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Elucidating the *CXCL12/CXCR4/CXCR7* chemokine axis in the pathogenesis of aggressive B cell lymphoma with implications for therapeutic intervention

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Was Geduld hat, kann alles überstehen.

François Rabelais (1494 - 1553)

AFFIDAVIT

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DANKE

KURZFASSUNG

Hintergrund: Die Chemokinrezeptoren, *CXCR4* und *CXCR7*, mit ihrem Liganden *CXCL12* wurden kürzlich identifiziert, an verschiedenen Prozessen in Tumorzellen einschließlich Vermehrung, Überleben, Dissemenierung/Invasion und Metastasierung in mehr als 20 unterschiedlichen Arten von Krebs beteiligt zu sein. Unsere bisherigen Ergebnisse belegten, dass hohe *CXCR4* und *CXCR7* Expression mit Knochenmarksinfiltration und einem schlechteren klinischen Ausgang in aggressiven Lymphomen mit schlechterem klinischem Stadium assoziiert waren.

Methoden: Um unsere bisherigen Daten zu bestätigen, untersuchten wir mittels Sanger Sequenzierung, ob die hohe Expression durch genetische Veränderungen in der kodierenden Sequenz der Rezeptoren, *CXCR4* und *CXCR7*, verursacht waren. Des Weiteren wurden immunhistochemische Analysen von CXCR4 und CXCL12 durchgeführt. Zusätzlich wurden mRNA Genexpressionsanalysen von *CXCR4*, *CXCR7*, *CXCL11* und *CXCL12* von Knochenmarksbiopsien von Patienten mit diffus großzelligen B-Zell Lymphomen (DLBCL) durchgeführt. Um die Rolle der CXCL12/CXCR4 Achse zu verdeutlichen, wurde die Wirksamkeit von CXCR4 Antagonisten, AMD070 und dessen Seitenketten modifizierte Variante (WK1) in Lymphomzellen *in vitro* untersucht.

Ergebnisse: Unsere mRNA Genexpressionsanalyse von Knochenmarksbiopsien von DLBCL-Patienten zeigte eine signifikant positive Korrelation der *CXCL12* Expression mit Knochenmarksinfiltrationsgraden von DLBCL Patienten. Des Weiteren fanden wir eine signifikante Abnahme der *CXCR4* Expression im Knochenmark nach absolvierter Chemotherapie. Behandlung von Lymphomzellen *in vitro* mit WK1 und AMD070 hatte wachstumsinhibitorische Effekte, die einerseits durch Apoptose bzw. Zellzyklusarrest bedingt waren. Diese Effekte waren stark von der *CXCR4* Expression abhängig, wobei diese jedoch deutlich stärker bei WK1 ausgeprägt waren. Des Weiteren konnten wir zeigen, dass die höhere Expression von *CXCR4* und *CXCR7* nicht durch Mutationen bedingt waren.

Schlussfolgerung: Diese Ergebnisse unterstützen unsere Hypothese, dass CXCR4 und CXCL12 wesentlich zur Pathogenese von aggressiven B-Zell Lymphomen beitragen, als sinnvolle klinische prognostische Marker dienen und interessante therapeutische Ziele bieten. AMD070 und WK1 stellen eine Innovation für die Entwicklung vielversprechender neuartiger Wirkstoffe in der Lymphomtherapie dar.

ABSTRACT

Background: The two chemokine receptors *CXCR4* and *CXCR7* with their prime ligand *CXCL12* were recently identified to be involved in multiple key processes in tumour cells including proliferation, survival, migration, invasion and metastasis in more than 20 different types of cancer, providing evidence for the importance of this chemokine signalling pathway in cancer. Our previous findings of higher expression of *CXCR4* and *CXCR7* were associated with bone marrow infiltration and a worse clinical outcome in aggressive lymphomas with an advanced clinical stage.

Methods: To confirm our previous data, we performed Sanger sequencing to clarify whether the high expression is caused by genetic alterations in the coding sequence of the receptors *CXCR4* and *CXCR7* and also immunohistochemical expression analysis of CXCR4 and CXCL12 in primary lymphomas was performed. Further, we investigated the CXCR4/CXCR7/*CXCL12* axis by mRNA expression analysis of *CXCR4, CXCR7, CXCL11* and *CXCL12* of bone marrow biopsies of patients with diffuse large B cell lymphoma (DLBCL). To elucidate the role of CXCL12/CXCR4 axis, the *in vitro* efficacy of CXCR4 antagonists, AMD070 and a side chain modified AMD070 (WK1) in lymphoma cells were carried out.

Results: Our mRNA expression analysis revealed a positive correlation of *CXCL12* expression with increasing bone marrow infiltration levels in bone marrow biopsies of DLBCL samples. Furthermore, we found a significant decrease in *CXCR4* expression in bone marrow after chemotherapy. *In vitro* treatment of lymphoma cells with WK1 and AMD070 resulted in a CXCR4 dependent growth inhibition of aggressive lymphoma cells by induction of apoptosis or cell cycle arrest. The inhibitory effect of WK1 was higher compared to AMD070. We did not detect any association of *CXCR4* and *CXCR7* mutation and their over-expression.

Conclusion: These data strongly support our hypothesis whereupon receptor and ligand are substantially contributing to the pathogenesis of aggressive B cell lymphomas, additionally serving as useful clinical prognostic markers and constituting interesting therapeutic targets. AMD070 and WK1 represent a new source for developing promising novel agents for anti-lymphoma therapy.

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ABBREVATIONS

7-AAD	7-amino-actinomycin D
ABC	activated B cell like subtype
AID	activation-induced cytidine deaminase
AML	acute myeloid leukemia
APC	antigen representing cells
BCL2	B cell lymphoma 2
BCL6	B cell lymphoma 6
BCR	B cell receptor
cDNA	complementary DNA
CLL	chronic lymphocytic leukemia
CSR	class switch recombination
CXCL11	or I-TAC, interferon-inducible T cell α chemoattractant
CXCL12	or SDF-1α, stromal cell-derived factor 1
CXCR4	C-X-C chemokine receptor type 4
CXCR7	C-X-C chemokine receptor type 7
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunoassay
FBS	fetal bovine serum
FDA	Food and Drug Administration
FDC	follicular dendritic cell
FL III	Follicular lymphoma grade III
G-CSF	granulocyte-colony stimulating factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal centre
GCB	germinal centre B cell subtype
GEP	gene expression profiling
GPCR	G protein coupled receptor
HBSS	Hank’s balanced salt solution
HIV	human immunodeficiency virus
HL	Hodgkin lymphoma

HPRT1	hypoxanthine-guanine phosphoribosyltransferase 1
HSC	hematopoietic stem cells
ID3	inhibitor of DNA binding 3
Ig	immunoglobulin
IL	interleukin
IRF4	interferon regulatory factor 4 / MUM1
IRS	immune reactive score
LPS	lipopolysaccharide
MEF2B	myocyte enhancer factor 2
MM	multiple myeloma
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGCB	non germinal centre B cell subtype
NHL	Non Hodgkin lymphoma
NK-cell	natural killer cell
PAX5	paired box 5
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMBL	primary mediastinal B cell lymphoma
PPIA	peptidylprolyl isomerase A
PRDM1	PR domain zinc finger protein 1 / BLIMP-1
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SHM	somatic hypermutation
TCF3	transcription factor 3
TNF	tumour necrosis factor
TNFAIP3	tumour necrosis factor, alpha induced protein 3
WHIM	warts, hypogammaglobulinemia, infection, and myelokathexis
WHO	World Health Organisation
WM	Waldenström macroglobulinemia
XBP1	X-box binding protein 1

I. Introduction

A. Lymphoma

Lymphomas are types of white blood cell cancer that develop in the lymphatic system, a part of our immune system. This occurs when lymphocytes begin behaving abnormally and grow outer control due to genetic alteration. Lymphomas can develop in many parts of the body, including thymus, spleen, lymph nodes, bone marrow or other organs, and can arise from different cell types (B cells, T cells and natural killer cells). About 90% of all lymphomas evolve from B cells. Historically, there are two main categories in which B cell lymphomas are subdivided: Non Hodgkin Lymphoma (NHL) and Hodgkin Lymphoma (HL). HLs have a typical histologic morphology. In the histological stains Reed-Sternberg cells -giant tumour cells- are found in the light microscope. NHLs include all other types of lymphomas. According to the World Health Organisation (WHO) classification, which was published in 2001 and updated in 2008, lymphomas are divided into five groups defined by cell type and different molecular, phenotypic and cytogenetic characteristics (Table I-1, Küppers, 2005; Campo *et al.*, 2011).

Table I-1: WHO classification of tumours of hematopoietic and lymphoid tissues

WHO classification of lymphoid neoplasms
Mature B cell neoplasms
Mature T-cell and NK-cell neoplasms
Hodgkin lymphoma
Histiocytic and dendritic cell neoplasms
Posttransplantation lymphoproliferative disorders

More than 25 subtypes of mature B cell neoplasms are currently distinguished in the WHO classification (Campo *et al.*, 2011). Diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL) are the most common forms of B cell lymphomas and account for 30-40% and 20% of all lymphomas in adults, respectively (Küppers, 2005; Jong and Balague Ponz, 2011). After a diagnosis, the lymphoma is staged using the Ann Arbor staging system to determine the extent of the cancer (malignant cells), which is important for prognosis and treatment (Table I-2, Carbone *et al.*, 1971).

Table I-2: Ann Arbor Staging System

Stage	
I	Involvement of a single lymphatic region (I) or localized involvement of single extra lymphatic organ or site (IE).
II	Involvement of 2 or more lymphatic regions on the same side of the diaphragm (II) or localized involvement of a single extra lymphatic organ or site and of one or more lymphatic regions on the same side of the diaphragm (IIE).
III	Involvement of lymph node regions on both sides of the diaphragm.
IV	Diffuse or disseminated involvement of 1 or more extra lymphatic organs or tissues with or without associated lymph node enlargement.

The clinical outcome of DLBCL is highly various, with 5 year survival rates between 30% and 80% (Jong and Balague Ponz, 2011). Combination chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone treatment plus rituximab (R-CHOP) is the current standard (Pfreundschuh *et al.*, 2006). However, approximately one third of patients with DLBCL is unresponsive and relapses (Tilly *et al.*, 2012). Concerning the high heterogeneity in clinical presentation and outcome, and diverse involved oncogenic pathways in the DLBCL pathogenesis a new classification of aggressive B cell lymphoma was established using gene expression profiling (GEP). GEP has identified three different molecular subtypes based on similarity in expression pattern to their "cell of origin"; the germinal centre B cell (GCB)-like subtype originating from centroblasts, the activated B cell (ABC)-like DLBCLs deriving from plasmablasts, and the primary mediastinal B cell lymphoma (PMBL) originating from thymic B cells (Alizadeh *et al.*, 2000; Basso and Dalla-Favera, 2015).

B cell development and differentiation

The B cell development is initiated in the fetal liver and transferred to the bone marrow during maturation of embryos (Seifert *et al.*, 2013). During this irreversible process hematopoietic stem cells differentiate to lymphoid precursors and finally to mature B cells. The maturation process of B cells is accompanied by the presentation of a differentiation marker -so called *B cell receptors (BCRs)*- on their cell surface. The *BCR* is composed of a membrane-bound *immunoglobulin (Ig)* that, like all antibodies, has a unique and randomly determined antigen-binding site and it interacts with a specific antigen. *BCR* functions are required for normal antibody production and B cell development. To create a functional Ig, the B cells rearrange DNA segments which encode the heavy- and the light-chain regions of the variable genes. First, three gene segments, *V (variable), D (diversity)* and *J (joining)* are joined to encode the heavy-chain variable region (Figure I-1). This process is called VDJ recombination. During this process the DNA located between the rearranging gene segments is excluded by the enzymes recombinase-activating genes *RAG1* and *RAG2*. The B cell precursors perform first the D-J rearrangement (1) followed by the V-DJ recombination (2) in the heavy chain, resulting in the expression of a functional BCR. The V regions of the light chains are encoded by V and J gene segments (not shown). For a non-functional BCR, the B lymphocyte precursors will undergo apoptosis (Küppers, 2005).

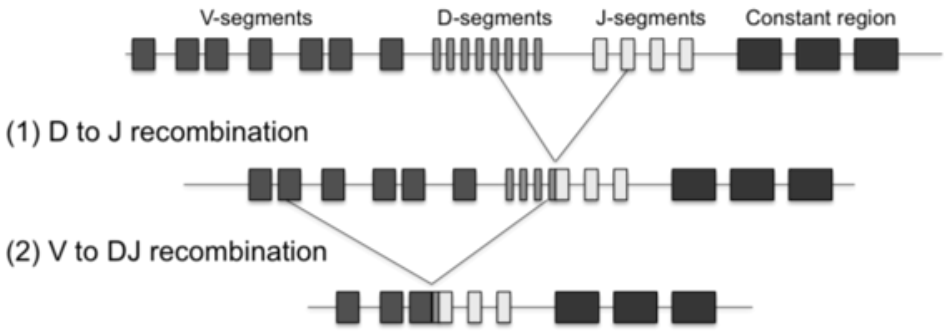

Figure I-1: VDJ recombination (adapted from Janeway, 2001)

The production of the variable heavy chain occurs in two steps. Initially, one of the J-segments is fused to the D-segment (1). Then, the combined DJ-sequence is added to one of the V-segments (2).

