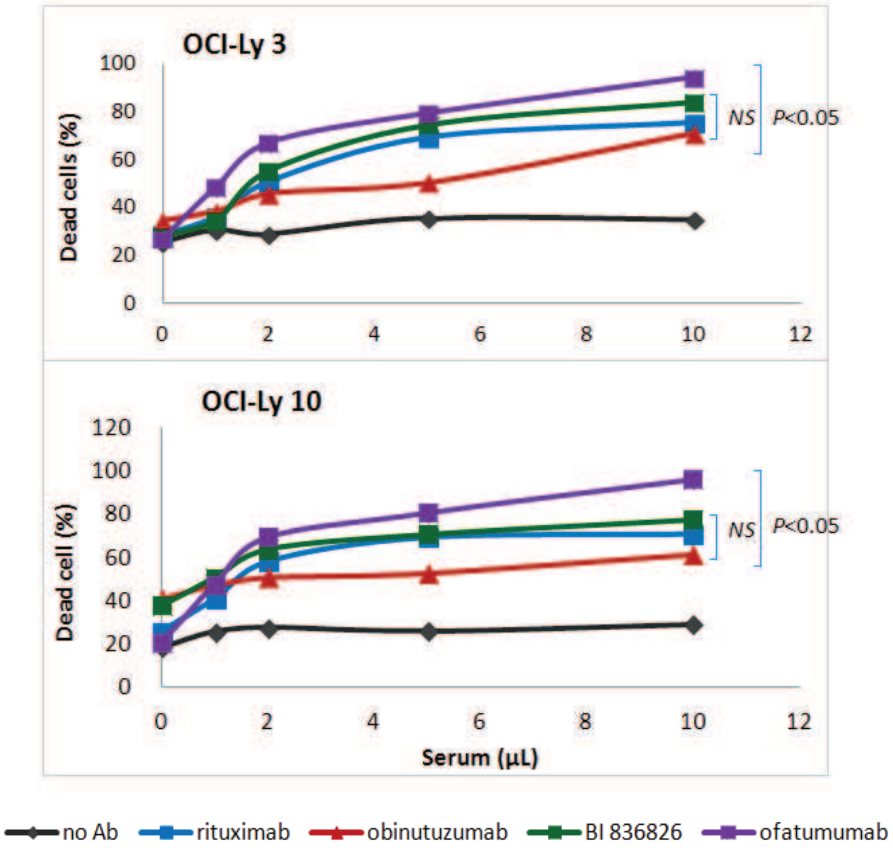

Fig. 33 Results of CDC as measured by PI assay using ABC type lymphoma cell lines incubated with healthy donor serum and rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

3.3.3 CDC with patient cells by PI staining cell death assay

3.3.3.1 Ofatumumab induces strong CDC with follicular lymphoma cells with serum from healthy donors or patients

The viability of follicular lymphoma target cells was 81.7±3.4% after 4 hours of incubation. Three independent experiments were done. Figure 34(A) shows that target cells labeled with ofatumumab and incubated with serum from healthy donors induces strong CDC. Between 50.2±8.2% and 71.8±6.8% of target cells were killed after incubation with serum at volumes of 2 µL and 10 µL. In contrast to ofatumumab, obinutuzumab did not induce high levels of CDC. Target cells labeled with obinutuzumab and incubated with serum from healthy donors induced weak CDC only. Between 16.9±2.7% and 30.0±4.3% of target cells were killed after incubation with serum at volumes of 2 µL and 10 µL. Rituximab induced intermediate cytotoxicity (33.0±5.7% and 61.2±4.9% of dead cells with serum at volumes of 2 µL and 10 µL). BI 836826 performed similar to obinutuzumab at serum volumes of 2 µL (18.5±1.2% dead cells); however, CDC elicited by BI 836826 increased to 45.4±7.1% when serum at a volume of 10 µL was used.

Figure 34(B) demonstrates CDC induced by autologous serum in follicular lymphoma cells labeled with different antibodies. Ofatumumab again showed high levels of CDC induction. The viability of follicular lymphoma cells decreased from 79.9±5.7% to 30.9±2.9% when the target cells were labeled by ofatumumab and incubated with 2 µL of autologous serum, and further dropped to 4.1±3.5% when incubated with 10 µL of serum. Obinutuzumab showed the lowest level of CDC. The viability of target cells decreased from 59.8±4.5% to 49.8±2.5% with 2 µL autologous serum and remained relatively high at 39.3±1.8% when 10 µL serum were used. Rituximab and BI 836826 induced similar levels of CDC. The viability of target cells labeled with these two antibodies decreased from 69.4±1.9% and 62.6±3.4% to 44.0±5.6% and 36.9±6.2%, respectively, when incubated with 2 µL serum and decreased to 29.5±1.6% and 22.9±3.0% when incubated with 10 µL serum. The strength of CDC induction in follicular lymphoma cells was as follows: ofatumumab > rituximab or BI 836826 > obinutuzumab.

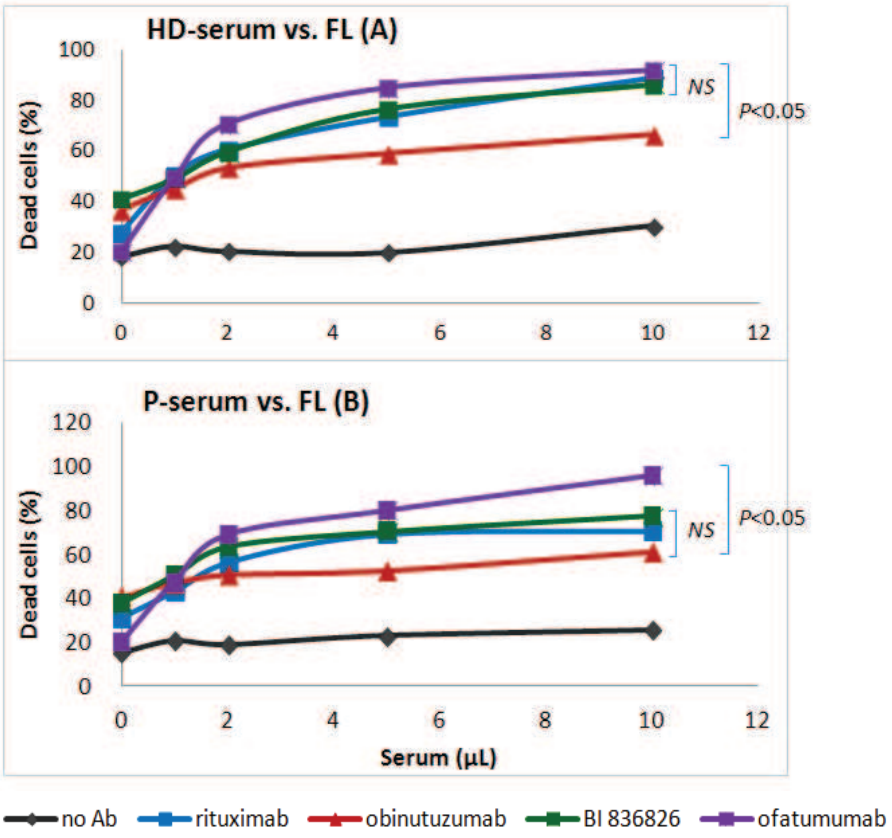

Fig. 34 Results of CDC as measured by PI staining cell death assay using follicular lymphoma cells as target cells labeled with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) and incubated with healthy donor serum (A) or patient serum (B) over 4 hours.

3.3.3.2 Serum from patients with MCL failed to induce CDC

The viability of mantle lymphoma cells was 65.9±4.3% after 4 hours of incubation. MCL cells labeled with ofatumumab and incubated with serum from healthy

donors showed induction of CDC at high levels (Figure 35A). The viability of MCL cells decreased from 64.0±5.2% to 29.1±6.1% when target cells were labeled with ofatumumab and incubated with 2 µL serum from healthy donors, and further dropped to 16.45±1.5% when incubated with 10 µL serum. There was no significant CDC induction when MCL cells were labeled with rituximab, obinutuzumab and BI 836826.

Figure 35(B) demonstrates CDC induced by autologous serum when MCL target cells were labeled with different antibodies. There was no significantly different CDC induction when MCL cells were labeled with rituximab, obinutuzumab, BI 836826 or ofatumumab.

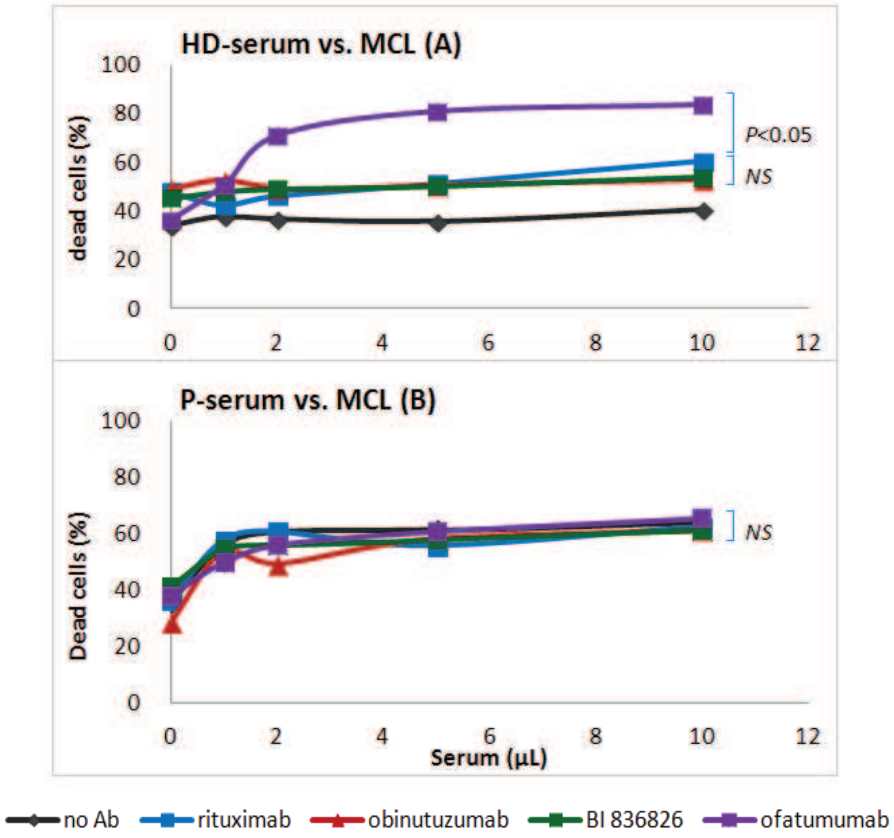

Fig. 35 Results of CDC as measured by PI staining cell death assay using MCL cells as target cells labeled with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) and incubated with healthy donor serum (A) or patient serum (B) over 4 hours.

3.3.3.3 Both rituximab and ofatumumab induce strong CDC with diffuse large B-cell lymphoma cells with serum from healthy donors or patients

The viability of diffuse large B-cell lymphoma cells was 74.4±4.1% after 4 hours incubation. DLBCL cells labeled with ofatumumab, BI 836826 or rituximab and incubated with serum from healthy donors showed high induction of CDC (Figure 36A). The viabilities of DLBCL cells labeled with these antibodies decreased from 74.3±2.0%, 59.3±3.1% and 61.5±2.7% to 22.5±1.9%, 30.4±3.7% and 32.3±2.9%, respectively, when incubated with 2 µL serum and further decreased to 3.6±0.8%, 6.6±1.0% and 11.41±2.1% when incubated with 10 µL serum. Obinutuzumab did not induce CDC when DLBCL cells were incubated with serum from healthy donors.

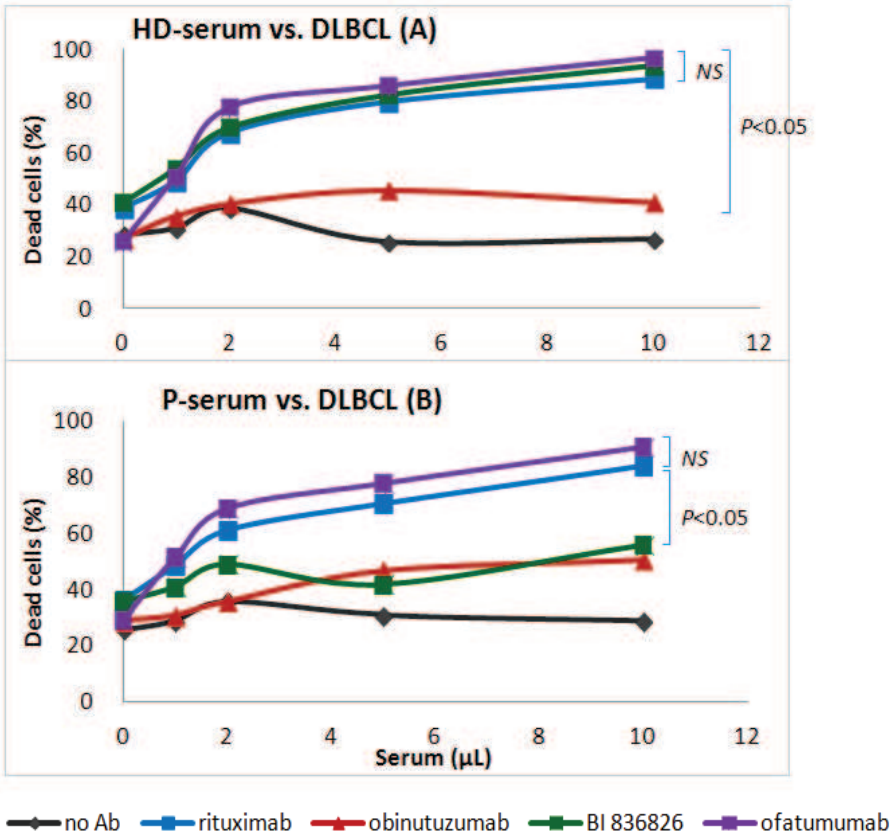

Fig. 36 Results of CDC as measured by PI staining cell death assay using DLBCL cells as target cells labeled with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) and incubated with healthy donor serum (A) or patient serum (B) over 4 hours.

Figure 36(B) demonstrates CDC induced by autologous serum when incubated with DLBCL cells labeled with different antibodies. Ofatumumab and rituximab showed high levels of CDC induction. The viability of DLBCL cells decreased from 71.0±5.4% and 64.3±6.1% to 31.7±3.9% and 39.5±4.8%, respectively, when target cells were labeled with ofatumumab or rituximab and incubated with 2 µL autologous serum and further dropped to 9.6±2.2% and 16.4±1.7%, respectively, when incubated with 10 µL serum. Obinutuzumab and BI 836826 showed lower levels of CDC. The viability of target cells decreased from 71.3±5.9% and 64.6±4.7% to 64.6±3.6% and 51.3±2.6%, respectively, when incubated with 2 µL autologous serum and dropped to 49.6±3.4% and 44.4±2.6% when incubated with 10 µL serum.

3.4 Programmed cell death (PCD)

To evaluate PCD induced by various monoclonal antibodies, a flow cytometry-based cytotoxicity assay, namely the Annexin V / PI staining programmed cell death assay, was used.

3.4.1 Establishment of the Annexin V / PI staining PCD assay

To investigate programmed cell death evoked by different antibodies, we applied the Annexin V and PI staining cell death assay to various lymphoma cell lines to differentiate early stage of apoptosis and late stage of apoptosis or necrosis (Figure 37).

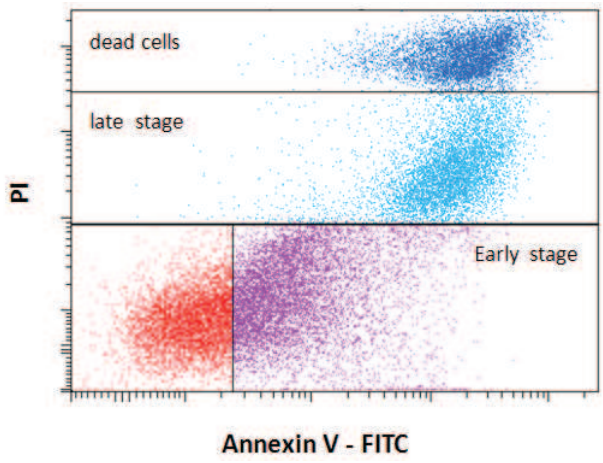

Fig. 37 Gating strategy to differentiate early stage of apoptosis from late stage of apoptosis or necrosis as reflected by the Annexin V / PI staining cell death assay.

3.4.2 PCD with lymphoma cell lines

Different stages of PCD (total PCD, early stage apoptosis, late stage apoptosis) were analyzed separately.

3.4.2.1 Total PCD with lymphoma cell lines

To compare PCD using different antibodies in vitro, GCB-type lymphoma cell lines (SU-DHL-4, Ramos, and Karpas 422) and ABC-type lymphoma cell lines (OCI-Ly 10 and OCI-Ly 3) served as target cells. Target cells at a concentration of $1x10^6$ cells/mL were labeled separately with rituximab, obinutuzumab, ofatumumab or BI 836826 (10 µg/mL each) and incubated over 4 hours. The cells were stained with PI and Annexin V in Annexin V binding buffer before measurement.