Mature naive B cells leave the bone marrow and migrate into secondary lymphoid organs by the time they encounter a cognate antigen and participate in T cell dependent

immune response. Antigen-activated B cells undergo clonal expansion and differentiate into centroblasts in the dark zone of the germinal centre (GC) (Figure I-2), where the variable regions of immunoglobulins are modified by somatic hypermutation (SHM). The GC B cells with the modified antigen receptor migrate into the light zone of the germinal centre and become centrocytes. Unfavourable aberrations in the *Ig* lead to reduced antigen affinity and induce cells to undergo apoptosis. This mechanism serves to delete ineffective cells. Cells with an increased antigen affinity are selected with the help of T cells and follicular dendritic cells (FDCs) and undergo an immunoglobulin class switch recombination (CSR). After several cycles of proliferation, mutation and selection, the GC B cells differentiate into memory B cells or plasma cells and leave the microenvironment (Küppers, 2005; Seifert *et al.*, 2013; Basso and Dalla-Favera, 2015).

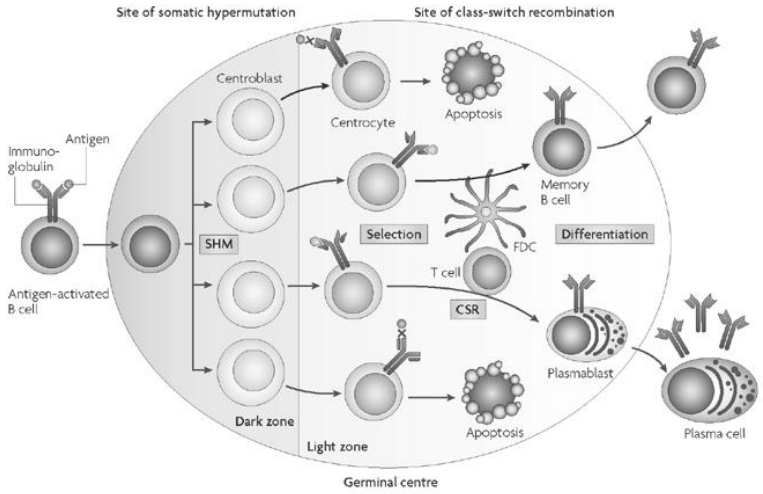

Figure I-2: B cell differentiation in the germinal centre reaction (adapted from Klein and Dalla-Favera, 2008)

Two modifications of the immunoglobulin occur during the germinal centre reaction, the SHM which introduces point mutations, deletions, or duplications into the rearranged variable regions of the *immunoglobulin* gene, and the CSR which changes the heavy chain class from IgM to IgG, IgA, or IgE. Both processes are dependent on the enzyme *activation-induced cytidine deaminase* (*AID*). In the SHM mechanism *AID* converts the cytidine into uracil to generate a U-G mismatch in the DNA strand. These mismatches are repaired by error prone DNA repair mechanism and generate somatic mutations. These genetic alterations are essential for a normal immune response, but they also involved in the malignant transformation of B cells (Küppers, 2005; Lenz and Staudt, 2010; Seifert *et al.*, 2013).

Lymphomagenesis

GCs are not only involved in the differentiation of B cells but also in the formation of B cell lymphomas. Recent studies have discovered transcriptional factors and signalling pathways that are hijacked during lymphomagenesis (Figure I-3).

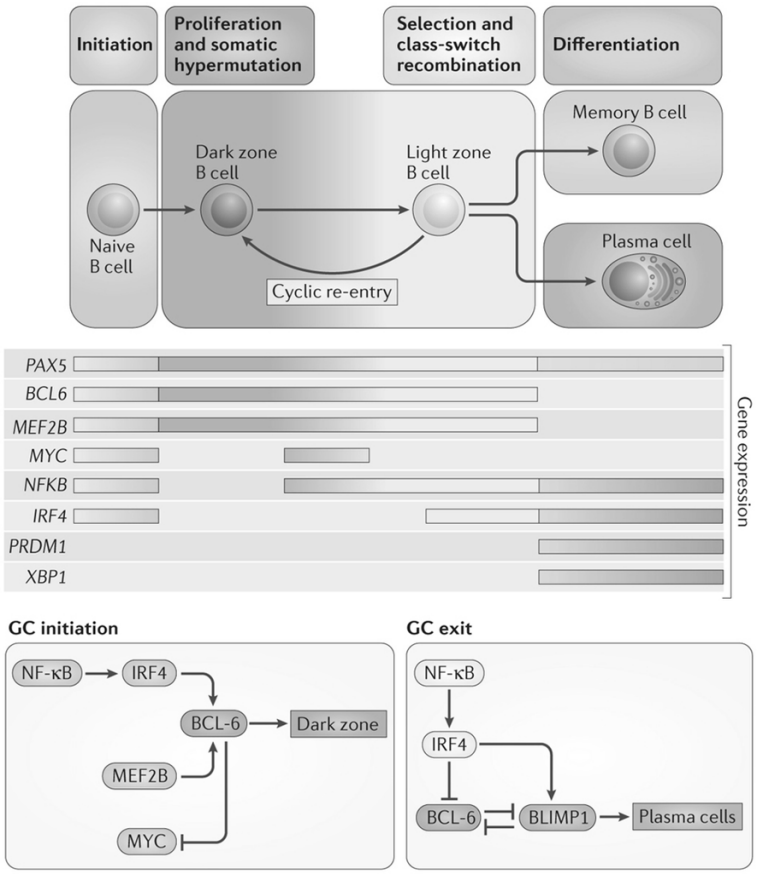

Figure I-3: Transcription factors regulating the germinal centre reaction (adapted from Basso and Dalla-Favera, 2015)

Paired box 5 (PAX5) is expressed throughout the development of a mature B cells except in the germinal centre exit, where the loss of *PAX5* promotes the plasma cell differentiation. The expression of *B cell lymphoma 6 (BCL6)* and *myocyte enhancer factor 2B (MEF2B)* play a role in the germinal centre initiation and expansion stage. *MYC (v-myc avian myelocytomatosis viral oncogene homolog)* is expressed in initiation and in the re-entry cycle from the light zone to the dark zone. *Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)* and *interferon regulatory factor 4 (IRF4)* also known as *MUM1* are required in the GC initiation and reappear in the light zone B cells. The *PRDM1* gene which encodes the B lymphocyte induced maturation protein 1 (BLIMP1) and the X-box binding protein 1 (XBP1) are involved in the plasma

cell differentiation. During the germinal centre initiation *NF-κB* induced *IRF4* expression and *MEF2B* contribute to the *BCL6* induction, but in the germinal centre exit it downregulates *BCL6* and *PRDM1* expression. *MYC* and *PRDM1* are negatively regulated by *BCL6* in both GC stages (Basso and Dalla-Favera, 2015).

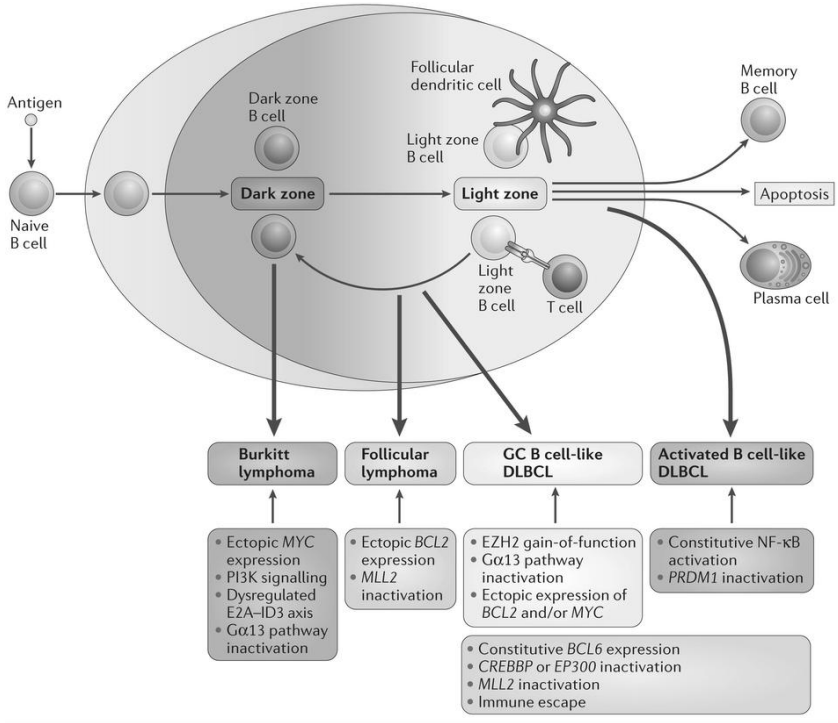

Figure I-4: The germinal centre origin of B cell lymphoma (adapted from Basso and Dalla-Favera, 2015)

Aggressive B cell lymphomas including Burkitt lymphoma, follicular lymphoma (FL), and diffuse large B cell lymphoma (DLBCL), which are derived from GC B cells (Figure I-4). Burkitt lymphomas originate from the dark zone B cells, FL and GC B cell like DLBCL show characteristics of light zone B cells, and activated B cell like DLBCL derive from late germinal centre B cells, that are obliged to plasma cell differentiation. Although the lymphomas share identical elements, each B cell derived subtype is defined by unique genetic modifications in their phenotype (Basso and Dalla-Favera, 2015).

In Burkitt lymphomas the translocation of the proto-oncogene *MYC* into the immunoglobulin loci deregulates the oncogene expression in 100% of cases. Other mutations in the *transcription factor 3 (TCF3)* which encodes the *transcriptional factor E2A* or in the *inhibitor of DNA binding 3* (*ID3)* gene promote the BCR signalling and

the phosphoinositide 3-kinase (PI3K) pathway. The *Gα13* pathway in Burkitt lymphomas is inactivated, which is normally involved in the cell migration of germinal centre B cells (Seifert *et al.*, 2013; Basso and Dalla-Favera, 2015).

The FL is the most common indolent form of a non Hodgkin lymphoma, but becomes more aggressive by transforming into a DLBCL (Montoto and Fitzgibbon, 2011). Two main alterations are present in the pathogenesis of FL: the ectopic expression of *B cell lymphoma 2 (BCL2)* and *histone methyltransferase MLL2* inactivation. In more than 90% of follicular lymphoma *BCL2* is translocated into the heavy chain locus. This t(14;18) chromosomal translocation occurs during the early stages of B cell development and becomes pathogenic at later stages, where *BCL2* inactivates the apoptotic process for the malignant cells. Beside the inactivation of *MLL2* which is also detected in over 90% of cases, the inactivation of the acetyltransferase *CREB-binding protein (CREBBP)* and *E1A-bindung protein p300 (EP300)* likewise the activation of histone methyltransferase *enhancer of zeste homolog 2 (EZH2)* play a role in the alterations of the epigenome of follicular lymphomas and also in diffuse large B cell lymphomas (Seifert *et al.*, 2013; Basso and Dalla-Favera, 2015).

Both subtypes of diffuse large B cell lymphoma, GCB-DLBCL and ABC-DLBCL show distinct genetic alterations, however they exhibit identical aberrations, including the inactivation of the chromatin modifiers, *EP300/CREBBP* and *MLL2*, the *BCL6* dysregulation and the lack the *major histocompatibility complex class I (MHC I)*, which is important for the cytotoxic T lymphocyte and natural killer cell mediated immune recognition (Figure I-4). In the GC B cell subtype of diffuse large B cell lymphoma the *Gα13* pathway is inactivated, which promotes a dysregulation in cell migration and in the *PI3K* signalling. The activation of *EZH2* in cooperation with the activated *BCL6* leads to epigenetic changes. GCB-DLBCL is also associated with the MYC and/or BCL2 translocations. The *nuclear factor-κB pathway* in the activated B cell like DLBCL is affected by multiple genetic events including activating mutations of *CD79A* and *CD79B*, which encode parts of the BCR, *CARD11*, which encodes caspase recruitment domain-containing protein 11, and alterations in the gene encoding *myeloid differentiation primary response protein 88 (MYD88)*, inactivation of *tumour necrosis factor, alpha induced protein 3 (TNFAIP3)*, which encodes A20, and a blockade in differentiation mediated by *BLIMP1* (Basso and Dalla-Favera, 2015).

B. Chemokines and chemokine receptors

Chemokines

Chemokines, also known as chemotactic cytokines, are a large multifunctional superfamily of proteins that control leukocyte trafficking during homeostasis and also during inflammation (Laing and Secombes, 2004; Coelho *et al.*, 2005). In addition to act as chemotactic mediators in the immune system, it is known that chemokines play important roles in regulating homeostatic functions, angiogenesis, cellular differentiation and activation, tumour growth and metastasis, wound healing, homing and development of lymphoid tissues, and in controlling the balance of T helper type I cells versus T helper type 2 cells (Rossi and Zlotnik, 2000; Coelho *et al.*, 2005; Ebert *et al.*, 2005; Melik-Parsadaniantz and Rostene, 2008; Vandercappellen *et al.*, 2008). Up to date, there are 50 chemokines identified so far. They can be classified into four subgroups (C, CC, CXC, and CX3C) based on the arrangement of cysteine residues located near the N terminus (Figure I-5). The two major groups of the chemokine family are CC and CXC, consisting of 28 (*CCL1-28*) and 17 (*CXCL1-17*) members, respectively. The C and the CX3C chemokines are much smaller with two (*XCL1-2*) and one (*CX3CL1*) members per family (Ono *et al.*, 2003; Barbieri *et al.*, 2010).

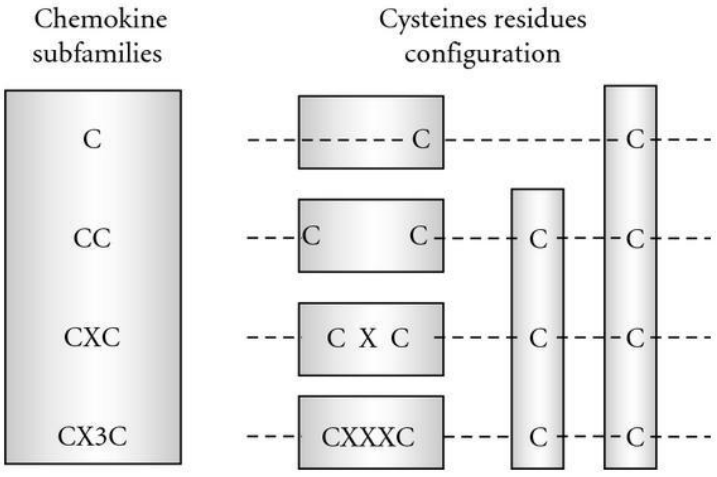

Figure I-5: Classification of chemokine subfamilies (adapted from Barbieri *et al.*, 2010)

Further, the CXC family can be divided into the two main categories, ELR^+ and ELR^- based on the presence or absence of the tripeptide Glu-Leu-Arg (ELR) before the CXC motif in the N-terminal domain (Laing and Secombes, 2004; Le *et al.*, 2004; Barbieri *et al.*, 2010). The presence of this tripeptide transmits angiogenic functions, while the ELR^--CXC chemokines have angiostatic properties, with the exclusion of CXCL12, also known as stromal cell-derived factor 1 (SDF-1), which is angiogenic. Generally, the chemokines elicit appropriate classes of leukocytes; C chemokines attract T cells, NK cells and monocytes, CC chemokines are involved in attracting mononuclear cells, eosinophils and basophils, the subfamily of CXC chemokines act as chemoattractant for neutrophils in the ELR^+ subgroup and for lymphocytes in the ELR^- subtype, and the CX3C chemokine also known as fractalkine, acts on T cells (Le *et al.*, 2004). Chemokines can also be classified in either constitutive or inducible based on their expression pattern. Constitutively expressed chemokines play an important role in leukocyte homing in the secondary lymphoid organs, whereas inducible chemokines are mainly involved in the recruitment of cells to the sites of infection, which are most of the chemokines. These chemokines are highly induced by inflammatory stimuli for instance tumour necrosis factor (TNF)-α, lipopolysaccharide (LPS) and interleukin (IL)-1 (Le *et al.*, 2004; Coelho *et al.*, 2005).

Chemokine receptors

Chemokine receptors are seven-transmembrane receptors, with their N terminus outside the cell surface, three extracellular, and three intracellular loops as well as a C terminus in the cytoplasm. One of the intracellular loops is associated with heterotrimeric G proteins. All chemokine receptors initiate signal transduction through the activation of these G proteins. When a chemokine ligand, e.g. *CXCL12*, binds to the extracellular side of the chemokine receptor, it changes the conformation of the receptor into a stabilized form which activates the heterotrimeric G proteins inside the cell. Another important motif for G protein coupling is the DRY (aspartic acid, arginine, tyrosine) box located on the second intracellular loop. The DRY box is also involved in β-arrestin binding and it conducts the ligand dependent internalization of the receptor. The G proteins consist of three subunits α, β and γ. Gα interacts directly with the G protein coupled receptor (GPCR) C-terminal domain, the intracellular loops two and three, and with the Gβ subunit, which forms a complex with the Gγ subunit. Gα binds GDP in the inactive state. After ligand binding the GDP dissociates from the Gα subunit, and is replaced by GTP. Then the Gα-GTP complex dissociates from the Gβγ subunits and the receptor. Both complexes, Gα-GTP and Gβγ, activate several downstream effectors and lead to physiological responses. Refraction to continued stimuli involves receptor desensitization and internalization by ligand dependent phosphorylation of the C-terminal tail of the GPCR by G protein receptor kinases (GRKs). The phosphorylation of the receptor promotes subsequently the binding of arrestins, which block further interaction with G proteins and mediate receptor internalization through clathrin-coated pits. In addition to their involvement in internalization, β-arrestins can function as signal transducers by activating pathways such as Akt, PI3K, MAPK and NF-κB (Figure I-6) (Holland *et al.*, 2006; Harrison and Lukacs, 2007; O'Hayre *et al.*, 2008).

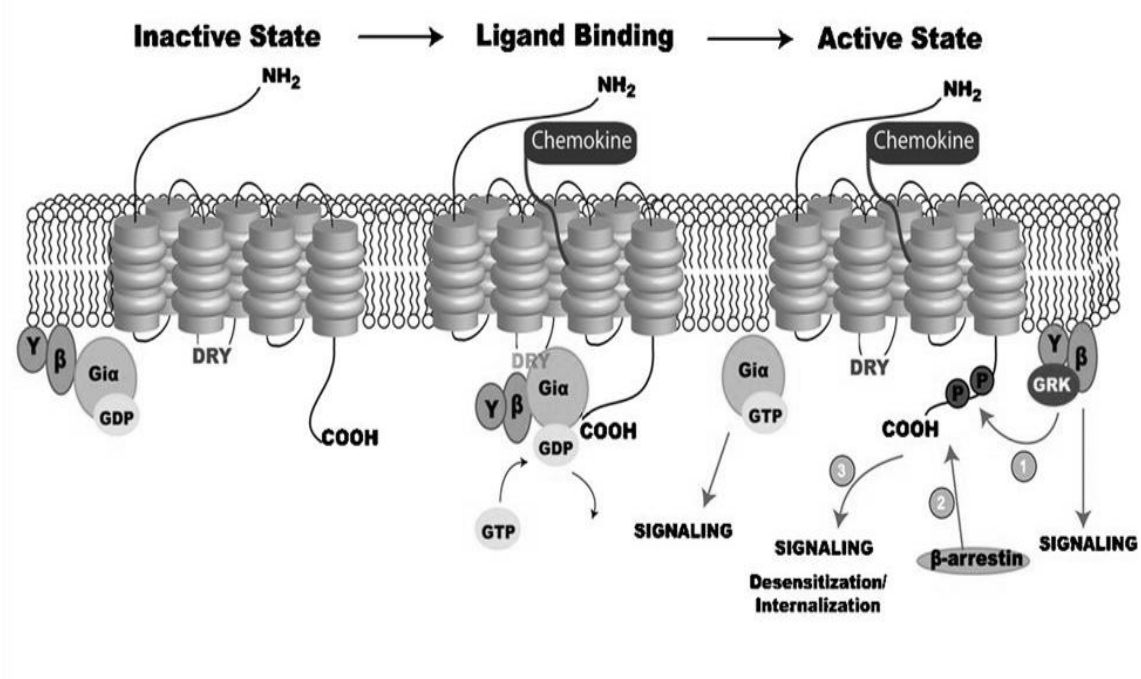

Figure I-6: Activation of chemokine receptor by ligand binding (adapted from O'Hayre *et al.*, 2008)

There exist numerous chemokines and chemokine receptors but no single chemokine is assigned to a single receptor. To date, at least 20 chemokine receptors (*CCR1-11*, *CXCR1-7*, *XCR1* and *CX3CR1*) have been identified and they are known to play an important role in inflammation, infection, tissue injury, allergy, and malignant tumours. It was shown, that chemokines and chemokine receptors play a crucial role in tumour cell proliferation, angiogenesis and metastasis, and they modulate senescence and cell survival (Kakinuma and Hwang, 2006; Sun *et al.*, 2010; Domanska *et al.*, 2013).

CXCR4 and CXCR7 in cancer

CXCR4 and *CXCR7* have been linked to multiple key processes in tumour cells, including proliferation, survival, migration, invasion and metastasis in more than 20 different types of cancer (Smith *et al.*, 2004; Kollmar *et al.*, 2007; Domanska *et al.*, 2013), providing evidence for the importance of this chemokine signalling pathway in cancer. *CXCR7* expression was described in malignant cell types, in fetal liver cells and in tumour associated blood vessels but not in normal vessels (Burns *et al.*, 2006; Miao *et al.*, 2007). The common ligand of *CXCR4* and *CXCR7* is *CXCL 12*, also known as SDF-1, which is broadly expressed by a variety of tissue types including the bone marrow, lymph nodes, liver and the brain (Sun *et al.*, 2010; Hattermann and Mentlein, 2013). Interaction of *CXCL12* with *CXCR7* promotes tumour growth, proliferation and pro-survival effects (Miao *et al.*, 2007; Mazzinghi *et al.*, 2008; Wang *et al.*, 2008) in contrast to *CXCR4*, which mediates chemotaxis and is associated with metastasis (Balkwill, 2004). *CXCR4* also plays a role in cell homing of malignant- and hematopoetic stem cells as well as cancer cells of solid tumours to the bone marrow (Sun *et al.*, 2003; Kyriakou *et al.*, 2008; Hayakawa *et al.*, 2009). *CXCR4* expression was detected in most B cell malignancies (Trentin *et al.*, 2004), whereas *CXCR7* expression analysis in lymphoma cells has not been investigated so far. The development of chemokine receptor antagonists and their use in clinical trials for a variety of diseases shows the potential of chemokine receptors for molecular targeted therapy (Golay and Introna, 2008).

CXCR4 antagonists AMD3100 and AMD070

For therapeutic blocking of *CXCR4* receptors, AMD3100 and AMD070 were found to be potent and selective inhibitors. The first antagonist, AMD3100; also known as plerixafor, a bicyclam with an aromatic linker (Figure I-7), was approved by the Food and Drug Administration (FDA) in 2008. AMD3100 has demonstrated effectiveness in stem cell mobilization from bone marrow for autologous transplantation in patients with leukemia and lymphoma and has fewer side effects than any other agent (Wong *et al.*, 2008; Sun *et al.*, 2010; Debnath *et al.*, 2013; Jacobson and Weiss, 2013; Clercq, 2015). In contrast, AMD070, a noncyclam small molecule that carries a primary amine (Figure I-7), is the first orally bioavailable *CXCR4* antagonist. AMD070 affects the migration, proliferation and survival of cancer cells through the *CXCL12*-mediated MAPK signalling (Wong *et al.*, 2008; Debnath *et al.*, 2013; O'Boyle *et al.*, 2013).

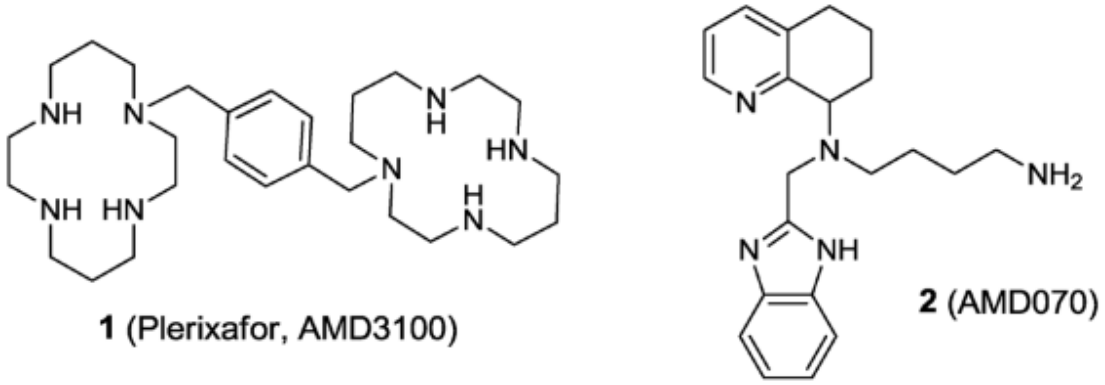

Figure I-7: Structures of *CXCR4* antagonists (adapted from Debnath *et al.*, 2013)

Currently the antagonists, AMD3100 and AMD070, are applied in several clinical trials for cancer therapy. Before AMD3100 was approved by the FDA, the antagonist was designed for treatment of human immunodeficiency virus (HIV). Finally, AMD3100 is given in combination with granulocyte-colony stimulating factor (G-CSF) to mobilize HSCs to the peripheral blood in patients with Non-Hodgkin Lymphoma and multiple myeloma (Blanco *et al.*, 2000; Burger *et al.*, 2005; Debnath *et al.*, 2013; Domanska *et al.*, 2013). Further, AMD3100 in combination with other chemotherapeutic agents to prevent growth and metastasis in cancers as acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) are also in clinical trials (Table I-3; Debnath *et al.*, 2013). The orally available AMD070 was under investigation in HIV infected patients with X4-tropic virus. The study was previously suspended in April 2009 because of hepatotoxicity and histological findings in long-term animal studies (Moyle *et al.*, 2009).

Table I-3: *CXCR4* antagonists under clinical investigation

Agent	Phase	Indication
AMD3100	FDA approved	Hematopoietic stem cell mobilization in patient with Non-Hodgkin Lymphoma and multiple myeloma
AMD3100	Phase I	Glioma, Acute Myeloid Leukemia, Chronic Lymphocytic Leukemia
AMD3100	Phase I.	Myelokathexis (WHIM Syndrom)
AMD070	Phase I / II	HIV Infections

CXCR4 and CXCR7 expression in lymphoma

In one of our previous studies, we showed that *CXCR4* was exclusively expressed on nodal lymphomas when comparing chemokine expression profiles of extranodal to nodal lymphomas and that it was associated with bone marrow infiltration (Figure I-8). Additionally, in extranodal gastric lymphomas *CXCR7* was associated with lymphoma progression (Deutsch *et al.*, 2013). However, the functional consequences of *CXCR4* and *CXCR7* expression in aggressive lymphoma cells are still unknown.