Target cells labeled with different mAbs all induce PCD but to different extents (Figure 38 and 39). Annexin V - positive cells measured by FACS represent the total percentage of PCD (early and late stage of apoptosis or necrosis). The percentage of Annexin V - positive cells from lymphoma cell lines without labeling antibody was in the range of 16.8±3.01% to 25.9±5.98%, except for OCI-Ly 3 cells which showed higher spontaneous labeling (33.4±5.39%). In general, obinutuzumab and BI 836826 led to higher PCD in all GCB-type lymphoma cell lines while ofatumumab showed low or no PCD. PCD in GCB-type lymphoma cell lines can be described as follows: obinutuzumab or BI 836826 > rituximab > ofatumumab.

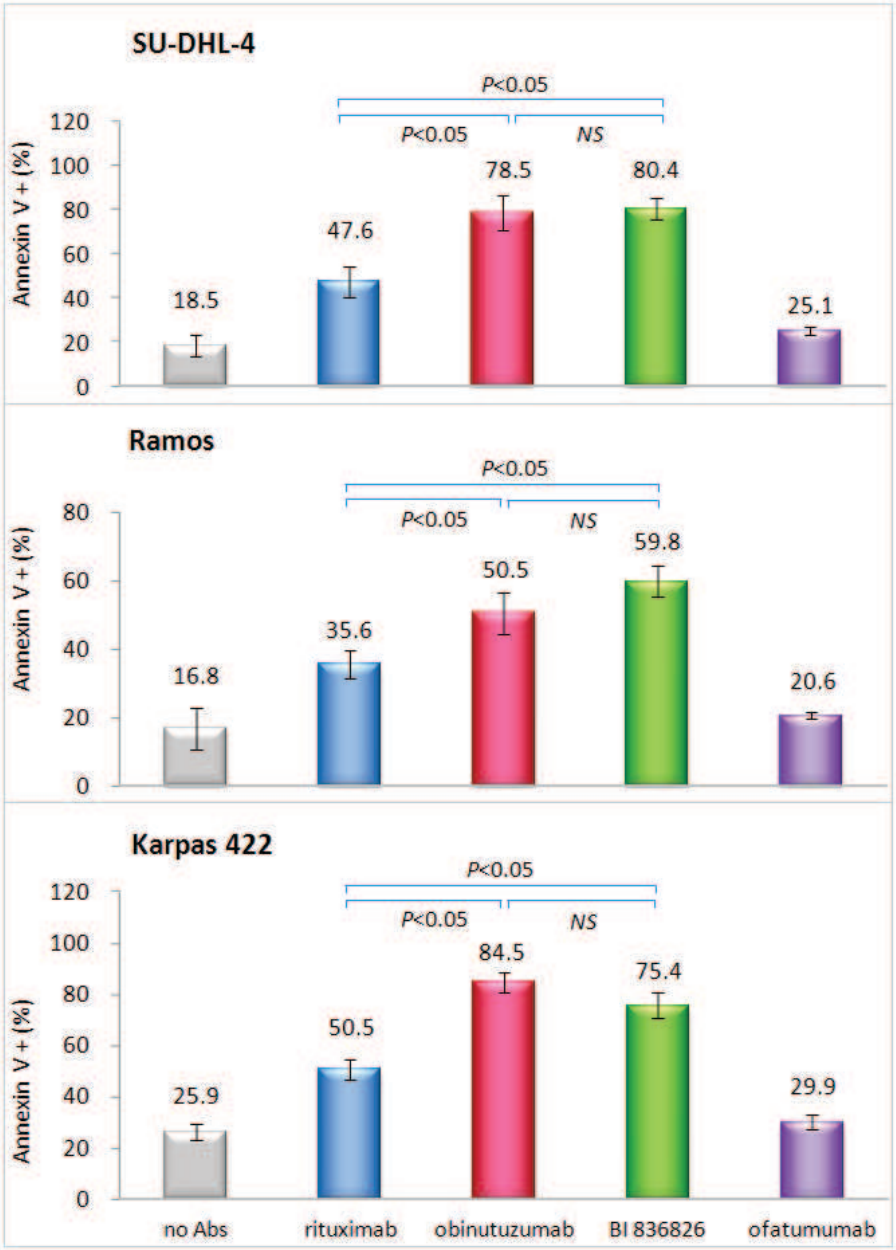

Fig. 38 Results of PCD as measured by Annexin V / PI assay using GCB-type lymphoma cell lines incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

For ABC-type lymphoma cell lines, again obinutuzumab showed strong PCD; no significant difference between ofatumumab and baseline was detected. Total PCD with OCI-Ly 3 cells was as follows: obinutuzumab > rituximab or BI 836826 >> Ofatumumab. When OCI-Ly 10 was labeled with obinutuzumab or BI 836826 both showed similar degrees of PCD and could kill 80.8±4.12% or 82.5±3.34% of target cells after 4 hours incubation.

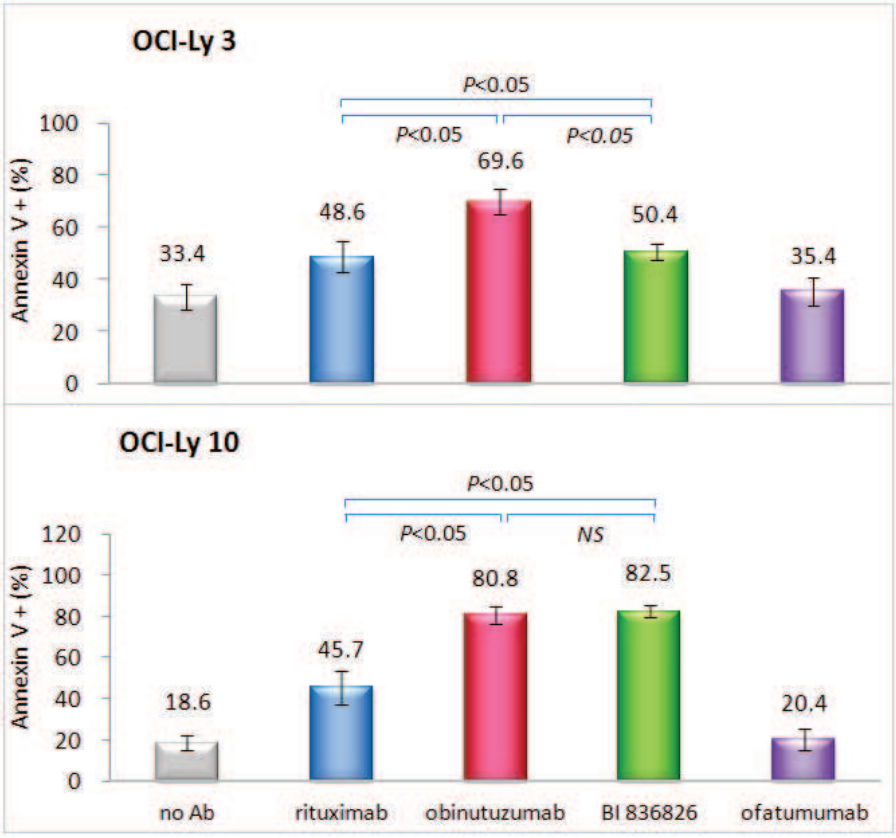

Fig. 39 Results of PCD as measured by Annexin V / PI assay using ABC-type lymphoma cell lines incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

3.4.2.2 Early stage apoptosis with lymphoma cell lines

Figure 40 and Figure 41 show early stage apoptosis induced in lymphoma cell lines by different antibodies. Cells undergoing early stage apoptosis are stained by Annexin V but not PI. Lymphoma cell lines not labeled with antibody were Annexin V-positive (0.4±0.1% to 7.9±4.99% positive cells) and PI - negative. No significant difference between ofatumumab and baseline was detected.

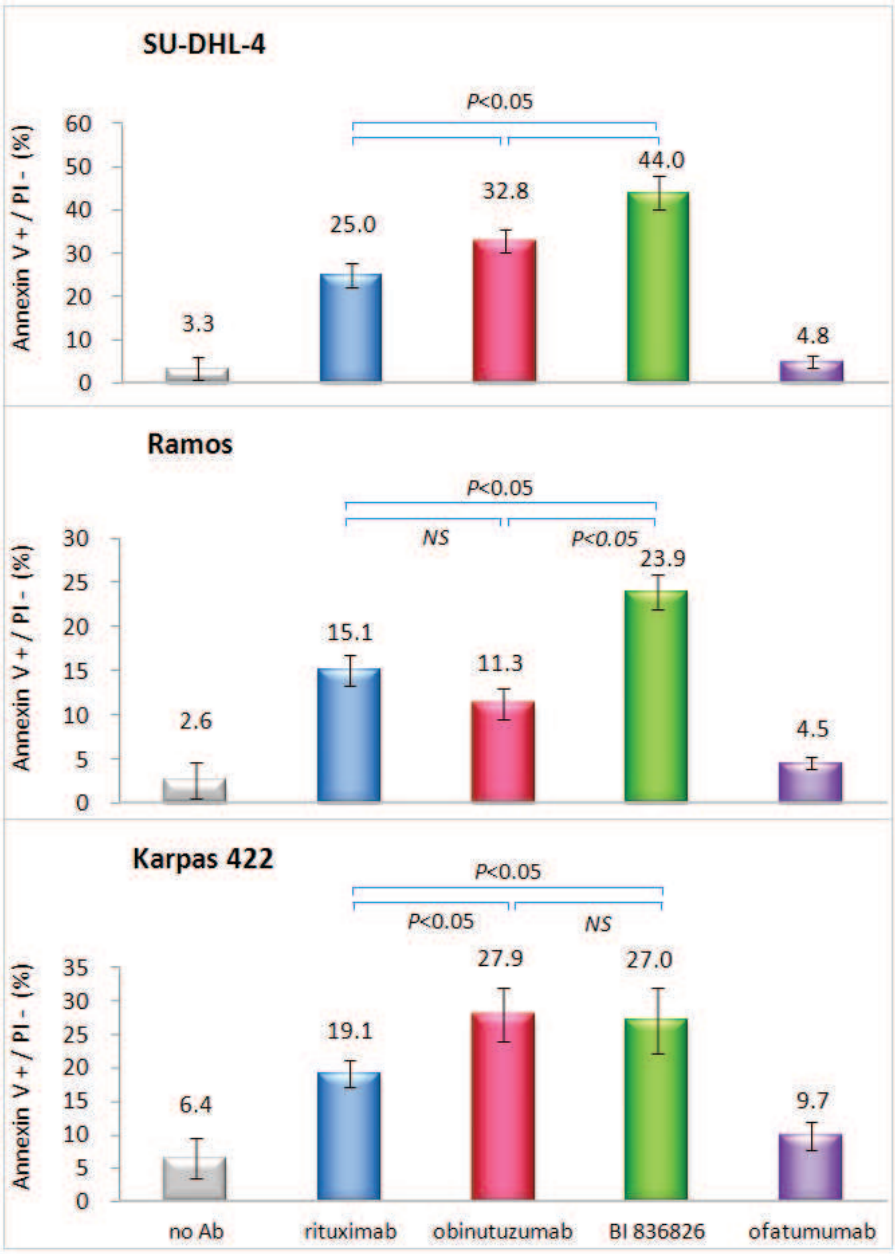

Fig. 40 Results of early stage apoptosis as measured by Annexin V / PI assay using GCB-type lymphoma cell lines incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

For Su-DHL-4 and Ramos, BI 836826 induced stronger early stage apoptosis (44.0±3.87% and 23.9±2.21% of Annexin V+/PI-) than rituximab (25.0±2.75%and 15.1±1.75%) or obinutuzumab (32.8±2.97% and 11.3±1.68%). Obinutuzumab showed the same degree of early stage apoptosis compared with BI 836826 (27.9±3.89% vs. 27.0±4.76%) in Karpas 422 lymphoma cell line.

For ABC-type lymphoma cell lines, again obinutuzumab showed the strongest early stage apoptosis. Apoptosis of OCI-Ly 3 cells was as follows: obinutuzumab (35.4±5.53%) > rituximab or BI 836826 (20.1±5.88% or 22.3±3.12%) >> ofatumumab (8.6±5.20%). When OCI-Ly 10 was labeled with obinutuzumab or BI 836826 (40.6±2.25% or 45.1±1.97%) both showed very similar degrees of early apoptosis.

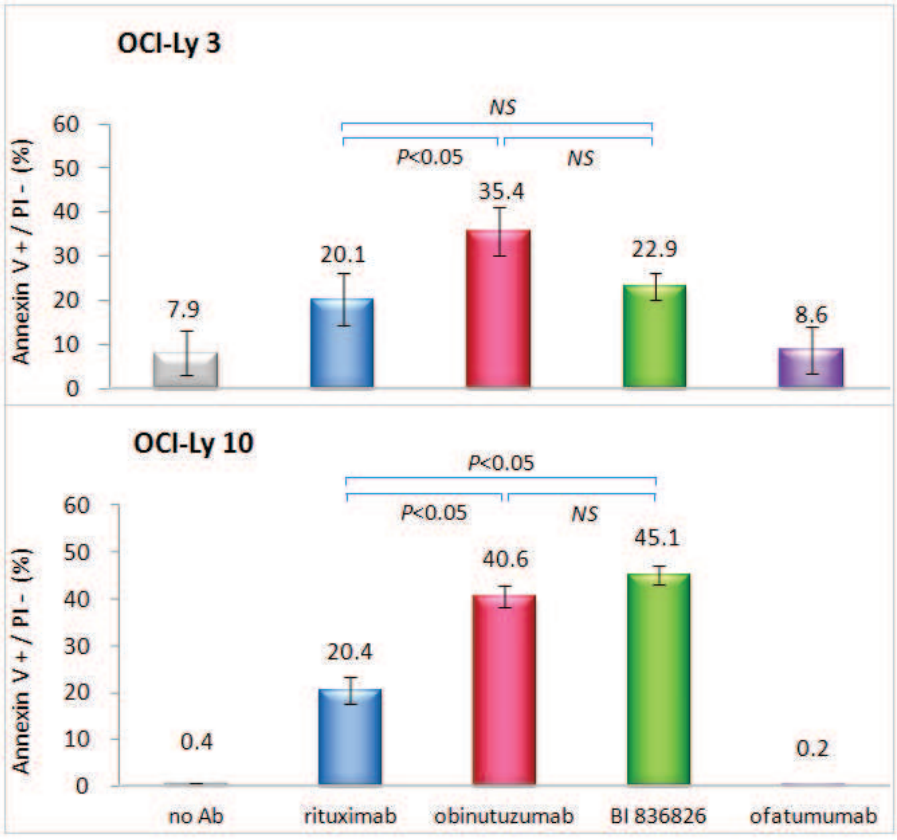

Fig. 41 Results of early stage apoptosis as measured by Annexin V / PI assay using ABC-type lymphoma cell lines incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

3.4.2.3 Late stage apoptosis or necrosis with lymphoma cell lines

Cells at late stage of apoptosis or necrosis are detected as Annexin V - positive and PI - positive (Annexin V + / PI +) (Figure 42 and 43). Fifteen to 20 % of cells of lymphoma cell lines not labeled with antibody were Annexin V +/ PI + except for OCI-Ly 3 cells (25.49±5.13% Annexin V +/ PI +).

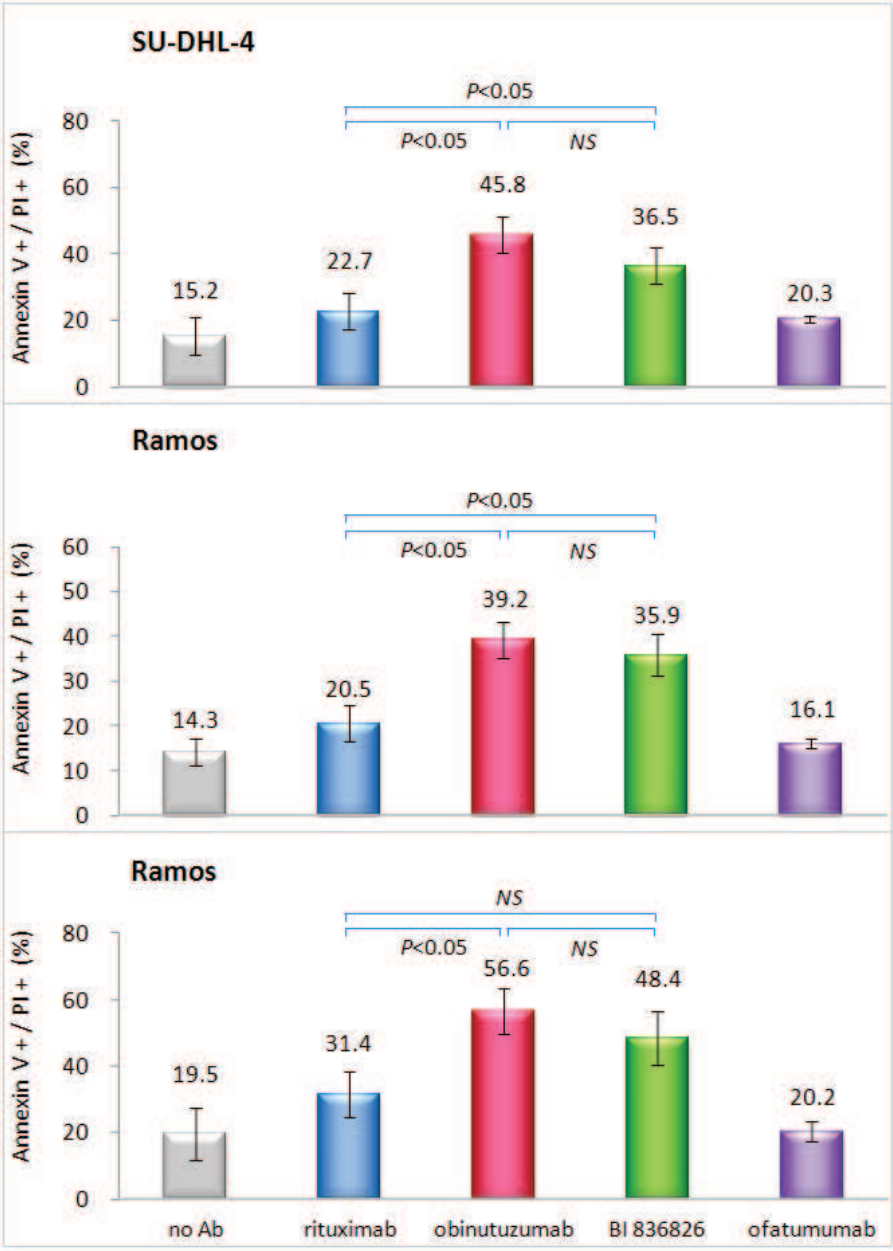

Fig. 42 Results of late stage apoptosis as measured by Annexin V / PI assay using GCB-type lymphoma cell lines incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

Obinutuzumab and BI 836826 showed stronger apoptosis at late stage or necrosis in all GCB-type lymphoma cell lines while rituximab or ofatumumab showed low or no apoptosis. The strength of late stage apoptosis or necrosis in GCB-type lymphoma cell lines was as follows: obinutuzumab or BI 836826 > rituximab or ofatumumab (Figure 42).