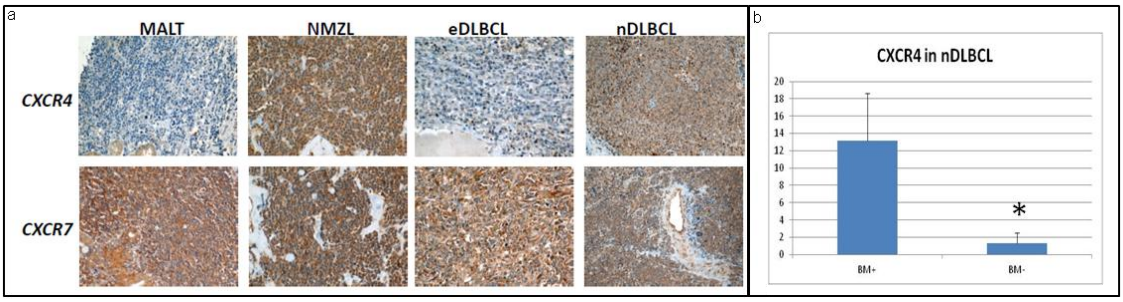

Figure I-8: *CXCR4* and *CXCR7* expression in extranodal and nodal lymphomas (Deutsch *et al.*, 2013)

a: immunohistochemical analysis of *CXCR4* and *CXCR7* in gastric MALT lymphoma (MALT), in nodal marginal zone B cell lymphoma (NMZL), extranodal DLBCL (eDLBCL) and nodal DLBCL (nDLBCL). *CXCR4* expression was exclusively found in nodal lymphomas.

b: *CXCR4* expression analysis comparing nodal DLBCL with and without bone marrow infiltration. Nodal DLBCL with bone marrow infiltration showed a significant higher *CXCR4* expression.

Preliminary data of our group on *CXCR4* and *CXCR7* expression in DLBCL, transformed follicular lymphomas (tFL) and germinal centre B cells on mRNA transcripts showed an at least two fold over expression of *CXCR4* in 22 of 52 (42%) DLBCL- and in 16 of 27 (59%) tFL-specimens and of *CXCR7* in three of 52 (6%) DLBCL- and in three of 27 (11%) tFL-specimens compared to their non-neoplastic controls – germinal centre B cells (Figure I-9a). Taking all lymphoma specimens together and comparing expression levels of both chemokine receptors in aggressive lymphoma patients with clinical stage I to lymphoma with an advanced stage (stage II-IV), a significant higher of *CXCR4* (fivefold, p=0.017) and a trend for a higher *CXCR7* (3.5 fold, p=0.087) expression were detected in lymphoma specimens with an advanced clinical stage (Figure I-9b). Additionally, a significant positive correlation of *CXCR4* and *CXCR7* expression and bone marrow infiltration of the lymphomas (15 of 71,

Spearman rho=0.550 and p<0.001 for *CXCR4* and rho=0.406 and p=0.004 for *CXCR7*, Figure I-9c) was observed. Furthermore, Kaplan–Meier analyses by using the median of both chemokine receptors revealed, that high *CXCR7* expression was associated with poor overall survival (Figure I-9d).

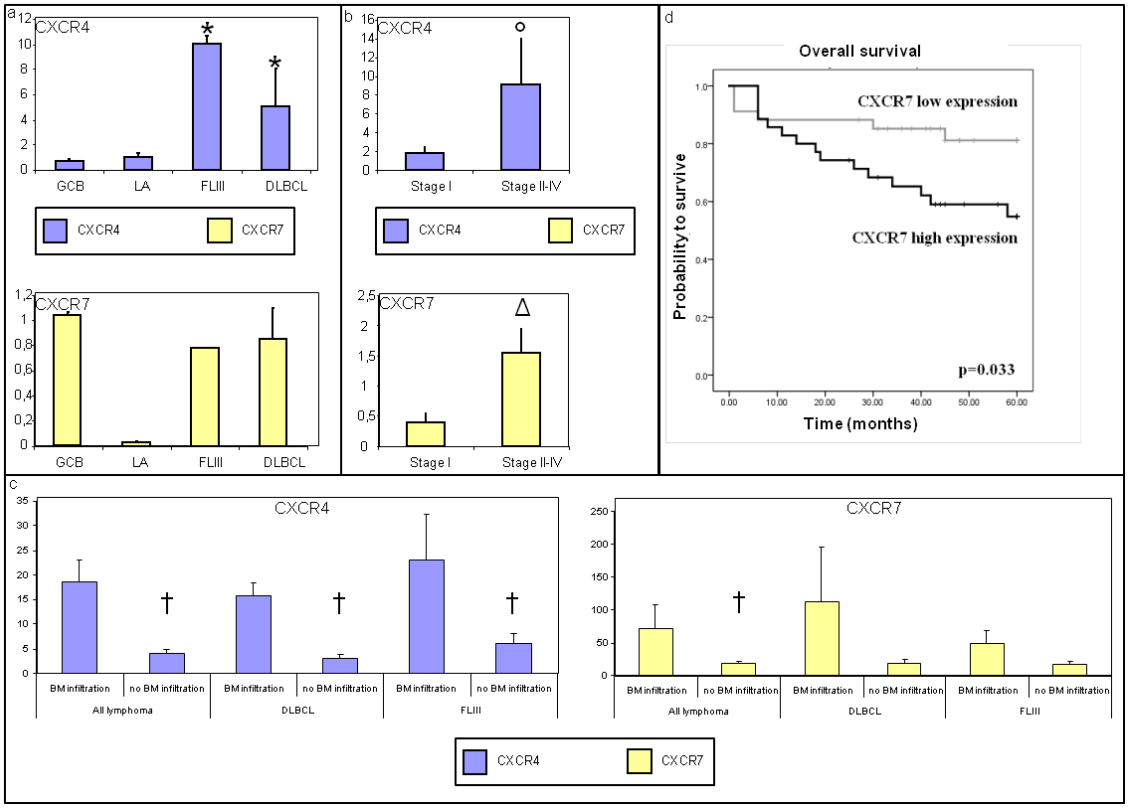

Figure I-9: *CXCR4* and *CXCR7* expression in aggressive B cell lymphoma

a: *CXCR4* and *CXCR7* expression in germinal centre B cells (GCB), lymphadenopathy (LA), FLIII and DLBCL. *CXCR4* was significantly higher expressed in FLIII and DLBCL compared to GCB. "*" indicates higher expression compared to GCB (p<0.01).

b: Comparison of *CXCR4* and *CXCR7* expression to clinical stage. *CXCR4* was significantly higher expressed in lymphoma with an advanced stage (stage II-IV) compared to lymphoma with stage I (indicated by circles, p=0.017). For *CXCR7* there was a tendency of a higher expression in lymphoma with an advanced stage (indicated by triangle, p=0.087).

c: *CXCR4* and *CXCR7* expression analysis comparing aggressive B cell lymphoma with and without bone marrow infiltration. High *CXCR4* and *CXCR7* expression was detected in aggressive lymphoma with bone marrow infiltration. Cross indicates significant reduced *CXCR4* and *CXCR7* expression (p<0.03).

d: Probability of cancer-specific survival in DLBCL patients stratified according to the *CXCR7* mRNA expression level. High *CXCR7* expression is associated with poor survival (p=0.033).

C. Aims

Based on the preliminary data of *CXCR4* and *CXCR7* in aggressive B cell lymphoma, we want to comprehensively study the function of both chemokine receptors to elucidate their function in the dissemination process -especially BM infiltration- of aggressive lymphomas.

In the first part of this study we want to clarify whether the high *CXCR4* and *CXCR7* mRNA expression occurred with a worse lymphoma specific survival is caused by genetic alterations in the coding sequence of the receptors using Sanger sequencing. Furthermore, we want to confirm the preliminary *CXCR4* expression by immunohistochemical analysis of primary aggressive lymphomas. In the third clinical part, mRNA expression analysis of *CXCR4*, *CXCR7* and their ligands *CXCL11* and *CXCL12* of bone marrow biopsies of aggressive lymphoma patients should be investigated.

In the second part we want to investigate the effects of the commercially available *CXCR4* antagonist AMD070 and a novel side chain modified derivative of AMD070, namely WK1, on lymphoma cells followed by *CXCL12* stimulation *in vitro*. Therefore, we will examine their ability to block *CXCL12* binding to stably transduced lymphoma cells expressing either *CXCR4* or *CXCR7* employing $CXCL12^{AF647}$ binding assays by FACS analysis. Furthermore, the *CXCR4* antagonists will be used to treat lymphoma cells followed by *CXCL12* stimulation, and their effects on migration, invasion, proliferation and cell viability will be estimated.

II. Material and Methods

Human Specimens

To investigate the mRNA expression levels of *CXCR4*, *CXCR7*, *CXCL11* and *CXCL12*, the genetic alteration of *CXCR4* and *CXCR7*, and the immunohistochemical expression of *CXCR4* in aggressive Non Hodgkin Lymphomas, in total 70 different patients were analysed. Frozen tissues -primary aggressive lymphoma specimens (53 specimens) as well as bone marrow biopsies (63 specimens)- were available at the Department of Hematopathology, Institute of Pathology, Medical University Graz.

For sequence analysis 25 primary lymphomas with an advanced stage (II-IV) were selected and for immunohistochemical analysis 40 primary lymphoma specimens were stained and analyzed. Table II-1 shows the different entities of the used DLBCL specimens.

Table II-1: Entities and number of primary lymphomas used for sequence and immunohistochemical analysis

Entities	Sequence analysis	Immunohistochemical analysis
DLBCL-NGCB	6	14
DLBCL-GCB	16	22
other classified	3	4
Total number of specimens	**25**	**40**

To determine the mRNA expression levels of the chemokine receptors and their ligands, in total 63 bone marrow biopsies of 52 patients were analyzed. From seven of the 52 patients two biopsies were used, and from two patients three biopsies were processed. Table II-2 classified the used bone marrow biopsies by their entities.

Table II-2: Entities and number of bone marrow biopsies used for mRNA expression analysis

Entities	Number of bone marrow biopsies	Number of initial diagnosis
DLBCL-NGCB	21	17
DLBCL-GCB	29	27
other classified	13	8
Total number	**63**	**52**

Lymphoma cell lines

In addition, 12 human lymphoma cell lines were examined for mutations. Table II-3 represents the different lymphoma subtypes. The cell lines Raji, RI-1, SU-DHL-4, U2932 and U937 were maintained in Roswell Park Memorial Institute 1640 (RPMI) (Gibco®/Life Technology, Carlsbad, California) Medium with 10% HyClone™ Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Waltham, Massachusetts) and 1% Antibiotic-Antimycotic 100x (Gibco®/Life Technology, Carlsbad, California). All other cell lines were maintained in RPMI1640 with 20% FBS and 1% Antibiotic-Antimycotic. The cells were kept and split as recommended by Leibnitz Institute DSMZ-German Collection of Microorganism and Cell Cultures and cultured by 37°C under 5% CO_2 conditions. All cell lines were checked for mycoplasma regularly and were examined as negative.

Table II-3: Cell lines used for sequence analysis

Name of cell line	Origin
NU-DUL-1	ABC DLBCL (activated B cell)
RI-1	ABC DLBCL (activated B cell)
U-2932	ABC DLBCL (activated B cell)
Karpas-422	B-NHL (diffuse large cell)
OCI-LY1	B-NHL (diffuse large cell)
OCI-LY3	B-NHL (diffuse large cell)
OCI-LY19	GCB DLBCL (germinal centre B cell)
SU-DHL-4	GCB DLBCL (germinal centre B cell)
SU-DHL-6	GCB DLBCL (germinal centre B cell)
U937	histiocytic lymphoma
Raji	Burkitt’s lymphoma
BL-2	Burkitt’s lymphoma

Sequencing of CXCR4 and CXCR7

Fifteen to twenty ten micrometer thick sections from fresh frozen tissues from the above mentioned specimens (see Table II-1) were micro dissected and prepared for DNA isolation. To eliminate cross contamination of specimens, a new microtome blade was used after each tissue. The pieces were put into a 2ml sterile tube.

DNA from 25 human specimens and 12 cell lines (see Table II-3) was extracted with the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For polymerase chain reaction the peqGOLD PCR Master-Mix and Hot Start-Mix S (Peqlab, Erlangen, Germany) was used. The total reaction volume was 25µl/well containing 1x Hot Start-Mix S, forward and reverse primer (Table II-4; Eurofins MWG Synthesis GmbH; Ebersberg, Germany) in final concentration of 10pmol/µl and 2µl of the extracted DNA. The following cycling protocol was used: one initial step at 95°C for 5min, followed by 40 cycles consisting of DNA denaturation at 95°C for 30s, annealing of primers, *CXCR7* exon1 at 54°C, *CXCR4* exon2 at 55°C and *CXCR4* exon1 at 59°C for 30s, and elongation at 72°C for 60s, concluding with one final step at 72°C for 7min.

Mutational screening of *CXCR4* and *CXCR7* were performed by direct sequencing by standard Sanger sequencing using BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The Reaction-Mix contained Big Dye™ Terminator v1.1 Cycle Sequencing RR-100, Big Dye™ Terminator v1.1 Cycle 5x Sequencing Buffer, 1µl of either forward or reverse primer (Table II-4) and 2µl of purified PCR product was added to a final volume of 20µl/well. The specimens were run with following cycling protocol with a ramp rate of 1°C/second: one incubation step for 1min at 96°C, followed by 25 cycles consisting of 10s denaturation at 96°C, 5s annealing at 50°C, and 4min extension at 60°C, closing with a holding step at 4°C before purifying. All purified cycle products were sequenced using the ABI Prism 3730 instrument at VBC Biotech in Vienna. The obtained sequences where analysed using the GeneMapper® software and SeqScape® software (Applied Biosystems, Carlsbad, CA, USA). All specimens were sequenced at least three times.