When OCI-Ly 10 representing ABC-type lymphoma was labeled with obinutuzumab or BI 836826 late stage apoptosis or necrosis was higher than with other antibodies (40.2±2.25% or 37.4±2.12%); no significant difference between ofatumumab, rituximab or no antibody for late stage apoptosis / necrosis was detected. Apoptosis or necrosis with OCI-Ly 10 cells was as follows: obinutuzumab or BI 836826 > rituximab or ofatumumab. For OCI-Ly 3, there was no significant difference among all different antibodies and baseline after 4 hours of incubation (Figure 43).

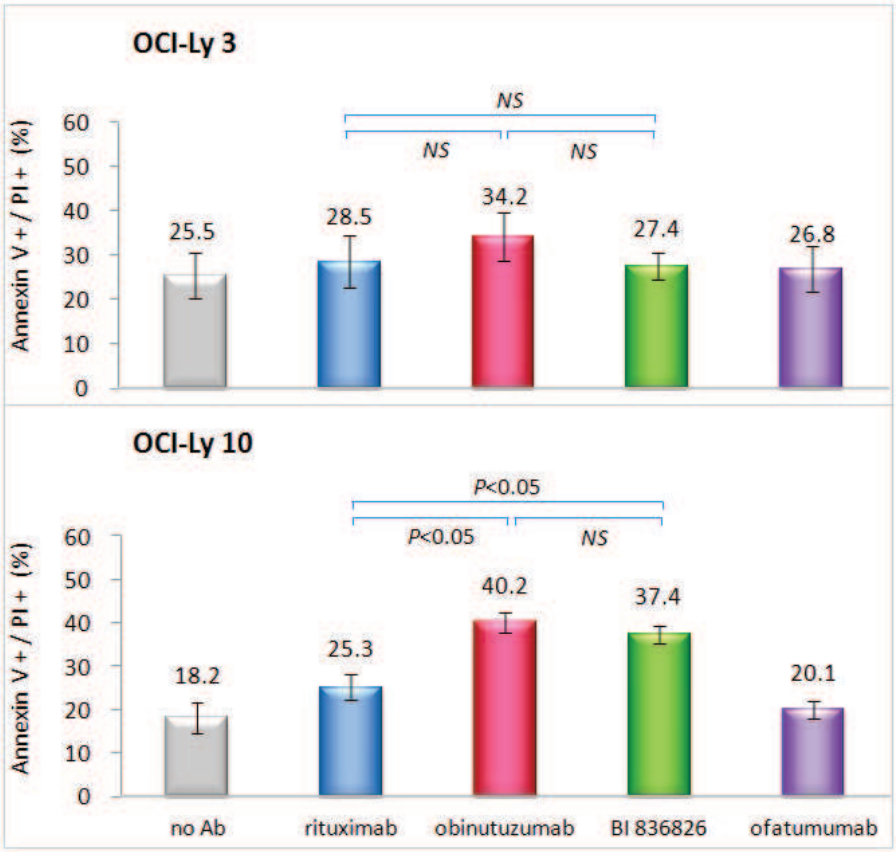

Fig. 43 Results of late stage of apoptosis as measured by Annexin V / PI assay using ABC-type lymphoma cell lines incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours.

3.4.3 PCD with patient cells by Annexin V/PI assay

3.4.3.1 Obinutuzumab and BI 836826 induce stronger PCD than rituximab in follicular lymphoma cells

We found that follicular lymphoma cells labeled with different antibodies induce PCD to different extents (Figure 44). Follicular lymphoma cells labeled with obinutuzumab and BI 836826 showed the strongest total PCD (78.5±7.50% and 87.4±6.25% of Annexin-positive cells) while ofatumumab showed no difference compared with baseline. PCD in follicular lymphoma cells was as follows: obinutuzumab or BI 836826 > rituximab > ofatumumab. However, BI 836826 induced stronger early stage apoptosis than obinutuzumab and rituximab.

3.4.3.2 Obinutuzumab and BI 836826 induce stronger PCD than rituximab in mantle cell lymphoma cells

MCL cells labeled with obinutuzumab and BI 836826 also showed strong PCD (78.2±7.47% and 83.5±7.66% Annexin V-positive cells) while ofatumumab showed no difference compared with baseline (Figure 45). There was no significant difference among different antibodies, even when compared with baseline for late stage apoptosis. However, BI 836826 showed superior PCD induction at early stage of apoptosis.

3.4.3.3 Obinutuzumab and BI 836826 induce stronger PCD than rituximab in diffuse large B-cell lymphoma cells

Figure 46 shows that DLBCL cells labeled with obinutuzumab and BI 836826 showed strong PCD (72.1±4.89% and 81.1±7.38% of Annexin V – positive cells) while ofatumumab showed no difference compared with baseline. At late stage of apoptosis, obinutuzumab showed superior PCD induction while BI 836826 showed superior PCD induction at early stage of apoptosis.

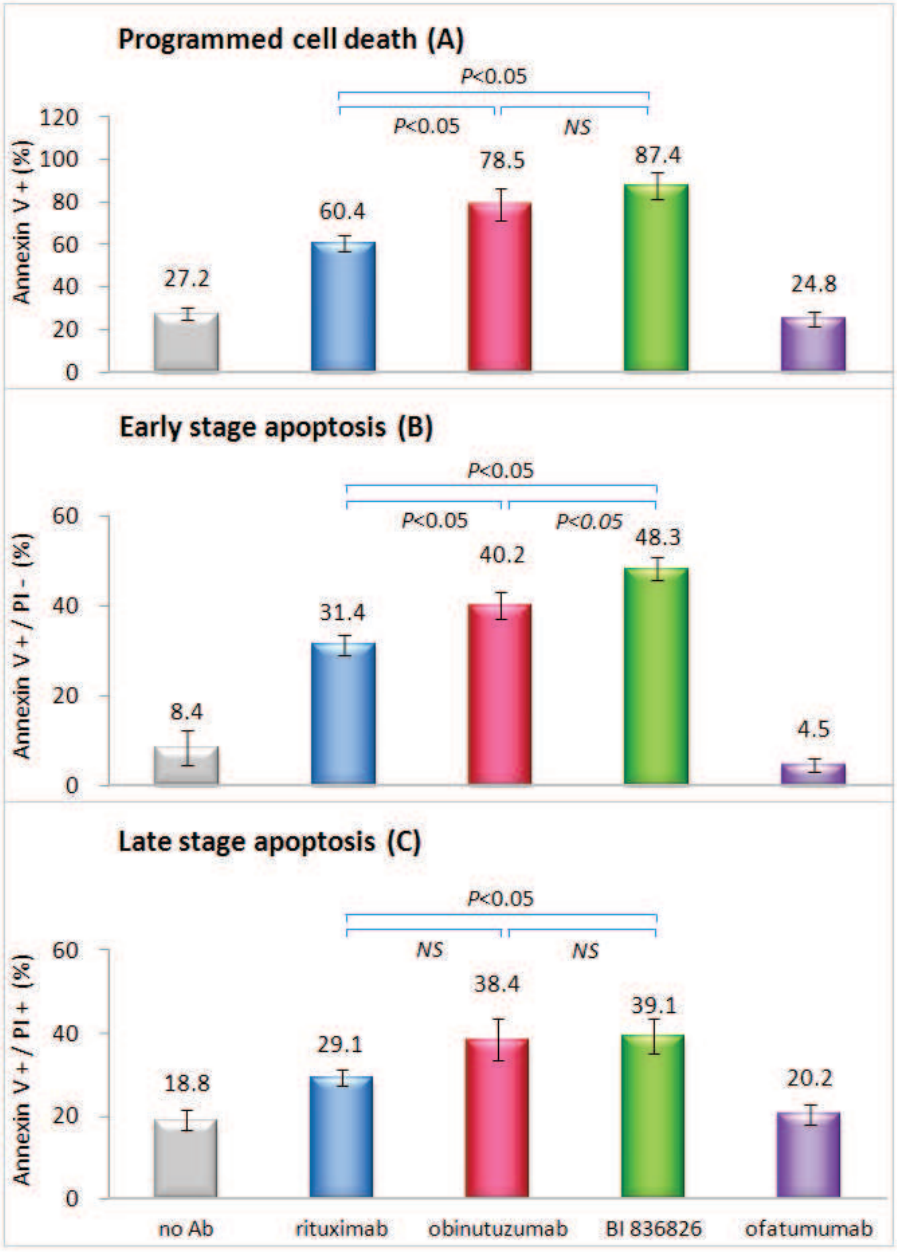

Fig. 44 Results of PCD as measured by Annexin V / PI assay using FL cells incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours. (A) total PCD (Annexin V +), (B) early stage apoptosis (Annexin V + / PI -), and (C) late stage apoptosis or necrosis (Annexin V + / PI +).

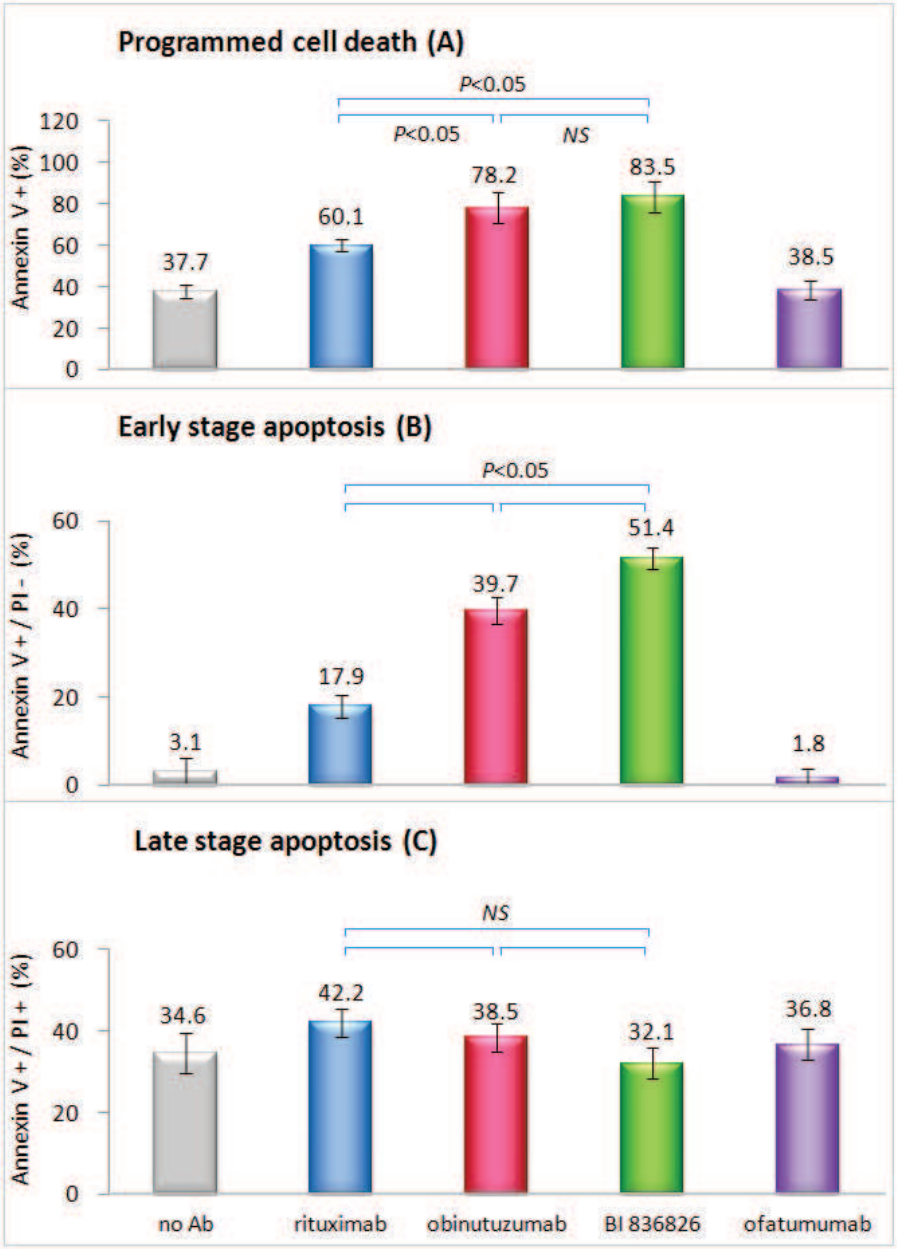

Fig. 45 Results of PCD as measured by Annexin V / PI assay using MCL cells incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours. (A) total PCD (Annexin V +), (B) early stage apoptosis (Annexin V + / PI -), and (C) late stage apoptosis or necrosis (Annexin V + / PI +).

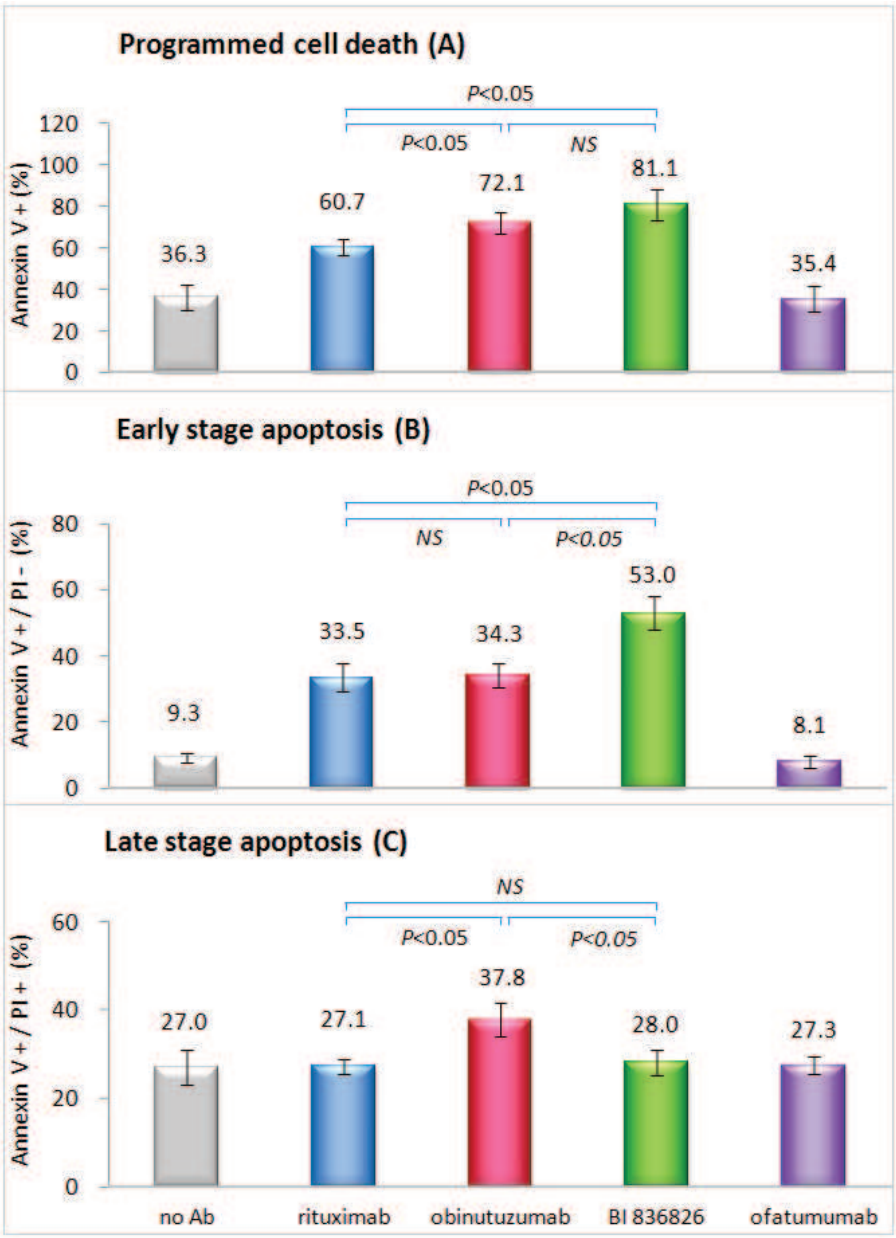

Fig. 46 Results of PCD as measured by Annexin V / PI assay using DLBCL cells incubated with rituximab, obinutuzumab, BI 836826 or ofatumumab (10 µg/mL each) over 4 hours. (A) total PCD (Annexin V +), (B) early stage apoptosis (Annexin V + / PI -), and (C) late stage apoptosis or necrosis (Annexin V + / PI +).