PCR products with *CXCR4* exon2 and *CXCR7* were purified with Hamilton Starlet instrument by using either NucleoFast® 96 PCR plates (Macherey-Nagel, Düren, Germany) before cycle sequencing reaction or afterwards using the Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Merck Millipore, Darmstadt, Germany). Protocols and instrument were provided from the Centre for Medical Research (ZMF). The *CXCR4* exon1 PCR products were purified with SigmaSpin™Sequencing Reaction Clean-Up, Post Reaction Clean-Up Columns (Sigma-Aldrich®, Vienna, Austria) according to the manufacturer's protocol.

Table II-4: Nucleotide acid sequences used for sequence analysis

Gene-Primer-ID	Length	Sequence
*CXCR4*_exon1_forward	19	CAGGTAGCAAAGTGACGCC
*CXCR4*_exon1_reverse	18	GAGGGGCTGCGCTCTAAG
*CXCR4*_exon2-1_forward	19	GCAAGCCTGAATTGGTTTT
*CXCR4*_exon2-1_reverse	20	CGTTGGCAAAGATGAAGTCG
*CXCR4*_exon2-2_forward	20	GTGGTCTATGTTGGCGTCTG
*CXCR4*_exon2-2_reverse	25	CTTTTATATCTGAAAAATGTGTAAC
*CXCR7*_exon1-1_forward	22	TGCTTGGTTTTCTCATAGGTCA
*CXCR7*_exon1-1_reverse	20	ACGGTCTTCAGGTAGTAGGT
*CXCR7*_exon1-2_forward	21	ACACCTACTACCTGAAGACCG
*CXCR7*_exon1-2_reverse	23	CTGTTCAAAAACAAGTAAACCCG

Immunohistochemical analysis of CXCR4 and *CXCL12*

Immunohistochemical analysis was investigated using 40 formalin-fixed paraffin embedded tissues from primary tumour specimens to detect *CXCL12* and/or *CXCR4* expression. These results were correlated with the preliminary results found by our group. Staining was performed after pre-treatment with Target Retrieval Solution (Dako, Glostrup, Denmark) using the DakoCytomation® (Glostrup, Denmark) automated immunostainer and iView detection system (Ventana Medical System, Tucson, AZ). Already established primary antibody to *CXCR4* (Abcam, Cambridge, Great Britain; dilution: 1:200) and *CXCL12* (R & D, Minneapolis, USA; dilution: 1:50) were used for expression analysis. For correlation analysis the immune reactive score (IRS) was calculated as described by Zhuang *et al*., and correlated with the *CXCR4* mRNA expression data .

Chemokine Expression Profiling

Thirty to forty five micrometer thick sections from 63 fresh frozen tissues from the above mentioned specimens (Table II-2) were micro dissected and prepared for RNA isolation. To eliminate cross contamination of specimens, a new microtome blade was used after each tissue. The pieces were put into a 2ml sterile tube.

Total RNA was extracted with the ReliaPrep™ FFPE Total RNA Kit (Promega, Fitchburg, WI, USA) including a DNase treatment according to the manufacturer's protocol with the exception of twice the amount of Lysis Buffer and 20times the volume of ProteinaseK and an overnight incubation at 56°C. The obtained total RNA was converted into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). For reverse transcription a total of 2.5 µg of template RNA and 0.4 µg of random hexamer primer were used.

The Real-Time PCR was performed in duplicates using an ABI Prism 7900 Detector (Applied Biosystems, Carlsbad, CA, USA). The mastermix contained 2x KAPA PROBE FAST qPCR Master Mix ABI Prism™ (Peqlab, Erlangen, Germany), 0.5µl of TaqMan® Gene Expression Assays (Table II-5; Applied Biosystems by Life Technology Foster City, California) and 4µl of diluted cDNA up to a volume of 10µl/well. The cycling protocol was as followed: one activation step for 3min at 95°C, followed by 40 cycles consisting of 2s denaturation at 95°C, 20s annealing at 60°C, and 1s extension at 72°C. *Glyceraldehydes 3-phosphate dehydrogenase (GAPDH)*, *hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1)* and *peptidylprolyl isomerase A (PPIA)* served as housekeeping genes. cDNA from buffy coat served as calibrator. Control specimens with either no reverse transcriptase or genomic DNA were included, to corroborate DNA decontamination. The results were expressed as relative units based on calculation $2^{-\Delta\Delta CT}$, which gives the relative amount of target gene normalized to the endogenous control (geometric mean of the three housekeeping genes).

Table II-5: TaqMan® Gene Expression Assays used for molecular analysis

Gene	Gene-ID	Length	Assay design
CXCR4	Hs00607978_s1	153	Primers and probe map within a single exon
CXCR7	Hs00604567_m1	129	Probe spans exons
CXCL11	Hs04187682_g1	96	Probe spans exons
CXCL12	Hs03676656_mH	88	Probe spans exons
GAPDH	Hs02758991_g1	93	Probe spans exons
HPRT1	Hs02800695_m1	82	Probe spans exons
PPIA	Hs99999904_m1	98	Amplicon spans exons and probe does not span

CXCR4 expression on cell lines

To determine the expression of CXCR4 on the cell line surface all cell lines were tested by using an anti-CXCR4 antibody. Therefore, $5x10^5$ cells of each lymphoma cell line were stained by either adding 3µl of anti-human CXCR4 or the isotype control conjugated with PerCP for 30 minutes on ice and FACS analysis were performed on a BD™ LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

CXCL12/SDF-1alpha binding assay and antagonist blocking

First, a CXCL12 binding assay was performed using an Alexa Fluor® labelled $CXCL12^{AF647}$ (BD, Franklin Lakes, USA) to determine the specificity of CXCL12 on *CXCR4* expressed cells. Therefore, cells were washed with HBSS buffer (Hanks' balanced salt solution with 20mM HEPES buffer and 1% bovine serum albumin, pH7.4) and incubated with $CXCL12^{AF647}$ for 30 minutes at room temperature. Then the cells were washed twice, fixed in phosphate buffered saline (PBS), and analyzed on the LSRII flow cytometer.

Further investigations were performed with the CXCR4 antagonists AMD070 (MCE, Stockholm, Sweden) and a chemical modified AMD070, namely WK1 (derivatized and provided from TU Graz, Austria; Figure II-1).

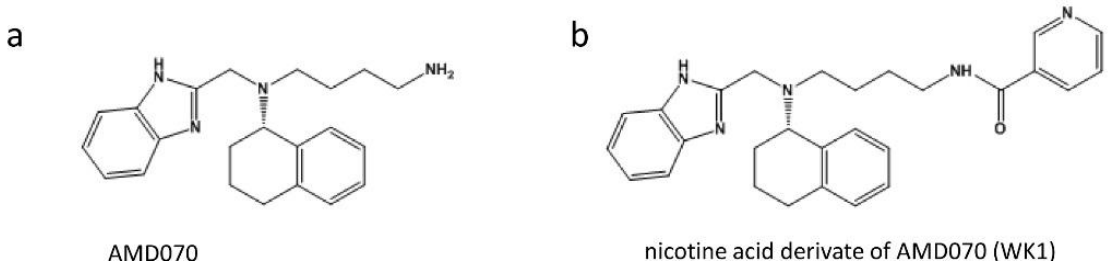

Figure II-1: Chemical structure of AMD070 (a) and derivatized AMD070 (b)

The CXCR4 antagonist AMD070 is commercially available. In order to enhance the therapeutic properties of these molecules, several chemical modifications of the parents structures have been introduced and their activity profiles have been evaluated (Jacobson and Weiss, 2013). Based on these findings, novel modifications were designed. In structure AMD070, the primary amine can be chemically addressed in a selective manner by reductive amination, acylation as well as sulfonylation reactions. The introduction of basic and/or aromatic systems as well as amino acid residues seems to be a starting point for the investigation of novel derivatives thereof. In this study we focused on the first residue, the nicotinic acid derivative of AMD070 (Figure II-2).

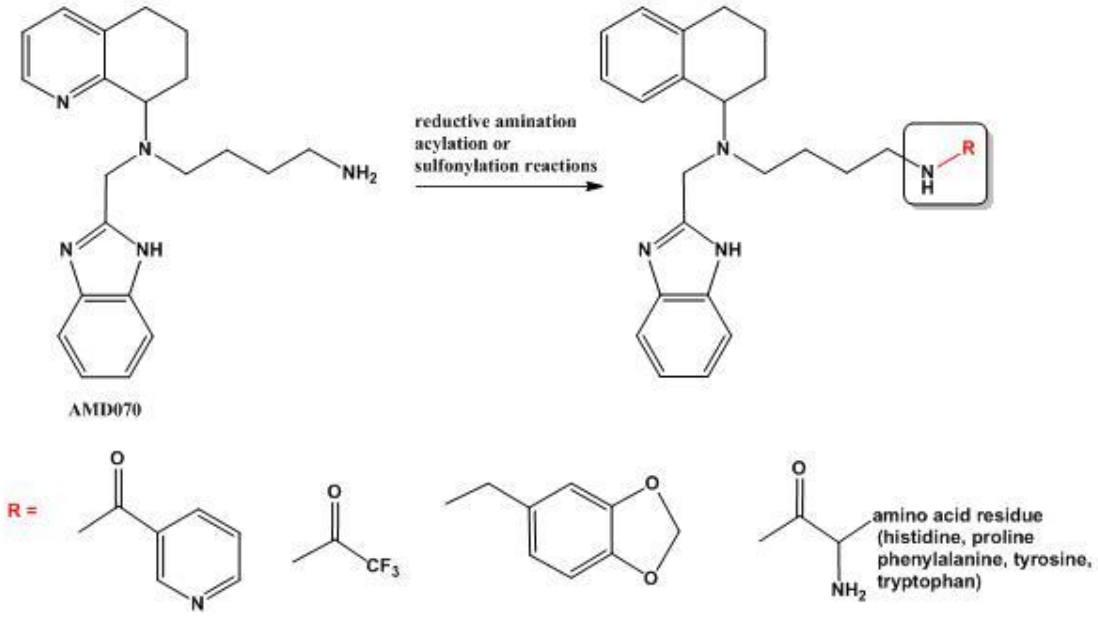

Figure II-2: Chemical modification of AMD070

Different concentrations of each antagonist, AMD070 and WK1 were tested to evaluate the inhibitory effect on the binding of the labelled CXCL12. Therefore, $1x10^6$ BL-2 cells were centrifuged and resuspended in 100µl HBSS, and the antagonists in different concentrations were added (10µM, 5µM, 1µM, 0.5µM, 0.1µM, 0.05µM, 0.01µM, 0.005µM) and incubated for 45 minutes at 37°C. Then, 0.5µl $CXCL12^{AF647}$ was added and incubated for another 3 hours at 37°C. After incubation, the cells were washed and resuspended in 100µl PBS and measured using flow cytometry. The percentage of inhibition of $CXCL12^{AF647}$ binding were calculated as described by Hatse *et al.*

Quantikine human SDF-1 alpha Immunoassay

Supernatant of 10 different cell lines were taken after 24 and 48 hours, as well two controls including RPMI Medium (Gibco®) with 10% FBS and 20% FBS (Thermo Scientific Fisher) for the solid-phase ELISA using the Human CXCL12/SDF-1 alpha Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis; MN, USA) to measure human CXCL12 according to following instructions: 100µl of assay diluents was added in each well, then 100µl of Standard, controls and supernatant was added and incubated at room temperature for 2 hours on a horizontal micro plate shaker. The plate was aspirated and washed for a total of 4 washes. Then 200µl conjugate solution was added and incubated for another 2 hours on the shaker. The plate was aspirated and washed again 4 times. Thereafter 200µl of Substrate Solution was added and again incubated at room temperature for 30 minutes protected from light. Last 50µl of Stop Solution was added to each well and measured at 450nm at a spectrophotometer.

Multiplex Immunoassay

For the multiplex immunoassay supernatant of 8 different cell lines were taken after 24, 48 and 72 hours, as well three controls including RPMI Medium (Gibco®) without FBS or with 10% FBS and 20% FBS (Thermo Scientific Fisher). Human SDF-1 alpha Simplex and human I-TAC/CXCL11 Simplex were combined using the ProcartaPlex™ Multiplex Immunoassay (Affymetrix eBioscience, San Diego, CA, USA). Therefore, the antibody coated magnetic capture beads were successive added and washed using a hand- held magnetic plate washer. 50µl of Standards, controls and supernatants were added and incubated for 2 hours at room temperature at 500rpm on a horizontal plate shaker protected from light. The plate was washed two times and 25µl of Detection Antibody Mix was added and incubated for another 30 minutes with the same conditions and again washed two times. After addition of 50µl Streptavidin-PE the plate were incubated and washed again. Last 120µl of Reading Buffer was added to each well and measured on a Bio-Plex 200 multiplex system (BIO-RAD, Hercules, CA, USA).

Cell viability and apoptosis assay

Cells were stained with Annexin V-APC and 7-AAD (7-amino-actinomycin D) with the Annexin V Apoptosis Detection Kit APC (Affymetrix eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Cells were washed and centrifuged in binding buffer (0.1M HEPES/NaOH (pH7.4), 1.4M NaCl, 25mM $CaCl_2$), and the pellet was resuspended in 5µl Annexin V-APC, 5µl 7-AAD and 200µL binding buffer, followed by incubation for 15 minutes at room temperature in the dark. Measurement was performed by flow cytometer using the LSRII instrument (BD Biosciences). Percentage of double negative cells was taken to determine viability.

Activity of caspases 3 and 7 was determined by Caspase-Glo 3/7 assay (Promega, Fitchburg, WI, USA). 50µl of the luminogenic substrate was added to 50µl cell suspension and incubated for 1 hour. Luminescence was measured with the LUMIstar Omega (BMG Labtech, Offenberg, Germany). All experiments were performed in duplicates at two time points: 24h, 48h.

Cell cycle assay

$1x10^6$ cells were incubated with 1µM BrdU solutions for 1 hour at 37°C. BrdU and 7-AAD staining was performed according to the BrdU Flow kit manual from BD. A total of 10.000 events were collected on a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell growth assay

Cells and controls were plated at a density of 10000 cells/ml and cultured for 72 hours. Three replicates of the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, USA) were done using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) according to the manufacturer's protocol. The absorbance was recorded by a BioRad spectrophotometer at 490nm.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA). A $p<0.05$ was considered statistically significant. All estimated values are presented as mean values and error bars represent the standard deviation.

III. Results

A. Clinical Part

Sequence Analysis – primary aggressive lymphoma

Preliminary data from our research group indicated that *CXCR4* and *CXCR7* expression were associated with an advanced stage in aggressive B cell lymphomas. Further, a high *CXCR7* expression correlated with a poor lymphoma specific survival and clinical outcome. To clarify, whether this observation is caused by genetic alterations in the receptors, we directly sequenced 25 primary lymphoma specimens.

CXCR4 sequence was altered in three of the 25 lymphoma specimens and in two of the 12 lymphoma cell lines. All mutated specimens had a single base pair substitution, which was located at the 414 position in the coding exon2 (Table III-1; Figure III-1a). This led to a base exchange of Cytosine (C) to Thymine (T). The base exchange did not influence the amino acid sequence and is representing a synonymous mutation.

Table III-1: Results of the *CXCR4* sequencing analysis in 25 lymphoma specimens and 12 cell lines

		DLBCL	Cell lines
***CXCR4* exon2**	**mutated/tested**	3 / 25	2 / 12
	mutation	c.414 C > T	c.414 C > T
		silent	silent

Classification of the mutated specimens to their origin is shown in Table III-2. In *CXCR4* exon2 two of the three mutated DLBCL specimens were found in GCB subtype specimens and one in the NGCB subtype specimen. Cell lines with this alteration were NU-DUL-1 and U-2932; both have an NGCB-DLBCL origin. In the coding region of exon1 of *CXCR4* were no mutation found either in patient specimens nor in the 12 tested cell lines.

Table III-2: Classification in DLBCL subtypes of the *CXCR4* sequencing results

		DLBCL	DLBCL-GCB	DLBCL-NGCB	Other DLBCL
CXCR4 exon1	**mutated/tested**	0 / 25	0 / 16	0 / 6	0 / 3
	mutation	-	-	-	-
CXCR4 exon2	**mutated/tested**	3 / 25	2 / 16	1 / 6	0 / 3
	mutation	c.414 C > T	c.414 C > T	c.414 C > T	-
		silent	silent	silent	-

Direct sequencing of *CXCR7* was also performed in 25 patient specimens and 12 lymphoma cell lines. We found one silent variation located at 796 position in ten of 25 specimens and in six of 12 cell lines (Table III-3, Figure III-1b). Four of the 25 specimens and three of the cell lines additionally exhibited a second silent variation at the 189 nucleotide position (Figure III-1c). All mutations were a single base pair exchange of Cytosine (C) to Thymine (T) and had no effect at the amino acid sequence.

Table III-3: Results of the *CXCR7* sequencing analysis in 25 lymphoma s and 12 cell lines

		DLBCL	Cell lines
CXCR7	**mutated/tested**	10 / 25	6 / 12
	mutation	c.796 C > T	c.796 C > T
	mutated/tested	4 / 25	3 / 12
	mutation	c.189 C > T	c.189 C > T
		silent	silent

Table III-4 shows the ten mutated patient specimens classified to their origin. Three of the in total ten mutated DLBCL specimens in position 796 were found in germinal centre B cell subtype specimens, five in the non-germinal centre B cell subtype specimens, and two in other classified DLBCL specimens. The mutation in position 189 in the coding sequence of *CXCR7* was found in two GCB- and also in two NGCB patient specimens (Table III-4). The six cell lines with the alteration in position 796 were OCI-LY3, Karpas-422 (both B-NHL origin), OCI-LY19, SU-DHL-6 (both GCB-DLBCL origin), U937 (histiocytic lymphoma origin) and Raji (Burkitt's lymphoma origin). OCI-LY3, OCI-LY19 and U937 had an additional mutation at position 189.

Table III-4: Classification in DLBCL subtypes of the *CXCR7* sequencing results

		DLBCL	DLBCL-GCB	DLBCL-NGCB	Other DLBCL
CXCR7	**mutated/tested**	10 / 25	3 / 16	5 / 6	2 / 3
	mutation	c.796 C > T	c.796 C > T	c.796 C > T	c.796 C > T
	mutated/tested	4 / 25	2 / 16	2 / 6	0 / 3
	mutation	c.189 C > T	c.189 C > T	c.189 C > T	-
		silent	silent	silent	-

In all altered *CXCR7* specimens no mutation were found in the *CXCR4* exon2. All mutations were confirmed at least three times.

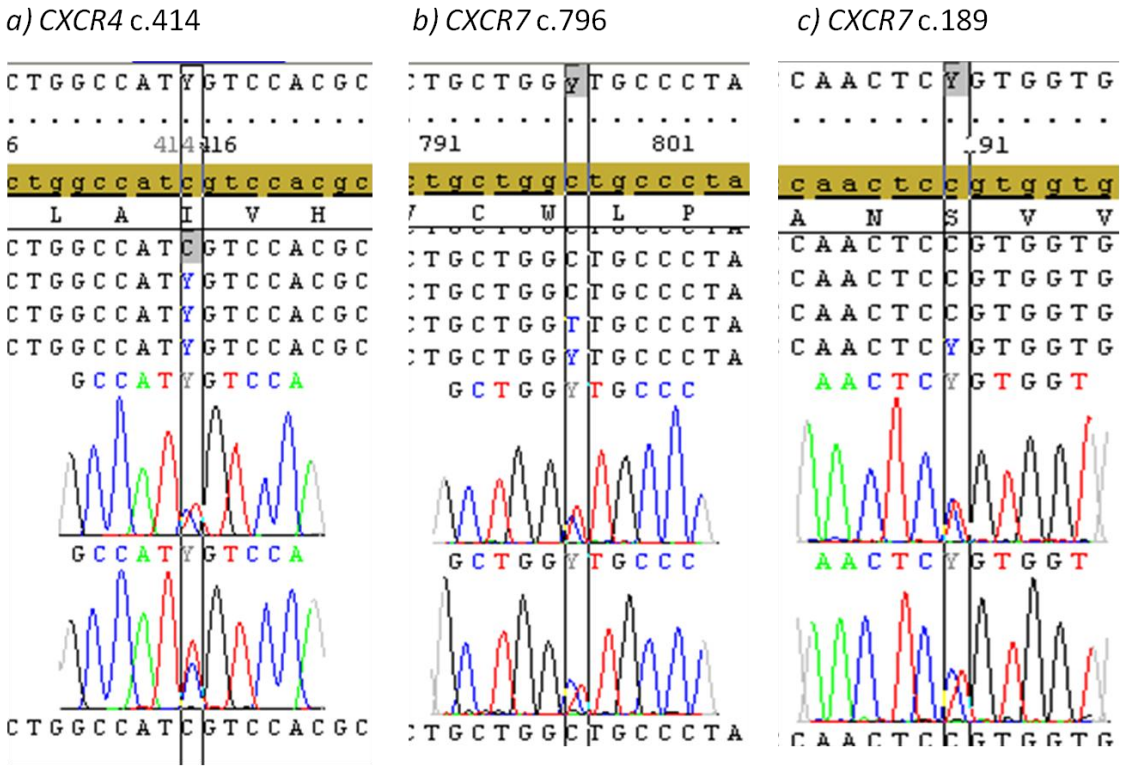

Figure III-1: Mutations found in *CXCR4* and *CXCR7* sequence analysis

All mutations had a single base pair substitution from Cytosine (C) to Thymine (T), which were located in the 414 position of the *CXCR4* coding exon2 (a), in the 796 position (b) and in the 189 position (c) in the *CXCR7* coding sequence.

CXCR4 and CXCL12 expression in primary aggressive lymphomas

In our previous study on *CXCR4* expression in DLBCL, we found an over expression of *CXCR4* compared to their non-neoplastic controls. We also compared the expression levels in aggressive lymphoma patients with clinical stage I to patients with an advanced stage (stage II-IV); a significant higher expression of *CXCR4* was found. Additionally, a significant positive correlation of *CXCR4* expression and bone marrow infiltration of the primary lymphomas was observed. Furthermore, we used the microarray data (GSE10846) published by Lenz *et al.*, 2008 of patients treated with R-CHOP (n=200) classified in NGCB-DLBCL and GCB-DLBCL to perform survival analysis (Lenz *et al.*, 2008). The DLBCL patients were divided into two groups (low and high *CXCR4* expression) using the median of *CXCR4* expression. A significant association between high *CXCR4* expression and poor cancer-specific survival was observed (Figure III-2; p=0.0225, log-rank test).

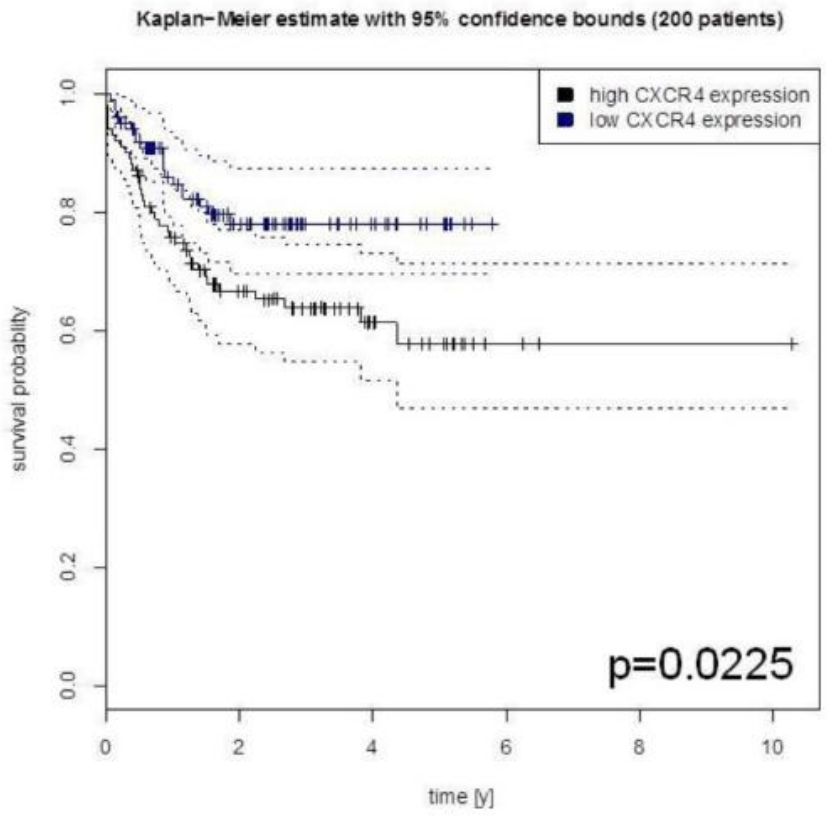

Figure III-2: Kaplan-Meier curve

Probability of cancer-specific survival in DLBCL patients stratified according to the *CXCR4* mRNA expression level. High *CXCR4* expression is associated with poor survival (p=0.0225).

To confirm the previous *CXCR4* expression data we performed immunohistochemical analysis with 40 primary lymphoma specimens including 14 NGCB-DLBCL, 22 GCB-DLBCL and 4 other classified DLBCL lymphomas (n=40). Comparing *CXCR4* expression levels to the clinical stage of this cohort a significantly decreased IRS (2 fold reduction) in primary lymphomas with an advanced stage (stage II-IV) was observed compared to the lymphoma with stage I (p=0.031; Figure III-3) and IRS did not correlate with our preliminary data of *CXCR4* mRNA expression in our cohort of primary lymphomas.