3.4.4 Obinutuzumab and BI 836826 evoke strong homotypic aggregation with lymphoma cell lines and primary lymphoma cells

To investigate homotypic aggregation with different lymphoma cells, wide field live imaging microscopy was used. Lymphoma cells were randomly settled in a µ-slide glass bottom well at concentration of $1x10^5$cells/ml and incubated with rituximab, obinutuzumab, ofatumumab or BI 836826 (10 µg/mL each) over 24 hours. Figure 47 shows the homotypic aggregation of SU-DHL-4 incubated with different antibodies. After injection of antibody into the wells, obinutuzumab and BI 836826 induced strong adhesion. Within one hour, several colonies were observed. After 24 hours of incubation, lymphoma cells incubated with obinutuzumab or BI 836826 showed stronger homotypic aggregation than cells incubated with rituximab. The homotypic aggregation of lymphoma cells was as follows: Obinutuzumab > BI 836826 > rituximab > ofatumumab.

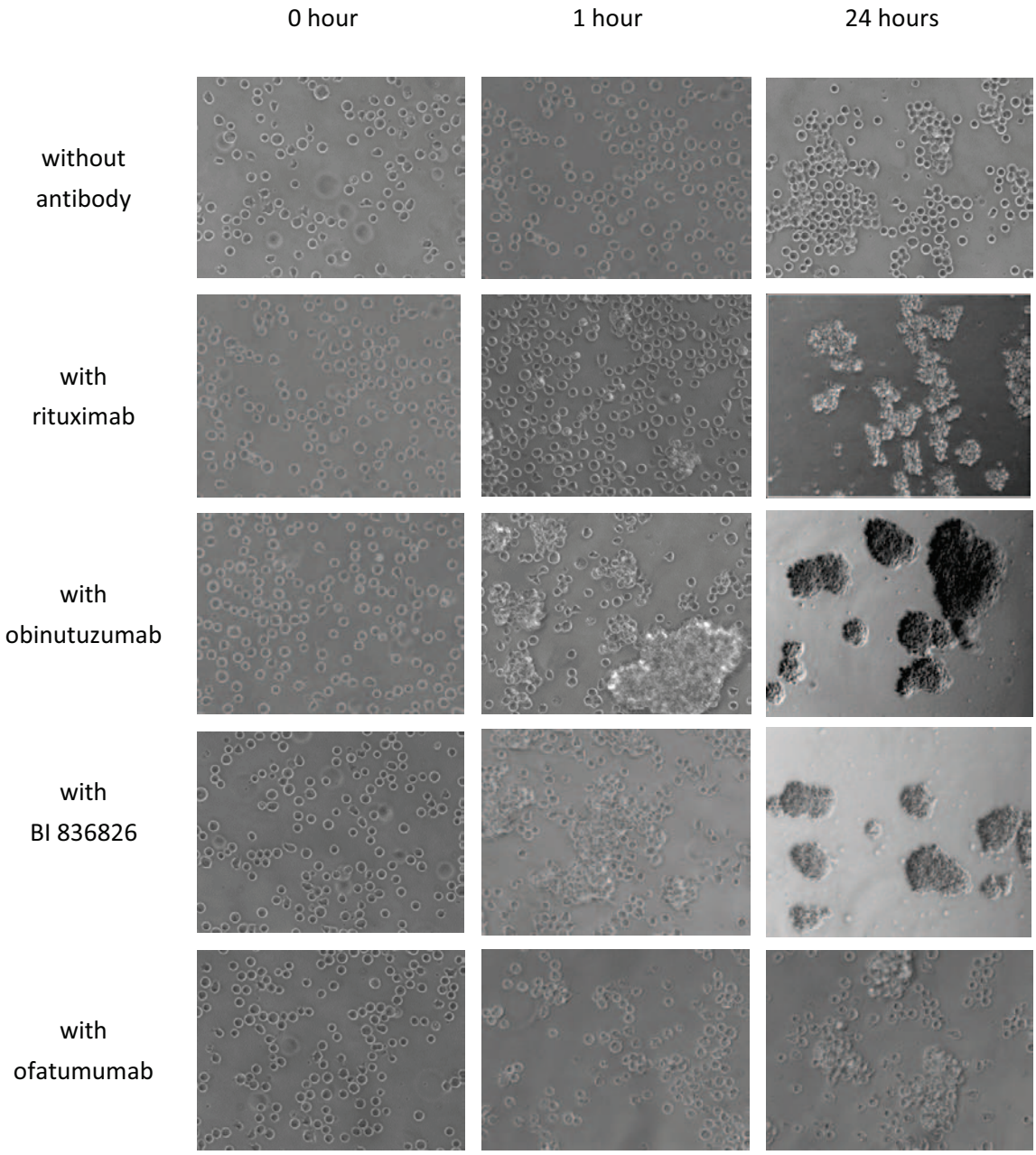

Fig. 47 Obinutuzumab and BI 836826 induce rapid and pronounced homotypic aggregation of SU-DHL-4 and OCI-Ly 10, as well as FL cells, MCL cells and DLBCL cells, whereas rituximab and ofatumumab induce only weak aggregation.

3.5 Additional results on treatment of T- and B-cell lymphoma

3.5.1 Allogeneic transplantation in relapsed and refractory T-cell lymphoma [109]

The first report on alloSCT for relapsed and refractory T-cell lymphoma was published almost ten years ago. Corradini et al. described 17 patients with PTCL, AITL or ALK-negative ALCL who had undergone alloSCT from an HLA-identical family donor (one unrelated donor) after reduced-intensity conditioning (RIC) with thiotepa, cyclophosphamide, and fludarabine. Prophylaxis of graft-vs.-host disease (GvHD) consisted of cyclosporine A (CyA) and short-course methotrexate (MTX). The estimated 3-year overall and progression-free survival rates (OS, PFS) were reported at 81 % and 64 %, respectively. More recently, the same group of investigators gave another report on 52 patients with all major T-cell subtypes, some of whom had been included in the previous report already. In the new publication the majority of patients still had an HLA-identical family donor (n=33); only13 donors were matched but unrelated and six donors were haplo-identical. The T-cell lymphoma subtypes were PTCL (n=23), AITL (n= 9), ALCL, ALK-negative (n=11) and other histologies (n=9). The median age of the patients was rather young (47 years), the same conditioning regimen as before was used but anti-thymocyte globulin (ATG) was added for patients having an unrelated donor. The 5-year OS and PFS was reported at 50 % and 40 %, respectively, with no significant differences between the various histological subtypes. Of note, 51 % of patients with chemo-sensitive disease but only 8 % with chemo-refractory disease were alive at the time of publication.

Older age (>45 years) was another factor negatively influencing OS. Relapse rates after RIC were high (49 % at 5 years) but TRM was rather low (12 %). Donor lymphocyte infusions (DLIs) were given to 12 patients for disease progression and 8 of these (66%) had a response (5 CR and 3 PR). The 5 CR patients were reported to be alive after a median observation time of 5 years (2-8 years) while the PR patients all progressed and died.

Recently, the Center for International Blood and Marrow Transplant Research (CIBMTR) published an overview on their experience with autologous and

allogeneic transplantation in patients with T-cell lymphoma. Covering the time period from 1996 to 2006 the analysis reported clinical results of 51 patients with ALCL (ALK-status unknown), 63 patients with PTCL NOS and 12 patients with AITL. The median age of the recipients at the time of transplantation was 38 years (range 5-60 years) and the median number of previous regimens was three. Sixty percent of recipients had an HLA-identical sibling donor, 24 patients had a matched unrelated donor and 16 patients had a mismatched unrelated donor. For all 126 patients who had been allografted PFS and OS at 3 years posttransplant was 36 % and 39 % after myeloablative conditioning and 33 % and 52 % after RIC. Results did not significantly differ for patients transplanted following myeloablative conditioning or RIC. Non-relapse mortality was 32 % after myeloablative conditioning and 27 % after RIC, relapse rates were 32 % after myeloablative conditioning and 40 % after RIC. There was no difference in outcomes between patients transplanted from HLA-identical siblings or MUDs. Neither acute nor chronic GvHD affected relapse or survival. Chemorefractory disease and higher number of chemotherapy lines before alloSCT negatively impacted on outcome.

Le Gouill et al. summarized the experience of the SFGM (Sociéte Française de Greffe de Moelle et Thérapie Cellulaire). Seventy-seven patients with a median age of only 38 years and various T-cell histologies [PTCL, n=27; ALCL (5 patients ALK- negative; 8 patients ALK-positive; 13 patients with unknown ALK-status) AITL, n=11; and other histologies, n=12] were transplanted from mostly HLA-identical family donors (n=60) or unrelated donors (n=10). Seven patients received transplants from mismatched unrelated donors. The conditioning regimen was myeloablative in 57 of 77 patients (74 %). With a median follow up of 43 months, the 5-year - OS and - EFS was 57 % and 53 %, respectively. Interestingly, 2 patients had received donor lymphocyte infusions (DLI) and achieved durable remissions afterwards. The 5-year transplant-related mortality was 34 %. Factors negatively influencing OS in multivariate analyses were grade 3 to 4 acute GvHD and chemoresistant disease at time of alloSCT. The only factor negatively influencing EFS was the disease status.

Kyriakou et al. reported the experience of the European Group for Blood and Marrow Transplantation (EBMT) in patients with AITL. Forty-five patients were

transplanted between 1998 and 2005 from HLA-identical siblings (n=26), matched unrelated donors (n=16) or mismatched donors (n=3). The median age of this group of patients was 48 years (range 23-68); 33 % of patients had failed a previous autograft. The conditioning regimen was total-body irradiation (TBI) and cyclophosphamide or etoposide in 16 patients, busulfan and cyclophosphamide or other chemotherapy in 8 patients; RIC regimens like fludarabine plus an alkylating agent or low-dose TBI (2 Gy) were used in 21 patients. GvHD prophylaxis was heterogeneous but mostly consisted of CyA alone, CyA plus methotrexate, or CyA plus Mycophenolate Mofetil (MMF). The non-relapse mortality (NRM) was 25 % at 1 year after transplantation, the relapse rate was estimated at 20 % at 3 years, PFS was 54 % and OS was 64 % at 3 years after transplantation. Again, patients with chemosensitive disease had a significantly better OS than patients with refractory disease (81 % vs. 37 % at 3 years). Interestingly, none of the 19 patients developing chronic GvHD relapsed after transplantation, whereas 57 % of patients (n=15) not developing chronic GvHD relapsed. Two of four patients experiencing relapse after alloSCT responded to DLIs.

Glass et al. reported on 63 patients with PTCL (n=25), AITL (n=12), ALCL, ALK-negative (n=11), T-lymphoblastic lymphoma (n=6) and T-PLL (n=5), who received an alloSCT from an HLA-identical sibling (n=22), a matched unrelated donor (at least HLA 8/8 loci compatible, n=41), after conditioning with fludarabine (125 mg/m^2), busulfan (12 mg/ kg) and cyclophosphamide (120 mg/ kg body weight). GvHD prophylaxis consisted of MMF and Tacrolimus. In contrast to other reports, 53 % of patients had active disease immediately prior to transplantation. OS at 3 years was 42 % and PFS was 43 %. TRM was 32.1 % and the relapse rate was 35 % for the whole group of patients. No significant differences in outcome for the different histologies were seen, only the International Prognostic Index (IPI) before transplantation significantly influenced treatment results. Patients with CR, PR or SD prior to alloSCT had very similar outcome, patients with progressive disease fared less well. Patients with acute GvHD > grade 2 showed better results than patients with no GvHD or GvHD grade 1.

Wulf et al. (manuscript submitted) recently updated the German results in a larger cohort of 97 patients and confirmed the aforementioned results.

Obviously, conditioning with fludarabine, busulfan and cyclophospamide followed by transplantation of matched (10/10) related or unrelated grafts can overcome chemo-resistance in a sizeable fraction of patients. GvHD prophylaxis without ATG led to frequent and severe acute GvHD and high TRM. Consequently, our next study will include ATG into the conditioning regimen for all patients.

Jacobsen et al. examined 52 adult patients who underwent allogeneic transplantation at the Dana-Faber Cancer Institute between 1997 and 2009. The T-cell histologies varied broadly but most patients suffered from PTCL (38 %), mycosis fungoides / Sezary syndrome (13 %), ALCL (12 %) or AITL (10 %). The median age of the patients was 46 years, only 21 % of patients had failed a previous autograft. TRM at 3 years was 27 % (36 % for patients after myeloablative conditioning and 14 % after RIC). The cumulative incidence of relapse at 3 years was 43 % [33 % with myeloablative and 57 % with RIC (p=0.049)]. OS at 3 years was 41 %, PFS was 30 %. In multivariable competing risks regression analysis of NRM and relapse, RIC was a significant factor for relapse (p=0.00005), conditioning intensity was not associated with NRM. Interestingly, nine patients received DLI after relapse. Five of these nine patients were still alive at the time of this report and the median OS had not been reached.

Delioukina et al. reported on 27 patients treated with alloSCT after RIC with fludarabine and melphalan. Median age of patients was 50 years and 56 % of patients had an HLA-identical sibling donor. Eleven of these patients (41 %) suffered from a cutaneous T-cell lymphoma. The 2-year-probability of OS was 55 %; PFS was 47 %. The cumulative incidence of relapse / progression was 30 %, NRM was 22 %, respectively.

Czajczynska et al. recently reported the single center experience of the University of Kiel, Germany. Twenty-four patients with a median age of 53 years and all subtypes of T-cell lymphomas received standardized salvage therapy followed by BEAM chemotherapy together with alemtuzumab for conditioning. The donors were matched and unrelated in the majority of cases. Six patients (25 %) died of

NRM, and six patients relapsed. Twenty of 22 evaluable patients reached CR after alloSCT, 50 % of patients were alive and in CR at last follow up. The OS at 3 years was reported at 42 %. (Table 7)

Table 7 Results of allogeneic transplantation in relapsed / refractory T-cell

Author	Patients	Conditioning	NRM	REL	PFS	OS	
Dodero (2011)	52 (33)	TT/Cy/Flu	12%	49%	40%	50%	
Le Gouill (2008)	77 (60)	Myeloablative	34%	N.E.	N.E.	57%	
Kyriakon (2009)	45 (26)	Various	25%	20%	20%	64%	AITL only
Glass (2011)	63	Flu/Bu/Cy	32%		43%	42%	29 refractory pts
Jacobsen (2011)	52 (24)	TBi/Cy (31) Bu/Flu (21)	36% 14%	33% 57%	30%	41%	Myeloablative RIC
Delioukina (2012)	27 (15)	Flu/Mel	22%	30%	47%	55%	CTCL in 11 pts
Czajczynska (2013)	24 (5)	BEAM/ALEMT	25%	25%	N.E.	42%	
Smith (2013)	74 45	MAC RIC	32% 27%	32% 40%	36% 33%	39% 42%	

lymphomas. NRM= non-relapse mortality, REL= relapse, PFS= progression-free survival, OS= overall survival, MAC=myeloablative conditioning, RIC=reduced intensity conditioning.

3.5.2 Hematopoietic Stem Cell Transplantation in Patients with Lymphomatoid Granulomatosis[110]

The major patient characteristics, lines of treatment, and outcomes are summarized in Table 7. The patient cohort finally consisted of five females and five males aged between 19 and 53 years at the time of transplantation. Eight of ten patients had been diagnosed with stage IV disease, the lungs were the most frequently involved extranodal site (eight of ten patients) but involvement of liver, CNS, kidneys, adrenals and bone marrow was also described. All patients

had received at least two lines of therapy before transplantation including Rituximab in 7 patients the median time interval from diagnosis to SCT being 9.5 (range 5-52) months. All patients had active disease at the time of SCT: six patients were in partial remission (PR) immediately prior to their first transplant, two had stable disease (SD) and two presented with progressive disease (PD). Eight patients received HDT / ASCT as a first transplant; one of these patients (patient 1) received a second autologous transplant after HDT including ibritumomab tiuxetan (Zevalin®), another patient (patient 6) had a planned double transplant procedure: BEAM / ASCT was followed by reduced-intensity conditioning (RIC) and alloSCT from a matched unrelated donor (MUD). Three more patients (patients 3, 5, 7) received allografts after RIC, one of them because of relapse after HDT / ASCT (patient 3). The stem cell donor was an HLA matched sibling in one and a MUD in the remaining two. Five of the eight ASCT patients were transplanted after BEAM, the other patients received other HDT including thiotepa, melphalan and carboplatinum; fludarabine, busulfan and cyclophosphamide; or total body irradiation in combination with thiotepa and cyclophoshamide. All conditioning regimens prior to allogeneic transplantation were of reduced intensity (see table 8).

With a median follow up of 5.1 (range 1.4-6.3) years for surviving patients six of ten patients are alive with no evidence of disease. Notably, only two patients relapsed after transplantation; one of these patients is in continuous complete remission (CCR) for almost 4 years after a second ASCT, the other patient died three months after allogeneic transplantation from severe GvHD of the gut; at this time, histology was diffuse large B-cell lymphoma rather than LG.

Two ASCT patients died of septicemia on days +8 or +9 after transplantation, respectively; no evidence of LG was found at the autopsy in one of these patients, the other patient had evidence of PD after ASCT. Unfortunately, patient 8 committed suicide 19 months post ASCT while being in remission from LG.

Table 8 Patients Characteristics.

Patient	Age*/ Gender	Stage†/Grade	Sites of Disease†/Yr of Diagnosis	Lines of Therapy	Preparatory Regimen	Transplant/Time from Diagnosis to Transplantation (mo.)	Disease Status†	Best Response after Transplantation	Relapse	Outcome
1	38/F	IV A/3	Lung/2005	R-CHOP R-I, MTX, E	BEAM	Auto/7	PR	PR	Yes	Received second auto
					Z-BEAM	Auto	Relapse	CR	No	A&W, d +1456
2	48/M	I_E B/1	Lung/2007	Pred R R-CHOP	BEAM	Auto/16	SD	Autopsy: no evidence of LG	No	Died, d +8: septicemia, VOD
3	37/F	IV/not known	Lung, liver, lymph nodes/2004	CHOP DHAP ICE	BEAM	Auto/7	PR	CR	Yes	NA
					FM	Allo	NE	NA	No	Died, d +1567: GvHD
4	53/F	IV B/2	Lung, liver, lymph nodes/2007	Cy-Dex R-CHOP DHAP α-IFN HD-Cy	FBC[12]	Auto/9	SD	CR	No	A&W, d +1864
5	31/F	II A/not known	Lymph nodes/2002	V-CAMP R-ICE	FBC	Allo/52	PR	CR	No	A&W, d +1554
6	19/M	IV A/3	Bone marrow, lymph nodes/2001	COPADM-COPAD R DHAP-VIM-DHAP	BEAM	Auto/52				
					FM[18]	Allo	PR	CR	No	A&W, d +2119
7	34/M	IV B/2	Lung, CNS/2004	R HD-MTX AraC[16]	FM[18]	Allo/13	PD	not known	No	A&W, d +2314
8	31/M	IV A/2/3	Lung/2000	Pred Cy CHOP	BEAM[5]	Auto/10	PR	CR	No	Died, d +576: suicide
9	53/M	IV B/2/3	Lung/2005	R-CHOP ASHAP	TMC	Auto/5	PD	PD	not known	Died, d +9: PD, sepsis
10	49/F	IV B/1	Lung, liver, CNS, adrenals, kidney/2011	R-CHOP MTX R-IE, AraC	TC/TBI	Auto/5	PR	PR	No	A&W, d +502

R indicates rituximab; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; I, ifosfamide; MTX, methotrexate; E, etoposide; BEAM, BCNU, etoposide, cytosine-arabinoside, melphalan; Z-BEAM, Zevalin+BEAM; Pred, prednisone; DHAP, dexamethasone, cytosine-arabinoside, adriamycin, methotrexate, prednisone; ICE, ifosfamide, cyclophosphamide, etoposide; Cy, cyclophosphamide; IFN, interferon; FBC, fludarabine, busulphan, cyclophosphamide; V-CAMP, vincristine, cyclophosphamide, adriamycin, methotrexate, prednisone; COPADM-COPAD, cyclophosphamide, vincristine, prednisone, doxorubicin, dexamethasone, methotrexate; VIM, VP-16, ifosfamide, methotrexate; AraC, cytosine-arabinoside; ASHAP, adriamycin, solumedrol, cytosine-arabinoside, platinum; FM, fludarabine, melphalan; TMC, thiotepa, melphalan, carboplatinum; TC/TBI, thiotepa, cyclophosphamide, total body irradiation; A&W, alive and well; PR, partial remission; PD, progressive disease; d, day; GvHD, graft-versus-host disease; VOD, veno-occlusive disease.

* At time of transplantation.

† At diagnosis.

3.5.3 Risk of CNS Recurrence and Role of Prophylaxis in Diffuse Large B-Cell Lymphoma[111]

3.5.3.1 Risk of CNS disease[111]

Table 9 summarizes findings of larger studies (> 200 patients) in patients with aggressive B-cell lymphoma. Some of these studies randomized patients to R-CHOP or CHOP; for these studies Table 9 reports the number of patients on R-CHOP only; the risk factors mentioned are those found by multi-variate analyses in patients treated with R-CHOP. Altogether, the data demonstrate that secondary CNS involvement is a relatively rare complication of DLBCL occurring in 2.3 – 8.4 % of patients analyzed. The variation in frequencies of CNS disease reported by different authors most probably reflect the different patient characteristics, especially the differences in age and distribution of IPI factors. It remains controversial if the addition of Rituximab to CHOP decreased the incidence of CNS disease. If any, the effect of Rituximab is moderate and the problem of CNS disease is by no means solved. Risk factor analyses presented in Table 9 as well as those from other smaller or less well documented studies of the Rituximab- and pre-Rituximab era show that individual factors of the International Prognostic Index (IPI) (age>60, high LDH, poor performance status, advanced stage and more than 1 extranodal site) as well as various combinations thereof significantly impact on the risk of CNS disease. In addition, involvement of testis, kidney, adrenals, breast, bone marrow, and bone have been reported to increase the risk of CNS disease. In the largest study done so far including 2164 patients treated with R-CHOP or R-CHOE(etoposide)P on prospective trials conducted by the German High-Grade Lymphoma Study Group (DSHNHL) we found evidence that presence of any of the 5 IPI factors and involvement of kidneys/ adrenals increased the risk of CNS disease. However, even the roughly 6 % of patients with 4 to 6 risk factors had a 2-year-rate of CNS disease of no more than 10 %. This model has recently been validated on an independent data set from British Columbia.

Biological risk factors like MYC translocation, double-hit lymphomas, or the presence of certain adhesion molecules on lymphoma cells have recently been

associated with an increased risk for CNS disease. These hypotheses have been derived from retrospective analyses of small cohorts of patients. Therefore, findings need to be validated and it seems premature to use any of these biological factors to change diagnostic procedures or treatment.

3.5.3.2 Prophylaxis of CNS disease[111]

Typically, prophylaxis of CNS disease in DLBCL consists of intrathecal (i.th.) injections of methotrexate (MTX), cytosine-arabinoside (Ara-C), predniso(lo)ne (PRED) or combinations of these drugs. This practice is based on the positive experience gained in patients with ALL where CNS involvement was frequent but almost always is restricted to the CSF. In contrast, the percentage of patients with DLBCL developing CNS disease is much lower and about 50 % of patients present with isolated or combined involvement of the brain parenchyma. Because i.th. chemotherapy does not reach measurable concentrations in the brain parenchyma except for areas directly adjacent to the brain the concept of i.th. prophylaxis in patients with DLBCL must generally be questioned. In practical terms, and particularly for patients treated with R-CHOP there is increasing evidence from almost all recent studies summarized in Table 10 that i.th. prophylaxis is not effective. Table 2 also shows that in some studies CNS relapses seem to have occured more frequently in patients with than in patients without i.th. prophylaxis. This, however, must be considered an artifact because the patient cohorts who received prophylaxis were enriched for patients deemed at high risk for CNS disease. Given the major toxicities (leucopenia, infections, mucositis) seen with i.th. injections of cytotoxic drugs especially in older and frail patients this practice should be restricted to very high-risk patients or completely be abandoned. The only exception should be patients with involvement of the testes where i.th. prophylaxis should strictly be administered in conjunction with specific systemic treatment and local radiotherapy. For all other organs (bone marrow, bone, paranasal sinuses, breast, skin) and risk groups defined by early clinical studies to indicate high-risk the evidence supporting the use of i.th. prophylaxis is very scarce particularly in patients treated with R-CHOP and omission of CNS prophylaxis should be strongly considered.

If alternative strategies like administration of systemic therapy with high-dose MTX (>1.5g/m^2) or liposomal Ara-C i.th. is more effective, in preventing CNS progression or relapse is currently under study. The results of the French studies with (R)-ACVBP and currently available phase II studies using 1 or 2 courses of high-dose MTX in patients treated with R-CHOP suggest that this treatment is more effective than i.th. prophylaxis and high-dose MTX should be the prophylaxis of choice in high-risk patients who are fit enough to tolerate the toxicities which undoubtedly accompany this treatment.

Table 9 Risk factors for CNS disease in patients with DLBCL[¶] using (R)-CHOP and variants.

Study	Patients [§]	IPI	↑LDH[†]	>1 ENS	Advanced Stage	Extranodal site	Other
Tomita	82/1221 (6.7%)	NS	NS	NS	NS	breast 10.5 adrenal 4.6 bone 2.0	age > 60 2.1
Schmitz	14/620 (2.3%)	Not Applicable	3.8	NS	5.4	NS	R 0.3, not in high-risk patients
Boehme	22/608 (3.6%)	NR	S	S	NS	NR	ECOG > 1
Tai	19/320 (6.0%)	NS	NS	NS	NS	kidney 20.1 testis 6.7 breast 6.1	ECOG > 1 2.0 non-CR 3.3
Villa	19/309 (6.1%)	NS	NS	NS	stage IV 8.0	kidney 3.3	
Shimazu	20/238 (8.4%)	NR	2.4	2.0	NS	marrow 2.1	age > 60 2.5
Guirguis	8/214 (3.7%)	NS	NS	NS	NS	testis 33.5	None
Yamamoto	81/203 (3.9%)	NS	NS	NS	NS	NS	
Chihara	9/203 (4.4%)	NS	NS	Any EN 2.9	NS	NS	Bulk > 7.5 cm 3.34 ALC < 1.0 x 10^9 / L 2.38
Feugier	11/202 (5.4%)	S	S	NS	NS	NS	ECOG > 1

[¶] studies by Schmitz et al and by Boehme et al contain about 15 % of patients with other aggressive B-cell lymphomas (blastoid mantle cell lymphoma, follicular lymphoma grade 3);

[§] patients with CNS disease / patients on study;

[†] numbers in columns LDH, > 1 ENS, advanced stage, extranodal site, and other are hazard ratios reported from multivariate analyses.

CNS, central nervous system; R, rituximab; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; IPI, International Prognostic Index; LDH, lactate dehydrogenase; ENS, extranodal site; NS, not significant; S, significant; ECOG, Eastern Cooperative Oncology Group performance status; CR, complete response; NR, not reported; ALC, absolute lymphocyte count.

Table 10 Prophylaxis of CNS relapses in studies using (R)-CHOP and variants for treatment of DLBCL.

Study	Number of Patients	Systemic / i.th. treatment	CNS prophylaxis	CNS relapses (%)	P value
Schmitz	2196 (1576 w/o R, 620 w/ R)	(R)-CHO(E)P* i.th. MTX	BM, testis, head, sinuses, orbits, oral cavity, tongue and salivary glands	2.6% (all pts)	$p = 0.386$
Boehme	1222 (612 w/o R, 610 w/ R)	(R)-CHOP i.th. MTX	BM, testis, head, sinuses, orbits, oral cavity, tongue and salivary glands	2.5% (w/o prophylaxis) 4.4% (w/ prophylaxis)	NS
Kumar	989 (all w/ R)	R-CHOP i.th. MTX ± Ara-C, i.v. MTX	at the discretion of investigator	2.1% (w/o prophylaxis) 10.9% (w/ prophylaxis)	$p = 0.007$
Tai	499 (179 w/o R, 320 w/ R)	(R)-CHOP i.th. MTX	>1 ENS, orbits, sinuses, breast, testis, bone, BM	5% (w/o prophylaxis) 11% (w/ prophylaxis)	NR
Tomita	322 (all w/ R)	R-CHOP i.th. MTX	↑LDH, bulk >10, PS ≥ 2, BM, nasal, bone, breast, skin, testis	2.8% (w/o prophylaxis) 7.5% (w/ prophylaxis)	$p = 0.14$
Arkenau	259 (177 w/o R, 62 w/ R)	(R)-CHOP (R)-PmitCEBO i.th. MTX ± Ara-C	BM, testis, sinuses, orbits, bone, blood	1.1% (CI 0-2.5%) 2 pts w/o prophylaxis 1 pt w/ prophylaxis	NR
Guirguis	214 (all w/ R)	R-CHOP i.th. MTX (25 pts), i.v. MTX (17 pts)	↑ LDH, > 1 ENS, testis, epidural, sinuses or skull	2% (w/o prophylaxis) 1.9% (w/ prophylaxis)	NR

i.th., intrathecal; R-PMitCEBO, rituximab, prednisolone, mitoxantrone cyclophosphamide, etoposide, bleomycin, vincristine; E, etoposide; MTX, methotrexate; Ara-c, cytarabine; BM, bone marrow; NR, not reported; i.v.: intravenous.

*:includes patients treated with higher doses of cyclophosphamide, adriamycin, and etoposide and patients treated with dose-escalated sequential high-dose therapy and Rituximab.

4 Discussion

4.1 Anti-B-cell antibodies

Monoclonal antibodies directed against antigens present on the surface of malignant and normal B-cells have revolutionized the treatment of virtually all types of B-cell lymphoma. Rituximab, a chimeric IgG1 anti-CD 20 antibody, was first described in 1994[104] and after extensive pre-clinical testing entered the clinic approximately 16 years ago.[24] Mostly in combination with classical chemotherapy, this antibody improved survival of patients with many subtypes of B-cell lymphoma by 10 – 20 % when compared to the effects of chemotherapy only (shown in Figure 4).[8,10,23,24]

Rituximab displays several unique properties which may explain the clinical success of this antibody. First of all, it binds to the CD 20 antigen, a non-glycosylated protein of 33 to 35 kDa, which is highly expressed through all stages of B-cell development from the early pre-B-cell stage to mature B-cells. It is lost during final B-cell maturation to plasma cells.[46-48] Furthermore, the CD 20 antigen is unique as it is not shed from the cell surface into the circulation probably because it is deeply anchored in the cell membrane with a very short extracellular segment (43 residues between 3rd and 4th transmembrane region). It is also not internalized into the B-cell after antigen-antibody binding and therefore appears an ideal target for the recruitment of effector cells expressing Fc gamma receptors.[50-52]

Following the clinical success of rituximab as part of treatment of all B-cell lymphomas at all stages, other anti-CD 20 antibodies were developed. After understanding the most important mechanisms of action of rituximab and other first-generation antibodies Fc-engineered variations of anti-B-cell antibodies were constructed in order to improve binding to human Fc gamma receptors. New antibodies were designed but only few were finally approved for clinical use: until now ofatumumab, obinutuzumab and Ibritumomab tiuxetan, a

radiolabelled antibody, remain the only anti-CD 20 antibodies which were licensed in the US and Europe for treatment of lymphoma (Table 2). Tositumomab was withdrawn from the market in February 2014 due to a rapid decline in usage after pivotal studies had shown negative results and hence business deteriorated significantly.

4.2 Mechanisms of action

Comparative research looking into the mechanisms of action of various anti-CD 20 antibodies, elucidated that three types of function largely can explain the clinical efficacy of anti-B-cell antibodies: signaling in target cells on CD 20 binding leading to growth inhibition and (nonclassic) apoptosis (referred to as “direct cell death”), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC) mediated by cells displaying Fc gamma receptors such as NK cells and macrophages. The epitope and / or binding mode have been shown to dictate 2 major types of anti-CD 20 antibody effector function profiles, termed type I or type II. Although both types of antibodies bind bivalently to CD 20, they form distinct complexes with CD 20. Type I antibodies stabilize CD 20 on lipid rafts, leading to stronger C1q binding and potent induction of CDC. This binding mode triggers only low levels of direct cell death. In contrast, type II antibodies do not stabilize CD 20 in lipid rafts thus exhibiting reduced binding to C1q and lower levels of CDC, but they potently induce direct cell death.[33] The majority of anti-CD 20 antibodies, including the commercially available anti-CD 20 antibodies rituximab and ofatumumab are of type I, whereas the murine antibody B1 (tositumomab) and the humanized IgG1 antibody obinutuzumab are thought to represent type II antibodies. Obinutuzumab, previously known as GA 101, was under clinical development while the experimental studies reported here were performed. First data from early clinical studies in lymphoma[111] and comparative studies in chronic lymphocytic leukemia became available in 2014.[112] After publication of a phase III study comparing obinutuzumab with rituximab, obinutuzumab was recently licensed for treatment of CLL; it continues to be tested in large phase III trials comparing it to the standard anti-CD 20 antibody rituximab in major lymphoma subtypes (follicular lymphoma and DLBCL).

Simultaneously, other monoclonal antibodies have been described which are directed against alternative epitopes on immature and mature B-cells. Among others, the surface antigens CD 19, CD 22, CD 23, CD 37, CD 40 and CD 80 which are all present on normal B-cells at distinct stages of development and their malignant counterparts have been investigated.[35-46] While most antibodies raised against alternative B-cell antigens, lacked significant advantages over the antibodies already licensed and therefore were not further developed towards clinical application, the anti-CD 37 antibody (BI 836826) showed promising in vitro activity and is currently in phase I clinical studies in order to evaluate its safety profile and optimal dosing in man. We set out to investigate the effects of BI 836826 on a broader spectrum of cell lines and patient cells than originally reported[65] and compared its properties with licensed anti-CD 20 antibodies including the recently marketed antibody obinutuzumab.