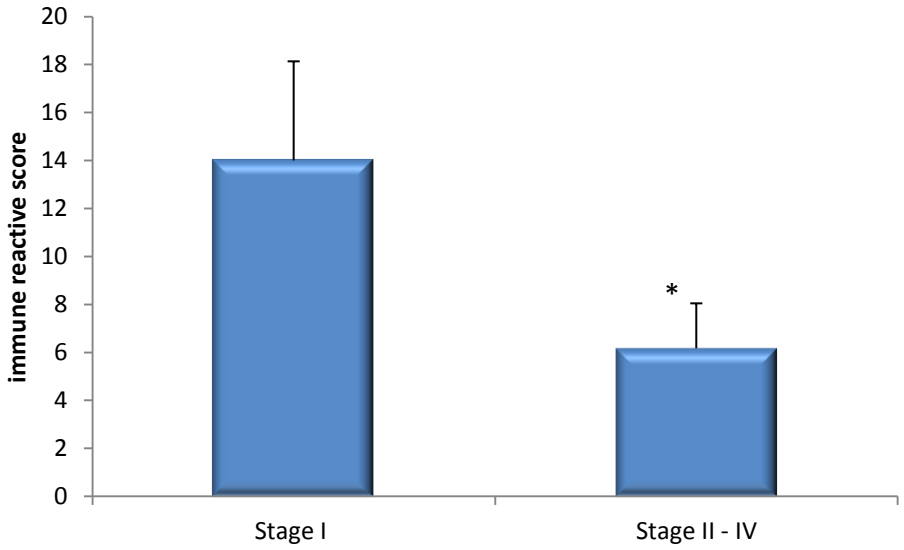

Figure III-3: Immune reactive score of *CXCR4*expression

Comparison of *CXCR4* immune reactive score (IRS) in a cohort of patients (n=40) with a clinical stage I to an advanced stage (stage II-IV), showed a 2 fold reduction in patients with an advanced stage (II-IV). Each bar represents the mean values of IRS ± standard deviation (SD). * indicates reduced expression ($P<0.05$)

Though, immunohistochemical analysis of *CXCR4* did not correlate to our mRNA expression in primary aggressive lymphomas of patients with DLBCL stage I compared to patients with an advanced stage (stage II-IV), two specimens with an advanced stage (stage II-IV) exhibited a strong *CXCR4* expression (Figure III-4, II and III), whereas the specimen with stage I showed a weaker reactivity for *CXCR4* (Figure III-4, I).

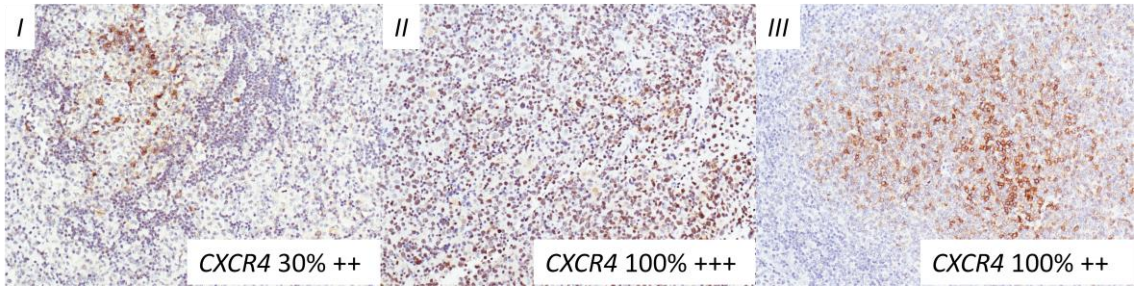

Figure III-4: Immunohistochemical analysis of *CXCR4*

Immunohistochemical analysis of *CXCR4* in primary aggressive lymphomas, in NGCB DLBCL (magnification x20 for panel I and panel II), in GCB DLBCL (magnification x20 for panel III). All images were captured by using an Olympus BX51 microscope and an Olympus E-330 camera.

CXCL12 expression in primary aggressive lymphoma exhibited no significant difference in patients with stage I compared to patients with stage II-IV (Figure III-5).

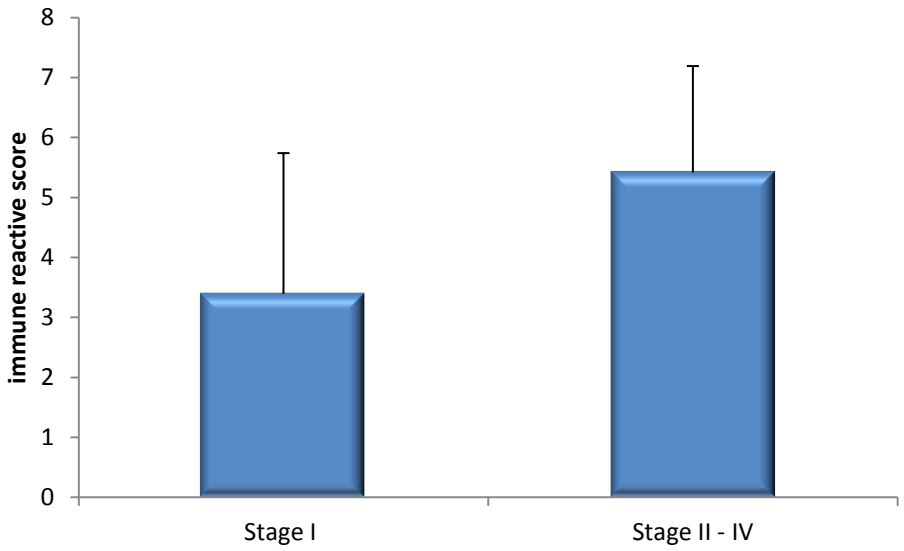

Figure III-5: Immune reactive score of *CXCL12* expression

Comparison of *CXCL12* immune reactive score (IRS) in a cohort of patients (n=40) with a clinical stage I to an advanced stage (stage II-IV) showed no significant difference. Each bar represents the mean values of IRS ± standard deviation (SD).

Although the IRS exhibited no significant difference in *CXCL12* expression in patients with an advanced stage (stage II-IV), the immunohistochemical staining of *CXCL12* showed no to 30% expression compared to the same specimens with *CXCR4* expression (Figure III-6).

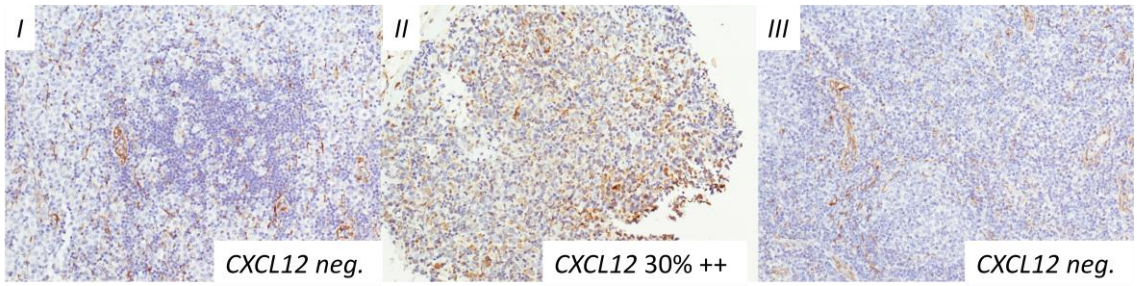

Figure III-6: Immunohistochemical analysis of *CXCL12*

Immunohistochemical analysis of *CXCL12* in primary aggressive lymphomas, in NGCB DLBCL (magnification x20 for panel I and panel II), in GCB DLBCL (magnification x20 for panel III). All images were captured by using an Olympus BX51 microscope and an Olympus E-330 camera.

Chemokine Expression of bone marrow biopsies of aggressive lymphoma patients

To determine whether *CXCR4*, *CXCR7*, *CXCL11* and *CXCL12* expression have any effects on the bone marrow infiltration process of aggressive lymphomas, we performed Realtime PCR analysis on the corresponding bone marrow biopsy of your lymphoma patient cohort. Therefore, we used in total 63 bone marrow specimens. 52 bone marrow biopsies were taken at time of diagnosis, including 17 NGCBs, 27 GCBs, and 8 other classified DLBCL. 12 (5 NGCBs, 5 GCBs, and 2 other classified DLBCL) of the 52 patients had an infiltrated bone marrow at time of diagnosis. 11 patients had repeated biopsies during their course of disease, seven patients went into remission and four patients relapsed.

CXCR7 mRNA expression was just found in a minority of the bone marrow specimens and survival analysis in the published data set of Lenz *et al.*, 2008 did not confirm the association of CXCR7 expression as found in our cohort, therefore this chemokine recptors was excluded from all analysis and all further *in vitro* experiments (Lenz *et al.*, 2008). Comparing the expression levels of *CXCR4*, *CXCL12*, and *CXCL11* between bone marrow specimens with and without lymphoma infiltration at the time of diagnosis, a higher expression of *CXCL11* (60 fold higher) was found in bone marrow specimens without lymphoma infiltration (Figure III-7). No significant difference of *CXCR4* and *CXCL12* expression was observed in the comparison of uninfiltrated and infiltrated specimens.

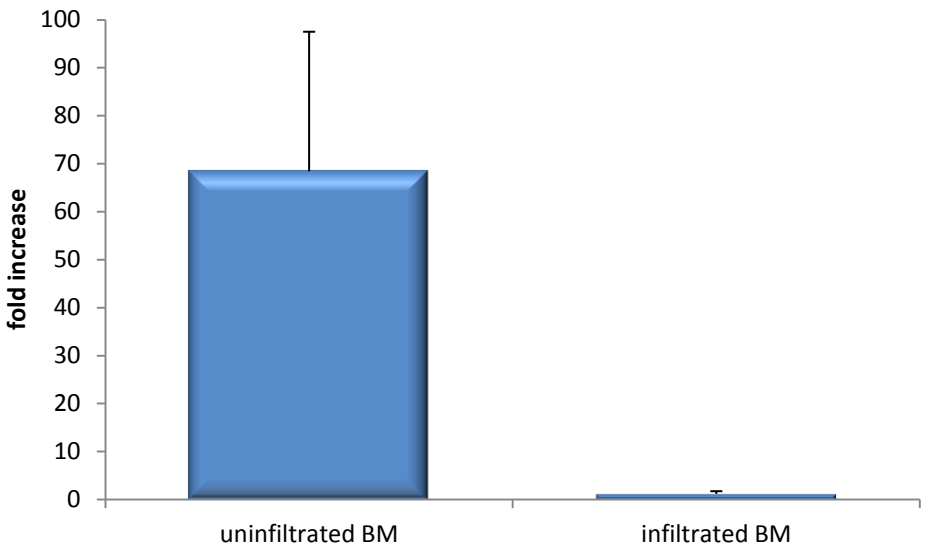

Figure III-7: Comparison of uninfiltrated and infiltrated bone marrow expression levels in *CXCL11*

A 60 fold higher *CXCL11* expression was found in uninfiltrated bone marrow compared to infiltrated bone marrow specimens at time of diagnosis was found. Each bar represents the mean values of expression levels ± standard deviation (SD).

By correlating *CXCL12* expression levels of 16 infiltrated bone marrow biopsies, consisting of 12 specimens at time of diagnosis and 4 relapsed specimens, we observed a significant positive correlation between *CXCL12* expression and the percentage of infiltration levels (Spearman-Rho=0.764; p=0.001) (Figure III-8).

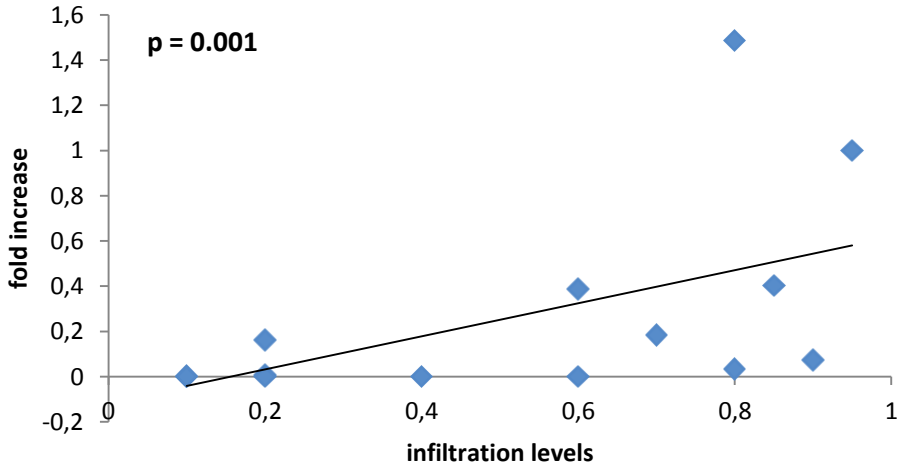

Figure III-8: Regression analysis of *CXCL12* expression and infiltration rates/levels

A significant positive correlation between *CXCL12* expression and percentage of infiltration levels was found in infiltrated bone marrow specimens (Spearman-Rho=0.764 p=0.001).

Further we analyzed 7 previously infiltrated BMs losing their infiltration following chemotherapy (BM under remission) and compared *CXCR4* expression to the untreated paired biopsies. Remission of the BM infiltration caused a 3-fold reduction of *CXCR4* expression (p=0.075, Figure III-9). For *CXCL11* and *CXCL12*, no differences were detected (data not shown).

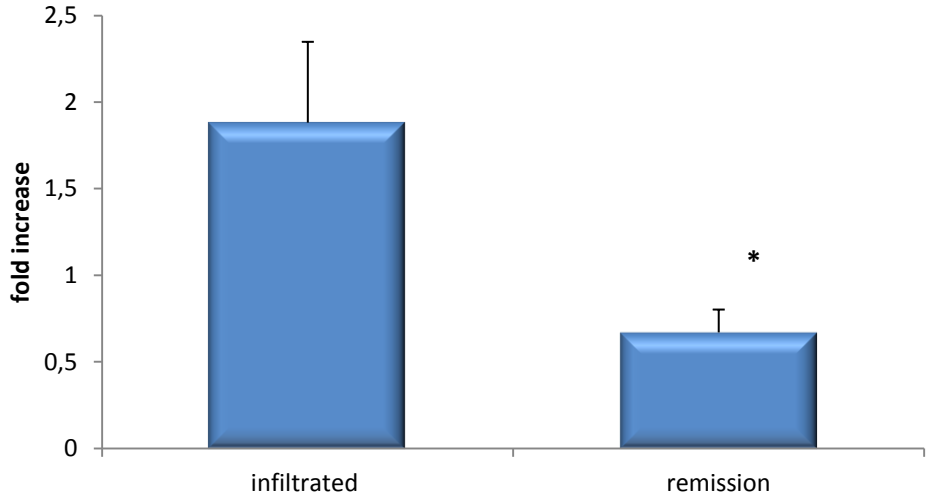

Figure III-9: Comparison of *CXCR4* expression levels of infiltrated bone marrow specimens with patient specimens in remission

Patient in remission showed a 3-fold lower *CXCR4* expression compared to the infiltrated bone marrow biopsies at time of diagnosis (Wilcoxon-Test p=0.075). Each bar represents the mean values of expression levels ± standard deviation (SD). * indicates reduced expression

B. In vitro analysis

CXCR4 expression on cell surface and CXCL12 binding of lymphoma cells

To determine the *CXCR4* expression on the surface of the cells we performed FACS analysis using an anti-CXCR4 antibody by incubation following cell lines, NU-DUL-1, RI-1, and U-2932 as ABC-DLBCL, SU-DHL-4 as GCB-DLBCL, Raji and BL-2 as Burkitt's lymphoma, Karpas-422 and OCI-LY1 as B-NHL. All investigated lymphoma cell lines except Karpas-422 exhibited a *CXCR4* expression (Figure III-10). BL-2, NU-DUL-1, OCI-LY1 and RI-1 showed strong *CXCR4* expression, Raji and SU-DHL-4 moderate expression, and U-2932 weak *CXCR4* expression.