The tetraspanin CD 37 is predominantly expressed on mature B-cells, with highest expression levels on peripheral blood B-cells. Low level expression has been reported on plasma cells, T-cells, granulocytes, and monocytes. Strong and homogeneous CD 37 expression has been demonstrated on the surface of B-cell lymphomas and leukemias.[57,62-65] In our experiments we used a chimerized high-affinity mouse antibody to CD 37 which has been engineered at the CH2 domain to improve binding to human Fc gamma receptors.[65]

The work presented here aimed at answering the following important questions which arise when anti-B-cell antibodies are investigated and compared:

a. How many and which B-cell lymphoma cell lines should be tested in order to produce results which reliably predict the performance of new antibodies ?

b. Can cell lines - which are much more readily available than patient material - grown from various types of lymphoma act as antibody target cells and substitute for true lymphoma cells which need to be obtained fresh from individual patients?

c. Which of the commercially available anti-CD 20 antibodies performs best in vitro? Do the experimental data correlate with results obtained in clinical trials?

d. How does BI 836826, a new antibody not targeting the CD 20 antigen but the CD 37 antigen on B – cells, perform in tests established to compare anti-CD 20 antibodies? Do the results of in vitro testing look promising enough to support further clinical development of BI 836826?

4.3 Evaluation of cell lines

The first question was approached by using various B-cell lymphoma cell lines as target cells for the assays described in the Methods section. The selection of the cell lines investigated was based on their cells of origin[113-115]; in particular we sought for cell lines representing the most common form of B-cell lymphoma (DLBCL) and its variants recently defined by gene expression profiling (GEP): the GCB- and the ABC-type DLBCL. Using five different cell lines (SU-DHL-4, Ramos, Karpas 422, OCI-Ly 3 and OCI-Ly 10) a broad spectrum of lymphoma was ultimately covered.

Under strictly identical culture conditions and experimental design (effector to target cell ratio, incubation periods, antibodies concentration, etc.) comparison of the killing efficacy showed unexpectedly homogenous patterns of antibody activity with all methods established to investigate the major mechanisms of action described for anti-B-cell antibodies. Thus, although absolute percentages of cell kill by ADCC measured by the CD 107a degranulation assay and the Europium release assay, by programmed cell death, early and late apoptosis measured by Annexin V / PI assay, or the strength of ISF varied from cell line to cell line, the patterns of antibody activity were similar for all cell lines tested. For example, obinutuzumab and BI 836826 performed better than rituximab or ofatumumab with ADCC, PCD, early and late apoptosis regardless of which cell line was used to represent the lymphoma target cell. Probably because of the limited number of experiments which could be done due to the complex

experimental design some of the results obtained with different cell lines differed significantly while others did not. The comparative patterns obtained with various cell lines, however, were similar suggesting that the use of one or two cell lines may suffice if testing of a broader variety of cell lines is not considered practical.

Not unexpected, the results of CDC were different from those obtained with the other assays (summarized as non-CDC assays) because ofatumumab and rituximab are considered type I antibodies while obinutuzumab is considered a type II antibody.

In line with previous reports on rituximab and ofatumumab, these antibodies gave best results for CDC while BI 836826 performed intermediate. CDC of obinutuzumab varied but overall was only 20 to 30% because without addition of serum as a source of complement, already 20 – 30 % of cells were found dead. This might be explained by PCD induced without complement. Nevertheless, variations in CDC depending on the cell lines used and the origin of lymphoma cells occurred. The finding demonstrates that exceptions to the general patterns do exist and testing of more cell lines may give more precise information than one or two cell lines can provide.

In practical terms, because of constraints in time and financial resources restricting in vitro testing to 1 or 2 cell lines instead of 5 or more is highly desirable. Therefore, our recommendation would be to in first place use SU-DHL-4 representing GBC-type DLBCL and OCI-Ly 10 representing ABC-type DLBCL because these two cell lines are relatively easy to grow and to keep healthy; both cell lines also turned out to be robust with regard of the reproducibility of the experimental results. If results with these 2 cell lines look promising it may be useful to expand experiments with other cell lines thought to represent other lymphoma subtypes.

4.4 Evaluation of patient lymphoma cells

In order to obtain information if cell lines, e.g. SU-DHL-4 or OCI-Ly 10, can be used to obtain reliable information on the performance of different anti-B-cell antibodies with patients' lymphoma cells we compared the results of all established assays (ADCC, PCD, CDC) using all cell lines or cells from patients with different lymphoma subtypes (DLBCL, FL and MCL). We also wanted to investigate if results might differ if individual antibodies were used to treat different lymphoma subtypes like DLBCL cells, FL cells, or MCL cells.

Generally speaking, results obtained with rituximab, ofatumumab, obinutuzumab and BI 836826 when DLBCL, FL and MCL cells were used as target cells matched the results seen with different cell lines, and with SU-DHL-4 and OCI-Ly 10 in particular. Obinutuzumab and BI 836826 gave best results with ADCC (CD 107a and Europium assay) and early as well as late apoptosis while ofatumumab and to a lesser extent rituximab performed well in CDC. These diverging results were expected because obinutuzumab is considered a type II antibody, rituximab and ofatumumab are type I antibodies. Results obtained with fresh patient lymphoma cells were consistent with results obtained with different cell lines. It needs to be mentioned that the activity of all antibodies tested for CDC and non-CDC assays was significantly weaker with patient serum or cells as compared to healthy donor serum or cells. Because serum and effector cells with very few exceptions were obtained from patients with relapsed disease, this finding may indicate that previous therapy led to exhaustion of cytotoxic effector systems including complement.[110] Among the different lymphoma subtypes tested, MCL seemed to be the subtype which was most difficult to attack. Especially the results of ADCC and CDC using optimal experimental conditions were less impressive than for FL and DLBCL cells. Interestingly, these results correlate with clinical experience that treatment results of MCL with rituximab (and chemotherapy) are less favorable than in other lymphoma subtypes (Figure 48).[116]

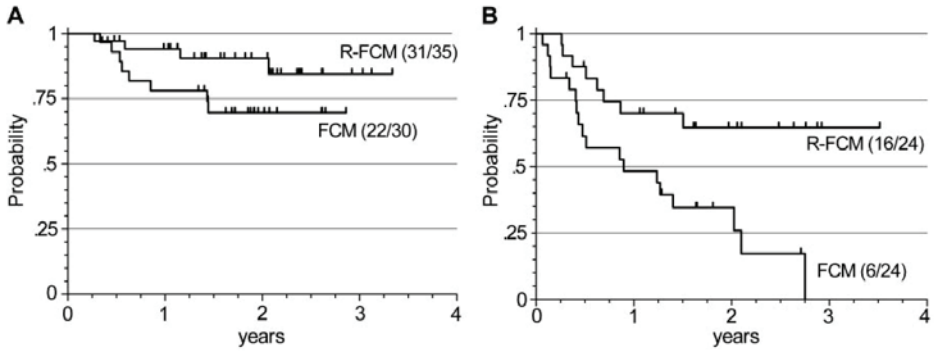

Fig. 48 Overall survival after start of therapy for patients with FL and MCL randomized for FCM* or R-FCM**.[116]

*FCM: fludarabine, cyclophosphamide, mitoxantroneon; R:rituximab

In conclusion, testing of new anti-B-cell antibodies against fresh patient lymphoma cells resulted in patterns of activity which resemble those obtained in cell lines. Therefore, testing new anti-B-cell antibodies against SU-DHL-4 (and other cell lines if early results are favorable) is a reasonable first step. Importantly, our results do not support the notion that early testing against fresh lymphoma cells is absolutely required. Relative activities of established and new antibodies against SU-DHL-4 and lymphoma cells were generally comparable. For MCL results of most assays (CDC and non-CDC) were significantly worse with fresh patient cells compared to any of the cell lines investigated. Therefore it might be worthwhile to investigate MCL cells separately.

4.5 Evaluation of tests required for in vitro testing of anti-B-cell antibodies

Another important question is if all tests performed here must be done in order to correctly judge the potency of different antibodies. Referring to the respective literature, almost all publications dealing with in vitro investigations on anti-B-

cell antibodies used one test for CDC, one for apoptosis, and one for ADCC.[65,72,74] Because it remains to be settled which of the three major mechanisms of action (ADCC, PCD or CDC) described for anti-B-cell antibodies are clinically most relevant, it seems logical to do at least one in vitro assay reflecting each postulated mechanism.

Before investigating ADCC, PCD, and CDC, we first looked at ISF, a very early event deemed necessary when NK- and T-cells are to attack lymphoma target cells. ISF gives a visional impression how effector and target cells get into contact and how intense the contact is. We saw clear differences in strength and velocity of ISF between different antibodies. Because no other reports on ISF for any of the antibodies tested here and the functional consequences have been published, it is not possible to compare our findings to those of other investigators. We found that obinutuzumab induces weak ISF (no cytoskeleton polarization was observed) compared to other antibodies but cannot comment on the functional consequences of this observation. However, obinutuzumab tends to induce homotypic aggregation (Figure 47). When target cells were incubated with antibody only, obinutuzumab induced strong polarization of the cytoskeleton (data not shown). This result is in line with the data published by Alduaij et al.[79] Obviously, this antibody has clinical effects without ISF being absolutely necessary. Regarding ADCC, we established the Europium release assay and the CD107a degranulation assay to specifically investigate the efficacy of Fc-engineered antibodies. While the CD 107a assay measures the activation of effector cells, namely NK-cells, by labeled target cells, the Europium release assay reflects the death of target cells as a result of ADCC. Thus, both assays are complementary if the major components of ADCC namely effector cell activation and the resulting cell death need to measured. If information on the decisive final step of ADCC (death of the target cell) is deemed most important, doing the Europium assay may suffice. Third generation antibodies have been manipulated in order to improve the binding affinity between the Fc receptor portion of antibody and the effector cell. We indeed found that Fc-engineered antibodies, such as obinutuzumab and BI 836826, resulted in high expression of CD 107a implying that NK cells were more activated and actively released lytic proteins, like perforin and granzymes. Third generation antibodies also induced death of

the target cells in higher percentages implicating that stronger activation of effector cells resulted in killing of more lymphoma cells.

Apoptosis was investigated by the Annexin V / PI assay. Using different gating strategies early stage apoptosis and late stage apoptosis can be described. Most investigators interested in apoptosis use the same assay with minor modifications. This assay is crucial in judging the efficacy of anti-CD 20 antibodies and must be performed in order to compare efficacy.

The CDC test performed here with few modifications matches the tests used by other investigators. Because a completely different and potentially very important mechanism of action of anti-CD 20 antibodies is monitored by this assay, CDC testing cannot be deleted from the test panel for anti-CD 20 antibodies.

4.6 Evaluation of anti-CD 20 antibodies

Rituximab has demonstrated clinical efficacy in thousands of patients worldwide and a wide range of ex vivo data were accumulated over time. Therefore, it seems adequate to use rituximab as a reference antibody and compare results obtained with other anti-CD 20 (and anti-B-cell) antibodies to those of rituximab.

When comparing the other anti-CD 20 antibodies which are clinically available, namely ofatumumab and obinutuzumab, to rituximab, ISF was significantly less strong than with rituximab. Similar results were obtained with cell lines and patient lymphoma cells (Figures 15 – 18).

ADCC measured by the CD 107a degranulation assay favored obinutuzumab when cell lines representing GCB- and ABC-type lymphoma cell lines or patient lymphoma cells were used as target cells. The only exception was that obinutuzumab seemed less effective when labeled MCL cells were lysed with patient effector cells. The Europium assay measuring ADCC in a different way gave comparable results: obinutuzumab performed best with cell lines and patient lymphoma cells.

Comparing the type I antibodies rituximab and ofatumumab with the type II antibody obinutuzumab, obinutuzumab showed less CDC compared with the type I antibodies. Interestingly, in CDC experiments (especially with SU-DHL-4, Ramos, OCI-Ly 10 and also with FL cells), 20 - 30 % more target cells were killed as compared to other antibodies before serum was added. This may be a consequence of the stronger PCD exerted by type II antibodies. Looking at CDC results with patient lymphoma cells ofatumumab and rituximab performed best (no significant difference between these two antibodies) while obinutuzumab showed inferior results in FL and DLBCL cells. In patients' MCL cells no difference between any of the antibodies tested was detected.

Overall, obinutuzumab performed best in all non-CDC assays while CDC results were inferior to those of type I antibodies. CDC with MCL cells was low but did not show any difference between the anti-CD 20 antibodies tested while with FL and DLBCL cells results with obinutuzumab were significantly worse than with type I antibodies.

Comparing the results of anti-CD 20 antibodies obtained with all in vitro tests, obinutuzumab seems preferable because of its strong performance in all non-CDC tests while showing at least some activity in CDC.

A review of the literature comparing rituximab with obinutuzumab gave results which are in line with our findings as far as cell lines are concerned.[74] Data with live lymphoma cells have not been reported by others. Therefore, a comparison of our data obtained with MCL, FL and DLBCL cells is not possible.

4.7 Evaluation of Anti-CD 37 antibody BI 836826

BI 836826 is a novel mouse-human chimeric IgG1 Fc-engineered monoclonal antibody which in contrast to the other antibodies tested binds to the CD 37 antigen which is highly expressed on B cells during pre-B to peripheral mature B-cell stage. Because extensive pre-clinical testing has shown favorable results;[65] BI 836826 is currently being evaluated in phase I clinical trials for patients with chronic lymphocytic leukemia and relapsed B-cell lymphomas.

Heider et al. investigated the binding of BI 836826 to the CD 37 antigen and to Fc gamma receptors. Apoptosis induction and ADCC was assessed on Ramos Burkitt lymphoma cells and cells derived from patients with CLL. While our results obtained with Ramos cells for apoptosis and ADCC were comparable to the results of Heider et al. we expanded apoptosis and ADCC testing using other methodologies (the CD 107a degranulation assay and the Europium release assay) and more cell lines (SU-DHL-4, Karpas 422). Furthermore, we also investigated ISF and CDC. ISF using SU-DHL-4 and OCI-Ly 10 labeled with BI 836826 was quick and strong; no other antibody tested showed comparable ISF ($p<0.05$). ADCC measured by the CD 107a degranulation assay and the Europium release assay gave results that were similar to obinutuzumab and significantly better than rituximab and ofatumumab. This pattern was observed with all cell lines tested. The CD 107a degranulation assay and the Europium release assay gave similar patterns of efficacy.

Also the Annexin V / PI assay (programmed cell death) showed best results for BI 836826 and obinutuzumab while all other antibodies performed significantly worse. CDC with SU-DHL-4, Ramos, and Karpas showed best results for the type I antibodies, ofatumumab and rituximab. However, CDC with BI 836826 was not significantly lower than CDC achieved with ofatumumab and rituximab using OCI-Ly 3, OCI-Ly 10, Ramos and Karpas cell lines. Only SU-DHL-4 cells were not effectively lysed in the presence of BI 836826. Using DLBCL cells, FL cells or MCL cells, ADCC and apoptosis was comparable to the type II antibody, obinutuzumab;

all other antibodies performed significantly less as well. CDC with MCL, FL, and DLBCL cells resulted in cell killing comparable to obinutuzumab in all combinations tested. Only with MCL using healthy donor serum as a source of complement BI 836826 performed less well.

Taken together, experimental results with BI 836826 compared favorably to those obtained with ofatumumab and rituximab. BI 836826 performed comparable to obinutuzumab in all non-CDC assays. For CDC, results varied from cell line to cell line and from lymphoma subtype to subtype. Therefore, BI 836826 is a promising new antibody for treatment of B-cell lymphoma. Because another antigen (CD 37) on malignant B-cells is targeted also a combination with an anti-CD 20 antibody might be an attractive possibility.

4.8 Additional discussions on treatment of T- and B-cell lymphoma

4.8.1 Allogeneic transplantation in relapsed and refractory T-cell lymphoma [109]

An overview of treatment strategies for T-cell lymphoma has been recently published. [121]

4.8.1.1 Allogeneic transplantation as part of first-line therapy in T-cell lymphoma

The poor prognosis of most patients with T-cell lymphoma after conventional chemotherapy and the favorable results of alloSCT in patients with relapsed and refractory T-cell lymphoma raised interest in using alloSCT earlier in the course of disease. Recently, Corradini et al. reported the first results of a prospective study following such an approach. The authors enrolled 64 patients mostly with PTCL-NOS (n=38) on a study which commenced treatment with 2 courses of CHOP-21 preceded by alemtuzumab (30 mg total dose). Treatment continued with 2 courses of Hyper-C-Hidam (high-dose methotrexate, hyperfractionated cyclophosphamide, and high-dose cytosine- arabinoside). Responding patients were to receive an autologous or an allogeneic graft based on donor availability (genetic randomization).

Disappointingly, only 62% of patients finally received a transplant; the other patients had either died of toxicity (5 patients) or suffered an early relapse (18 patients) before proceeding to transplantation. The conditioning regimen consisted of thiotepa, cyclophosphamide and fludarabine. Sixteen patients (70%) who received an alloSCT were alive in CR at the time of the report, 4 patients had died of disease and 3 died of toxicity. The authors conclude that the use of alemtuzumab and pre-transplant high-dose chemotherapy was unable to increase the number of patients proceeding to transplantation. Because of low patients numbers a statistically sound comparison of alloSCT and HDT / ASCT was not possible; however, no obvious differences in OS of patients receiving autologous or allogeneic transplantation was noted.

4.8.1.2 Allogeneic transplantation for patients with mycosis fungoides and sezary syndrome

After a number of case reports had documented the feasibility and had reported promising results the EBMT published the largest series of patients with Mycosis fungoides (MF) or Sezary syndrome (SS) who had received an allogeneic transplant for relapsed or refractory disease. A total of sixty patients with MF (n=36) or SS (n=24) received a first allogeneic transplant from matched related (n=45) or unrelated (n=15) donors. The median patient age was 46.5 years, 40 patients presented with advanced stage at the time of transplantation. Forty-four patients had been prepared with RIC and 16 patients with myeloablative conditioning. Twenty-five patients underwent T-cell depletion, 20 of them using alemtuzumab. The estimated OS and PFS at 3 years was 54 % and 34 %. Advanced disease and the use of an unrelated donor negatively influenced treatment results. NRM was 22 % at 2 years, myeloablative conditioning and poor performance status had independent adverse effects on NRM. The cumulative incidence of relapse was 47 % at 3 years. T-cell depletion was the strongest adverse factor influencing relapse rates. Chemosensitivity was not associated with the risk of relapse. These results have recently been updated.[20] With a median follow up of now 5 years, OS and PFS are reported at 48 % and 33 %, respectively. Disease progression remains the major problem with 26 patients having relapsed or progressed at a median of 3.8 months. Advanced disease, having an unrelated donor, and the use of myeloablative conditioning negatively impacted on survival.

4.8.1.3 Allogeneic transplantation in patients with NK / T-cell lymphoma

NK / T-cell lymphoma is a very rare disease with a highly skewed geographical distribution. Most cases are reported from Asia and South America; cases from other areas are very rare. Except for patients with localized disease restricted to the nasal cavity and non-nasal NK / T-cell lymphoma carry a very poor prognosis.

The recent use of MTX- and 1-Asparaginase containing regimens, have improved outcome.

A report from Japan summarized their experience on 28 patients with NK-cell neoplasms (22 extranodal NK / T-cell lymphoma, 3 blastic NK-cell lymphomas and 3 aggressive NK-cell leukemias). Twenty-two patients had matched related donors; conditioning was myeloablative in 23 patients. At 2 years, PFS and OS were 34% and 40% respectively.

4.8.1.4 Allogeneic transplantation in patients with adult T-cell leukemia

Almost all reports on alloSCT for adult T-cell leukemia/ lymphoma (ATLL) come from Asian countries and reflect the geographical distribution of this disease. By far the largest study on patients with adult T-cell leukemia, including patients with the lymphomatous subtype, was published by investigators from Japan. 386 patients received HLA-matched, related bone marrow or peripheral blood stem cells (n=154), mismatched related BMSC or PBSC (n=43) or unrelated bone marrow stem cells (n=99). Ninety patients received cord blood.

3-years-OS for the entire cohort was 33%. Multivariable analysis revealed 4 factors associated with lower survival: age >50 years, male sex, response status other than CR and use of cord blood compared with HLA-matched related grafts. TRM was rather high (37% at 3 years). Patients with an HTLV 1-seropositive donor had a higher risk of relapse after transplantation: 18 of 48 patients (38%) who had an HTLV 1- positive donor relapsed after transplantation in contrast to 16 of 65 patients (25%) with an HTLV 1-negative donor. The authors concluded that alloSCT is an effective treatment in selected patients with ATLL in particular if the extremely poor prognosis of such patients with any other treatment modality is considered.

4.8.1.5 Allogeeic transplantation in hepatosplenic T-cell lymphoma

So far only case reports of patients transplanted for alpha/ beta or gamma/ delta hepatosplenic T-cell lymphoma exist. These reports do not allow conveying a clear picture of how successful alloSCT in patients with hepatosplenic T-cell lymphoma might be. Clearly, one major problem for many patients is that the rapid progression of the underlying disorder does not allow finding a suitable donor and proceeding to transplantation on time. An EBMT project which at this time is ongoing identified not more than 20-25 patients who had been allografted for hepatosplenic T-cell lymphoma confirming the rarity of the disease and confirming the difficulty to bring such patients to transplant.

4.8.2 Hematopoietic Stem Cell Transplantation in Patients with Lymphomatoid Granulomatosis[110]

Six patients remain alive and well 5.1 (1.4-6.3) years after HDT / ASCT or RIC / alloSCT (three patients each). One patient committed suicide with no evidence of LG at that time and two patients died of transplant-related complications (septicemia) early after HDT / ASCT. One of these patients was reported as being refractory to salvage therapy prior to HDT / ASCT. Given the number and the nature of prior therapies (including Rituximab in seven patients) and the status of disease prior to transplantation we consider the results achieved with HDT / ASCT and/or RIC / alloSCT very promising in a disease believed to carry a poor prognosis at diagnosis but certainly in multiply relapsed or refractory patients.

This study represents the first albeit small series of patients transplanted for LG, a disease so exceedingly rare that until now only case reports for first-line and certainly for treatment of relapsed cases have been published.

With only ten patients reported here our series may be biased by overreporting of successful cases although we explicitly asked EBMT members to report all cases from their institutions. It also is not possible to decide if autologous or allogeneic transplants should be preferred or to comment on details of the transplant procedure like best choice of the preparatory regimen, donor selection, or GvHD prophylaxis for allografted patients. Nonetheless, HDT / ASCT and RIC / alloSCT showed excellent results in this small series of far advanced LG patients and should strongly be considered for relapsed and otherwise refractory patients fit enough to undergo any of these procedures.

4.8.3 Risk of CNS Recurrence and Role of Prophylaxis in Diffuse Large B-Cell Lymphoma[111]

Secondary involvement of the CNS is an early (median 8 months) and mostly fatal complication for patients with DLBCL. Accordingly, the interest in defining risk factors and prophylactic measures in order to spare patients this devastating experience continues to be high. With an overall incidence of about 5 %, CNS recurrence, however, is not frequent enough to justify specific diagnostic and prophylactic measures in every single patient. Recent efforts to better define the risk profile of individual patients has been met with limited success. While it seems possible to identify patients with a low risk of CNS disease (patients with IPI 0 and 1, and possibly 2), the question remains what to do with patients of IPIs 3 – 5 who run a risk of CNS relapse or progression between 5 and 10 %. Rigorous application of modern diagnostic tools like MRI of the brain and FACS analysis of the CSF will help to substantially improve diagnostic precision at the time of diagnosis. Patients with positive findings in imaging and/ or FACS analysis- although it is still undecided if every patient with single lymphoma cells in CSF has CNS disease- should be treated on protocols using drugs which cross the blood / brain barrier and possibly include high-dose therapy and autologous transplantation. For the remaining patients, the risk of CNS disease will be lower than previously reported because an undefined percentage of patients with ‘‘occult disease’’ will be shown to harbor lymphoma in the CNS at diagnosis leaving the treating physician with the “truly” CNS-negative patients verified by MRI-imaging and FACS analysis. As i.th. prophylaxis seems ineffective, these

patients would be candidates for systemic treatment with I.V. MTX or combinations of MTX with other drugs achieving cytotoxic concentrations in the brain. Such protocols are not without side effects and severe, sometimes lethal complications have been reported. Therefore, systemic therapy should be recommended only to patients who are highly likely to benefit from these procedures. Unfortunately, none of the risk models published so far achieved to unambiguously define a very high-risk group for CNS disease. With the exception of testicular involvement, there is few scientific evidence that any risk factor or combination of risk factors put patients at risk high enough to justify administration of I.V. MTX or other systemic therapy in order to prevent CNS disease. Rather than sticking to expert opinions generated 30 years ago and data coming from retrospective analyses of rather ill-defined patient populations we should treat patients on prospective trials strictly defining inclusion and exclusion criteria as well as diagnostic and prophylactic measures. Only these studies will identify science-based strategies in order to reduce or eliminate CNS disease. Before such data become available a proposal how to approach the problem is made.[111] In line with a recent analysis of the German High-Grade Lymphoma Study Group (DSHNHL) which has been validated on a large independent data set patients with 3 – 5 IPI factors and negative findings in MRI and FACS analysis of the CSF should be candidates for systemic MTX if an elevated LDH and involvement of more than one extranodal site is detected. In addition to testicular involvement where data supporting CNS prophylaxis are strongest involvement of the kidney and possibly the breast should trigger CNS prophylaxis. This or very similar algorithms have also been proposed by recent reviews but must be considered GRADE 2 C. Alternatively, also in high-risk patients adequately diagnosed with MRI and FACS analysis a “watch and wait policy” may be justified but data to support such a radical change of paradigm are not existing today.

Conclusion

We were able to show that the efficacy of various anti-B-cell antibodies can reliably and reproducibly be compared by using only 2 cell lines representing different B-cell lymphoma subtypes as target and healthy donor cells as effector cells. The early investigation of more cell lines or even patient lymphoma cells seems not really necessary. A state-of-the-art ADCC and a CDC assay may suffice to compare the potency of different antibodies. More cell lines or patient lymphoma cells and further assays can be used at a later stage of evaluation. Using this strategy — in combination with animal experiments necessary to retrieve pharmacokinetic and pharmakodynamic data as well as early information on safety — will allow to select which anti-B-cell antibody should go to clinical investigation. Obinutuzumab, which gave superior results in PCD and apoptosis tests, meanwhile has been licensed for treatment of B-cell lymphoma; BI 836826, an Fc-engineered anti-CD 37 antibody which also performed better than the licensed antibodies Rituximab and Ofatumumab in ADCC and apoptosis with moderate efficacy in CDC currently is being investigated in early clinical trials (phase I).

References

1. Swerdlow SH, Campo E, Harris NL, et al. WHO classification of tumours of haematopoietic and lympnoid tissues. IARC: Lyon 2008.

2. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed December 2013.

3. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(25):1937-47.

4. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med. 2008;359(22):2313-23.

5. Savage KJ, Johnson NA, Ben-Neriah S, et al. MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy. Blood. 2009;114(17):3533-7.

6. Barrans S, Crouch S, Smith A, et al. Rearrangement of MYC is associated with poor prognosis in patients with diffuse large B-cell lymphoma treated in the era of rituximab. J Clin Oncol. 2010;28(20):3360-5.

7. Cunningham D, Hawkes EA, Jack A, et al. Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisolone in patients with newly diagnosed diffuse large B-cell non-Hodgkin lymphoma: a phase 3 comparison of dose intensification with 14-day versus 21-day cycles. Lancet. 2013;381(9880):1817-26.

8. Pfreundschuh M, Trümper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol. 2006; 7(5):379-91.

9. Schmitz N, Nickelsen M, Ziepert M, et al. Conventional chemotherapy (CHOEP-14) with rituximab or high-dose chemotherapy (MegaCHOEP) with rituximab for young, high-risk patients with aggressive B-cell lymphoma: an open-label, randomised, phase 3 trial (DSHNHL 2002-1). Lancet Oncol. 2012; 13(12):1250-9.

10. Pfreundschuh M, Schubert J, Ziepert M, et al. Six versus eight cycles of bi-weekly CHOP-14 with or without rituximab in elderly patients with aggressive CD20+ B-cell lymphomas: a randomised controlled trial (RICOVER-60). Lancet Oncol. 2008; 9(2):105-16.

11. Rummel MJ, Niederle N, Maschmeyer G, et al. Bendamustine plus rituximab versus CHOP plus rituximab as first-line treatment for patients with indolent and mantle-cell lymphomas: an open-label, multicentre, randomised, phase 3 non-inferiority trial. Lancet. 2013;381(9873):1203-10.

12. Cameron F, and Sanford M. Ibrutinib: first global approval. Drugs. 2014;74(2):263-71.

13. Gopal AK, Kahl BS, de Vos S, et al. PI3Kδ inhibition by idelalisib in patients with relapsed indolent lymphoma. N Engl J Med. 2014;370(11):1008-18.

14. Li JY, Gaillard F, Moreau A, et al. Detection of translocation t(11;14)(q13;q32) in mantle cell lymphoma by fluorescence in situ hybridization. Am J Pathol. 1999;154(5):1449-52.

15. Barouk-Simonet E, Andrieux J, Copin MC, et al. TPA stimulation culture for improved detection of t(11;14)(q13;q32) in mantle cell lymphoma. Ann Genet. 2002;45(3):165-8.

16. Geisler CH, Kolstad A, Laurell A, et al. Long-term progression-free survival of mantle cell lymphoma after intensive front-line immunochemotherapy with in vivo-purged stem cell rescue: a nonrandomized phase 2 multicenter study by the Nordic Lymphoma Group.Blood. 2008;112(7):2687-93.

17. Schmitz N, Wu HS and Glass B. Aggressive B-cell lymphoma: modern diagnostics and treatment. Dtsch Med Wochenschr. 2014;139(41):2082-85.

18. Held G, Murawski N, Ziepert M, et al. Role of radiotherapy to bulky disease in elderly patients with aggressive B-cell lymphoma. J Clin Oncol. 2014;32(11):1112-8.

19. McKelvey EM, Gottlieb JA, Wilson HE, et al. Hydroxyldaunomycin (Adriamycin) combination chemotherapy in malignant lymphoma. Cancer. 1976;38(4):1484-93.

20. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med. 1979;300(19):1068-73.

21. Glass B, Hasenkamp J, Wulf G, et al. Rituximab after lymphoma-directed conditioning and allogeneic stem-cell transplantation for relapsed and refractory aggressive non-Hodgkin lymphoma (DSHNHL R3): an open-label, randomised, phase 2 trial. Lancet Oncol. 2014;15(7):757-66.

22. Fisher RI, Gaynor ER, Dahlberg S, et al. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med. 1993;328(14):1002-6.

23. Lenz G, Dreyling M, Hoster E, et al. Immunochemotherapy with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone significantly improves response and time to treatment failure, but not long-term outcome in patients with previously untreated mantle cell lymphoma: results of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). J Clin Oncol. 2005;23(9):1984-92.

24. Hiddemann W, Kneba M, Dreyling M, et al. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. Blood. 2005;106(12):3725-32.

25. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346(4):235-42.

26. Köhler G, Milstein C. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur J Immunol. 1976;6(7):511-9.

27. Coiffier B, Haioun C, Ketterer N, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. Blood. 1998;92(6):1927-32.

28. Jacobs SA, Harrison AM, Swerdlow SH, et al. Radioisotopic localization of (90)Yttrium-ibritumomab tiuxetan in patients with CD20+ non-Hodgkin's lymphoma. Mol Imaging Biol. 2009;11(1):39-45.

29. Zelenetz AD. A clinical and scientific overview of tositumomab and iodine I 131 tositumomab. Semin Oncol. 2003;30(2 Suppl 4):22-30.

30. Li D, Poon KA, Yu SF, et al. DCDT2980S, an anti-CD22-monomethyl auristatin E antibody-drug conjugate, is a potential treatment for non-Hodgkin lymphoma. Mol Cancer Ther. 2013;12(7):1255-65.

31. Polson AG, Yu SF, Elkins K, et al. Antibody-drug conjugates targeted to CD79 for the treatment of non-Hodgkin lymphoma. Blood. 2007;110(2):616-23.

32. Scott AM, Wolchok JD, and Old LJ. Antibody therapy of cancer. Nat Rev Cancer. 2012;12(4):278-87.

33. Pallasch CP, Leskov I, Braun CJ, et al. Sensitizing protective tumor microenvironments to antibody-mediated therapy. Cell. 2014;156(3):590-602.

34. Klein C, Lammens A, Schäfer W, et al. Epitope interactions of monoclonal antibodies targeting CD20 and their relationship to functional properties. MAbs. 2013;5(1):22-33.

35. Scott AM, Allison JP, and Wolchok JD. Monoclonal antibodies in cancer therapy. Cancer Immun. 2012;12:14.

36. Chan JK, Ng CS, and Hui PK. A simple guide to the terminology and application of leucocyte monoclonal antibodies. Histopathology. 1988;12(5):461-80.

37. Nadler LM, Anderson KC, Marti G, et al. B4, a human B lymphocyte-associated antigen expressed on normal, mitogen-activated, and malignant B lymphocytes. J Immunol. 1983;131(1):244-50.

38. Pezzutto A, Dörken B, Rabinovitch PS, et al. CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. J Immunol. 1987;138(9):2793-9.

39. Stashenko P, Nadler LM, Hardy R, et al. Characterization of a human B lymphocyte-specific antigen. J Immunol. 1980;125(4):1678-85.

40. Bourgois A, Kitajima K, Hunter IR, et al. Surface immunoglobulins of lipopolysaccharide-stimulated spleen cells. The behavior of IgM, IgD and IgG. Eur J Immunol. 1977;7(3):151-3.

41. Nadler LM, Stashenko P, Hardy R, et al. Characterization of a human B cell-specific antigen (B2) distinct from B1. J Immunol. 1981;126(5):1941-7.

42. Tedder TF, Clement LT, and Cooper MD. Expression of C3d receptors during human B cell differentiation: immunofluorescence analysis with the HB-5 monoclonal antibody. J Immunol. 1984;133(2):678-83.

43. Dörken B, Moldenhauer G, Pezzutto A, et al. HD39 (B3), a B lineage-restricted antigen whose cell surface expression is limited to resting and activated human B lymphocytes. J Immunol. 1986;136(12):4470-9.

44. Thorley-Lawson DA, Nadler LM, Bhan AK, et al. BLAST-2 [EBVCS], an early cell surface marker

of human B cell activation, is superinduced by Epstein Barr virus. J Immunol. 1985;134(5):3007-12.

45. Anderson KC, Park EK, Bates MP, et al. Antigens on human plasma cells identified by monoclonal antibodies. J Immunol. 1983;130(3):1132-8.

46. Anderson KC, Bates MP, Slaughenhoupt B, et al. A monoclonal antibody with reactivity restricted to normal and neoplastic plasma cells. J Immunol. 1984;132(6):3172-9.

47. Stashenko P, Nadler LM, Hardy R, et al. Characterization of a human B lymphocyte-specific antigen. J Immunol. 1980;125(4):1678-85.

48. Stashenko P, Nadler LM, Hardy R, et al. Expression of cell surface markers after human B lymphocyte activation. Proc Natl Acad Sci U S A. 1981;78(6):3848-52.

49. Nadler LM, Korsmeyer SJ, Anderson KC, et al. B cell origin of non-T cell acute lymphoblastic leukemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation. J Clin Invest. 1984;74(2):332-40.

50. van Meerten T, and Hagenbeek A. CD20-targeted therapy: a breakthrough in the treatment of non-Hodgkin's lymphoma. Neth J Med. 2009;67(7):251-9.

51. Anderson KC, Bates MP, Slaughenhoupt BL, et al. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. Blood. 1984;63(6):1424-33.

52. Einfeld DA, Brown JP, Valentine MA, et al. Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. EMBO J. 1988;7(3):711-7.

53. Press OW, Appelbaum F, Ledbetter JA, et al. Monoclonal antibody 1F5 (anti-CD20) serotherapy of human B cell lymphomas. Blood. 1987;69(2):584-91.

54. Uchida J, Lee Y, Hasegawa M, et al. Mouse CD20 expression and function. Int Immunol. 2004 Jan;16(1):119-29.

55. O'Keefe TL, Williams GT, Davies SL, et al. Mice carrying a CD20 gene disruption. Immunogenetics. 1998;48(2):125-32.

56. Cragg MS, Walshe CA, Ivanov AO, et al. The biology of CD20 and its potential as a target for mAb therapy. Curr Dir Autoimmun. 2005;8:140-74.

57. Polyak MJ, Ayer LM, Szczepek AJ, et al. A cholesterol-dependent CD20 epitope detected by the FMC7 antibody. Leukemia. 2003;17(7):1384-9.

58. Schwartz-Albiez R, Dörken B, Hofmann W, et al. The B cell-associated CD37 antigen (gp40-52). Structure and subcellular expression of an extensively glycosylated glycoprotein. J Immunol. 1988;140(3):905-14.

59. Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol. 2005;6(10):801-11.

60. van Spriel AB, Puls KL, Sofi M, et al. A regulatory role for CD37 in T cell proliferation. J Immunol. 2004;172(5):2953-61.

61. Knobeloch KP, Wright MD, Ochsenbein AF, et al. Targeted inactivation of the tetraspanin CD37 impairs T-cell-dependent B-cell response under suboptimal costimulatory conditions. Mol Cell Biol. 2000;20(15):5363-9.

62. Lapalombella R, Yeh YY, Wang L, et al. Tetraspanin CD37 directly mediates transduction of survival and apoptotic signals. 2012;21(5):694-708.

63. Link MP, Bindl J, Meeker TC, et al. A unique antigen on mature B cells defined by a monoclonal antibody. J Immunol. 1986;137(9):3013-8.

64. Maecker HT, Todd SC, and Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J. 1997 May;11(6):428-42.

65. Virtaneva KI, Angelisová P, Baumruker T, et al. The genes for CD37, CD53, and R2, all members of a novel gene family, are located on different chromosomes. Immunogenetics. 1993;37(6):461-5.

66. Heider KH, Kiefer K, Zenz T, et al. A novel Fc-engineered monoclonal antibody to CD37 with enhanced ADCC and high proapoptotic activity for treatment of B-cell malignancies. Blood. 2011;118(15):4159-68.

67. Deckert J, Park PU, Chicklas S, et al. A novel anti-CD37 antibody-drug conjugate with multiple anti-tumor mechanisms for the treatment of B-cell malignancies. Blood. 2013;122(20):3500-10.

68. Lim SH, Beers SA, French RR, et al. Anti-CD20 monoclonal antibodies: historical and future perspectives. Haematologica. 2010;95(1):135-43.

69. Cragg MS, Morgan SM, Chan HT, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. Blood. 2003;101(3):1045-52.

70. Deans JP, Robbins SM, Polyak MJ, et al. Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. J Biol Chem. 1998;273(1):344-8.

71. Polyak MJ, and Deans JP. Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure. Blood. 2002;99(9):3256-62.

72. Cragg MS, and Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. Blood. 2004;103(7):2738-43.

73. Beers SA, Chan CH, James S, et al. Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. Blood. 2008;112(10):4170-7.

74. Glennie MJ, French RR, Cragg MS, et al. Mechanisms of killing by anti-CD20 monoclonal antibodies. Mol Immunol. 2007;44(16):3823-37.

75. Mössner E, Brünker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. Blood. 2010;115(22):4393-402.

76. Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal

antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood. 2004;104(6):1793-800.

77. Cragg MS. CD20 antibodies: doing the time warp. Blood. 2011;118(2):219-20.

78. Niederfellner G, Lammens A, Mundigl O, et al. Epitope characterization and crystal structure of GA101 provide insights into the molecular basis for type I/II distinction of CD20 antibodies. Blood. 2011;118(2):358-67.

79. Chan HT, Hughes D, French RR, et al. CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. Cancer Res. 2003;63(17):5480-9.

80. Alduaij W, Ivanov A, Honeychurch J, et al. Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. Blood. 2011;117(17):4519-29.

81. Ivanov A, Beers SA, Walshe CA, et al. Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. J Clin Invest. 2009;119(8):2143-59.

82. Jak M, van Bochove GG, Reits EA, et al. CD40 stimulation sensitizes CLL cells to lysosomal cell death induction by type II anti-CD20 mAb GA101. Blood. 2011;118(19):5178-88.

83. Honeychurch J, Alduaij W, Azizyan M, et al. Antibody-induced nonapoptotic cell death in human lymphoma and leukemia cells is mediated through a novel reactive oxygen species-dependent pathway. Blood. 2012;119(15):3523-33.

84. Sung MW, Yasumura S, Johnson JT, et al. Natural killer (NK) cells as effectors of antibody-dependent cytotoxicity with chimeric antibodies reactive with human squamous-cell carcinomas of the head and neck. Int J Cancer. 1995;61(6):864-72.

85. Browning JL. B cells move to centre stage: novel opportunities for autoimmune disease treatment. Nat Rev Drug Discov. 2006;5(7):564-76.

86. Fischer K, Voelkl S, Berger J, et al. Antigen recognition induces phosphatidylserine exposure on the cell surface of human CD8+ T cells. Blood. 2006;108(13):4094-101.

87. Smyth MJ, Cretney E, Kelly JM, et al. Activation of NK cell cytotoxicity. Mol Immunol. 2005;42(4):501-10.

88. Boross P, and Leusen JH. Mechanisms of action of CD20 antibodies. Am J Cancer Res. 2012;2(6):676-90.

89. Bromley SK, Burack WR, Johnson KG, et al. The immunological synapse. Annu Rev Immunol. 2001;19:375-96.

90. Janeway, CA Jr; Travers P; Walport M; et al (2001). "The complement system and innate immunity". Immunobiology: The Immune System in Health and Disease. New York: Garland Science.

91. Glennie MJ, French RR, Cragg MS, et al. Mechanisms of killing by anti-CD20 monoclonal antibodies. Mol Immunol. 2007;44(16):3823-37.

92. Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood. 2004;104(6):1793-800.

93. Teeling JL, Mackus WJ, Wiegman LJ, et al. The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. J Immunol. 2006;177(1):362-71.

94. Manches O, Lui G, Chaperot L, et al. In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. Blood. 2003;101(3):949-54.

95. Weng WK, and Levy R. Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. Blood. 2001;98(5):1352-7.

96. Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, et al. Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. Biotechnol Bioeng. 2004;87(5):614-22.

97. Bursch W, Ellinger A, Gerner CH, et al. Programmed Cell Death (PCD): Apoptosis, Autophagic PCD, or Others? Ann NY Acad Sci. 2000; 926(1):1-12.

98. Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca et al. Features of apoptotic cells measured by flow cytometry. Cytometry. 1992;13(8):795-808.

99. Kroemer G, Petit P, Zamzami N,et al. The biochemistry of programmed cell death. FASEB J. 1995;9(13):1277-87.

100. Grakoui, A. et al. The immunological synapse: a molecular machine controlling T cell activation. Science 1999;285:221–27.

101. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. Nature 1998;395:82–6.

102. Orange JS, Harris KE, Andzelm MM, et al. The mature activating natural killer cell immunologic synapse is formed in distinct stages PNAS 2003; 100(24):14151-56.

103. Grakoui A, Bromley SK, Sumen C, et al. The immunological synapse: A molecular machine controlling T cell activation. Science 1999; 285: 221-27.

104. Kaizuka Y, Douglass AD, Varma R, et al. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. Proc Natl Acad Sci U S A. 2007;104(51):20296-301.

105. Reff ME, Carner K, Chambers KS, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994;83(2):435-45.

106. Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood. 2004;104(6):1793-800.

107. Beurskens FJ, Lindorfer MA, Farooqui M, et al. Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. J Immunol. 2012;188(7):3532-41.

108. Golay J, Da Roit F, Bologna L, et al. Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab. Blood. 2013;122(20):3482-91.

109. Schmitz N, Wu HS and Glass B. Allogeneic Transplantation in T-cell Lymphoma. Sem Hematol. 2014: 51 (1): 67-72.

110. Siegloch K, Schmitz N, Wu HS, et al. Hematopoietic Stem Cell Transplantation in Patients with Lymphomatoid Granulomatosis: A European Group for Blood and Marrow Transplantation Report. Biol Blood Marrow Transplant. 2013;19:1522-25.

111. Schmitz N, Wu HS and Zeynalova S. Risk of CNS Recurrence and Role of Prophylaxis in Aggressive Lymphoid Maliganacies. Blood. 2014 (in press).

112. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003;281(1-2):65-78.

113. Alter G, Malenfant JM, and Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods. 2004;294(1-2):15-22.

114. Waring MJ. Complex formation between ethidium bromide and nucleic acids. J Mol Biol. 1965;13(1):269-82.

115. Salles G, Morschhauser F, Lamy T, et al. Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. Blood. 2012;119(22):5126-32.

116. Goede V, Fischer K, Busch R, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. N Engl J Med. 2014;370(12):1101-10.

117. Davis RE, Ngo VN, Lenz G, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature. 2010;463(7277):88-92.

118. Salaverria I, Zettl A, Beà S, et al. Chromosomal alterations detected by comparative genomic hybridization in subgroups of gene expression-defined Burkitt's lymphoma. Haematologica. 2008;93(9):1327-34.

119. Pfeifer M, Grau M, Lenze D, et al. PTEN loss defines a PI3K/AKT pathway-dependent germinal center subtype of diffuse large B-cell lymphoma. Proc Natl Acad Sci U S A. 2013;110(30):12420-5.

120. Forstpointner R, Dreyling M, Repp R, et al. The addition of rituximab to a combination of fludarabine, cyclophosphamide, mitoxantrone (FCM) significantly increases the response rate and prolongs survival as compared with FCM alone in patients with relapsed and refractory follicular and mantle cell lymphomas: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. Blood. 2004;104(10):3064-71.

121. Schmitz N, Wu HS and Nickelsen M. Therapie peripherer T-Zell Lymphome. Uni-Med Verlag 2014 (in press).

Curriculum Vitae

Curriculum vitae entfällt aus datenschutzrechtlichen Gründen

Poster:

*__Wu HS__, Lunter AK, Spillner E, et al. A novel CD37 monoclonal antibody shows superior antibody-dependent cellular cytotoxic activity (ADCC) compared to rituximab against patients' lymphoma cells. DGHO Annual Meeting Abstracts 2013:P477.

*__Wu HS__, Lunter AK, Zobiak B, et al. A novel anti-CD37 antibody evokes homotypic adhesion and leads to enhanced antibody-dependent-cellular cytotoxic activity (ADCC) and programmed cell death (PCD) in B-cell malignancies. DGHO Annual Meeting Abstracts 2014:P243.

Publications:

*__Wu HS__, Lunter AK, Spillner E, et al. Effects of Rituximab and GA101 on in vitro killing of cell lines and patient lymphoma cells by normal donor or patient effector cells. Hematol Oncol 2013; 31: 285.

*Siegloch K, Schmitz N, __Wu HS__, et al. Hematopoietic Stem Cell Transplantation in Patients with Lymphomatoid Granulomatosis: A European Group for Blood and Marrow Transplantation Report. Biol Blood Marrow Transplant 2013; 19: 1522.

*Schmitz N, __Wu HS__ and Glass B. Allogeneic Transplantation in T-cell Lymphoma. Sem Hematol. 2014: 51 (1): 67.

*Schmitz N, __Wu HS__ and Glass B. Aggressive B-cell lymphoma: modern diagnostics and treatment. Dtsch Med Wochenschr 2014;139(41):2082.

*Schmitz N, __Wu HS__ and Zeynalova S. Risk of CNS Recurrence and Role of Prophylaxis in Aggressive Lymphoid Maliganacies. Blood 2014 (in press).

*Schmitz N, __Wu HS__ and Nickelsen M. Therapie peripherer T-Zell Lymphome. Uni-Med Verlag 2014 (in press).

Gefahrstoffe und Sicherheitshinweise

Folgende verwendete Reagenzien und Lösungsmittel waren mit Gefahrenhinweisen und Sicherheitsratschlägen gemäß §6 der Gefahrstoffverordnung versehen. Krebserzeugende, erbgutverändernde und fortpflanzungsgefährdende Stoffe (KMR-Stoffe) der Kategorie I und II sind fett dargestellt:

Verbindung	Gefahrensymbol	R-Sätze	S-Sätze
ABTS	Xi	36/37/38	26-36
Acrylamid	**T**	**45-46-20/21-25-36/38-43-48/23/24/25-62**	**53-45**
Ammoniumpersulfat	O, Xn	8-22-36/37/38-42/43	22-24-26-37
Ampicillin	Xn	36/37/38-42/43	22-26-36/37
Calciumchlorid Dihydrat	Xi	36	22-24
Chloroform	**Xn**	**22-38-40-48/20/22**	**36/37**
Diethylpyrocarbonat	Xn	20/22-36/37/38	26-36
Dimethylformamid	**T**	**61-20/21-36**	**53-45**
Dimethylsulfoxid	Xi	36/38	26
Dithiothreitol	Xi	36/37/38	36/37/39-22
EDTA-Dinatriumsalz-Dihydrat	Xn	22	-
Ethanol	F	11	7-16
Ethidiumbromid	**T+**	**22-26-36/37/38-40**	**26-28.2-36/37-45**
Glutardialdehyd, 25 %	T, N	22-23-34-42/43-50	26-26/37/39-45-61
Imidazol	C	22-34	22-26-36/37/39-45
Kanamycin Sulfat	T	61	26-36/37-39-45
Methanol	F, T	11-23/24/25-39/23/24/25	7-16-36/37-45
NaOH	C	35	26-37/39-45
NBT	Xn	20/21-33	22-45
Phenol (Tris-gesättigt)	**T**	**24/25-34**	**28.6-45**
2-Propanol	F, Xi	11-36-67	7-16-24/25-26
Salzsäure, konz.	C	34-37	26-36/37/39-45
SDS	Xn	22-36/38	22-24/25
TEMED	C, F	11-20/22-34	16-26-36/37/39-45
Triethylamin	F, C	11-20/21/22-35	3-16-26-29-36/37/39-45
Tris	Xi	36/38	-
Wasserstoffperoxid 30 %	C	34	3-28-36/39-45
Zitronensäure	Xi	36	24/25

Information on individual contributions to the publications included in this dissertation

Publications:

*Siegloch K, Schmitz N, **Wu HS**, et al. Hematopoietic Stem Cell Transplantation in Patients with Lymphomatoid Granulomatosis: A European Group for Blood and Marrow Transplantation Report. Biol Blood Marrow Transplant 2013; 19: 1522.
(Impact Factor 2014: 3.348)

*****Schmitz N, **Wu HS** and Glass B. Allogeneic Transplantation in T-cell Lymphoma. Sem Hematol. 2014: 51 (1): 67.
(Impact Factor 2014: 2.462)

*****Schmitz N, **Wu HS** and Glass B. Aggressive B-cell lymphoma: modern diagnostics and treatment. Dtsch Med Wochenschr 2014;139(41):2082.
(Impact Factor 2014: 0.55)

*****Schmitz N, **Wu HS** and Zeynalova S. Risk of CNS Recurrence and Role of Prophylaxis in Aggressive Lymphoid Maliganacies. Blood 2014 (in press).
(Impact Factor 2014: 9.775)

*****Schmitz N, **Wu HS** and Nickelsen M. Therapie peripherer T-Zell Lymphome. Uni-Med Verlag 2014 (in press).

Contributions: ~40% for all the publications including data collection, analysis, writing and submission.

Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Arbeit selbstständig und ohne fremde Hilfe sowie nur mit den angegebenen Hilfsmitteln und Quellen erstellt zu haben. Ergebnisse aus Schwerpunktarbeiten, die in diesem Arbeitskreis angefertigt wurden und die teilweise in diese Arbeit eingeflossen sind, wurden von mir initiiert und unter meiner Anleitung angefertigt.

Ebenfalls versichere ich, noch keinen weiteren Promotionsversuch an einer anderen Einrichtung unternommen zu haben.

Hamburg, den 05.11.2014

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Huei Shan Wu