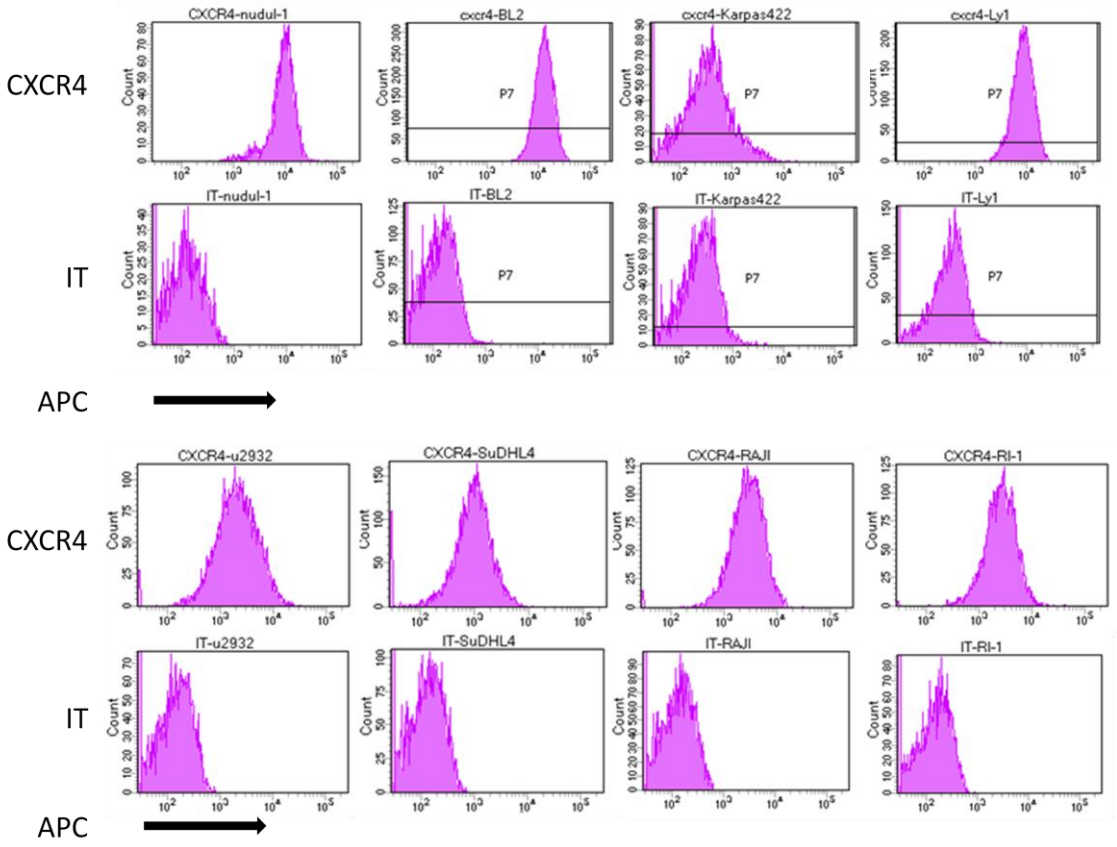

Figure III-10: *CXCR4* expression on the cell surface investigated by FACS analysis

The first and the third row showed the *CXCR4* expression on the cell surface after incubation with an anti-CXCR4 antibody and the second and fourth row demonstrated the corresponding isotype control, where no *CXCR4* expression was detected.

Further, we performed a CXCL12/SDF-1alpha binding assay using a fluorescent labelled $CXCL12^{AF647}$ to investigate the specificity of CXCL12 binding to CXCR4 positive lymphoma cells, NU-DUL-1, BL-2, and OCI-LY1, and Karpas-422 as CXCR4 negative lymphoma cells. No fluorescence shift was found for Karpas-422 cells, whereas the fluorescence of OCI-LY1, BL-2, and NU-DUL-1 showed higher intensity (Figure III-11). These data together with the FACS data of *CXCR4* expression indicate that lymphoma cells bind CXCL12 via CXCR4.

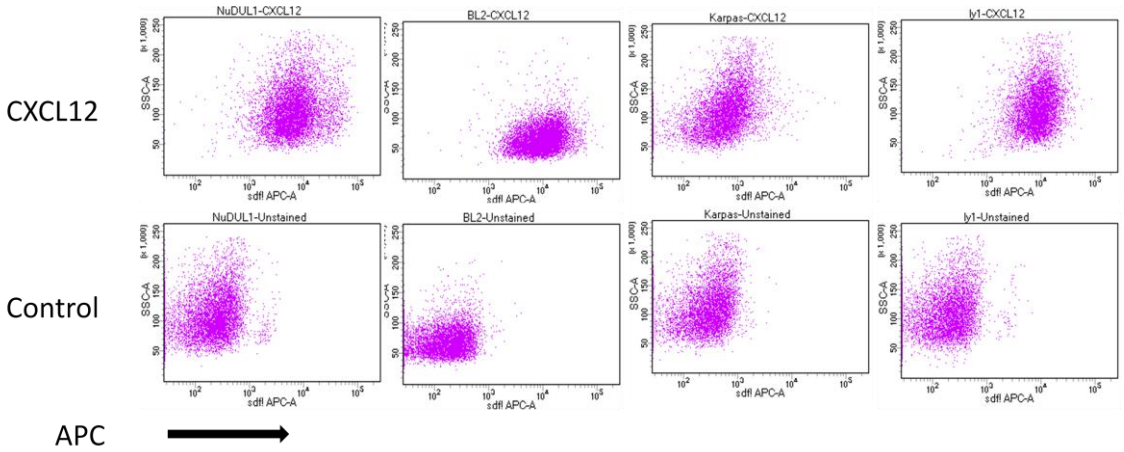

Figure III-11: Specific interaction of $CXCL12^{AF467}$ to $CXCR4^{+}$ and $CXCR4^{-}$ cells

The first row showed the $CXCL12^{AF647}$ binding on CXCR4 positive cells, NU-DUL1, BL-2 and OCI-LY1, and CXCR4 negative Karpas-422. The second row demonstrated the corresponding control, where no CXCL12 binding was observed.

BL-2 has been described by Beider *et al.*, 2013, to possesses a strong *CXCR4* expression and spread via CXCR4 into bone marrow *in vivo* within 4 weeks (Beider *et al.*, 2013). Therefore, we especially investigated BL-2 for functional characterization and all other experiments on CXCR4. Beside BL-2 we used also these three aggressive lymphoma cell lines, SU-DHL-4 as GCB-DLBCL, RI-1 as ABC-DLBCL, and Raji as Burkitt's lymphoma for further investigations.

Effect of different concentrations of CXCL12

To investigate the effect of CXCL12 on the lymphoma cells, we treated under starvation (without FBS) three lymphoma cell lines - BL-2, SU-DHL-4, and RI-1- in combination with and without different CXCL12 concentrations (0ng/ml, 500ng/ml, 750ng/ml and 1500ng/ml) and determined the cell viability in comparison to cells cultured in full media as control. The cell viability was measured with the TC20 cell counter (Bio-Rad, Hercules, CA, USA). No significant difference could be detected between the different concentrations of added CXCL12 or without CXCL12 for the starved cells. Cell viability of lymphoma cells under starvation was reduced, whereas cells in full media exhibited almost an equal percentage of viable cells (Figure III-12).

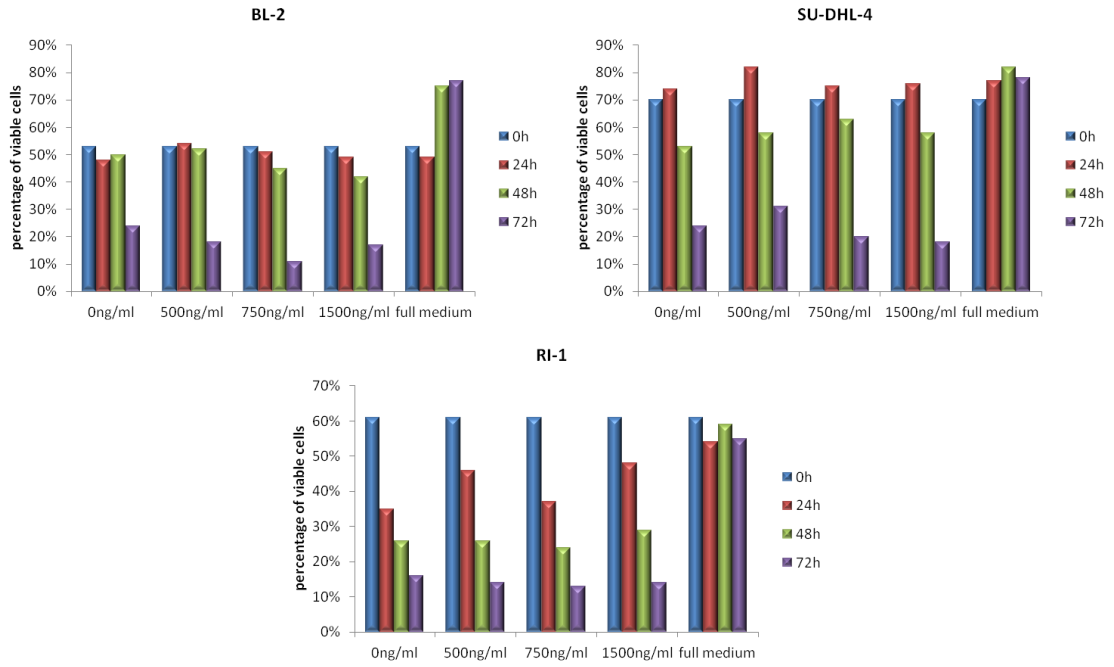

Figure III-12: Viability of cells with different concentrations of CXCL12

BL-2, SU-DHL-4, and RI-1 were treated under starvation without and with different CXCL12 concentrations (0, 500, 750, and 1500ng/ml) compared to cells cultured in full media (served as control) for 0, 24, 48, and 72 hours. Each bar represents the mean values of viable cell percentages.

These data indicate that CXCL12 treatment does not prevent lymphoma cells from induction apoptosis under starvation.

CXCL12 production by lymphoma cells

To observe human CXCL12 in cell supernatants an immunoassay (CXCL12/SDF-1 alpha Quantikine ELISA) was performed using ten different cell supernatants under starvation after 24 and 48 hours, and measured at 450nm at a spectrophotometer. We could not detect any CXCL12 protein in the analysed supernatants. The concentration of SDF-1alpha in our lymphoma cells might be low. Therefore, we performed another SDF-1alpha and I-TAC combined multiplex immunoassay – a more sensitive method (Leng *et al.*, 2008). Moreover, the remaining supernatants of 8 different cell lines were used for the ProcartaPlex™ Multiplex Immunoassay (Affymetrix eBioscience) for CXCL12 and CXCL11. According to the manufacturer's instructions we observed the standard curves for SDF-1alpha and I-TAC (Figure III-13). Both graphs showed very low fluorescence intensity up to a concentration of 100pg/ml.

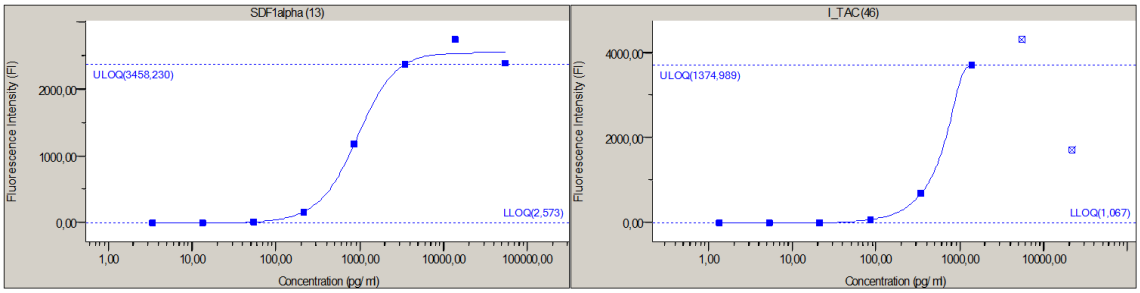

Figure III-13: Multiplex immunoassay standard curves: SDF-1alpha and I-TAC

Standard curves of SDF-1alpha/CXCL12 (left graph) and I-TAC/CXCL11 (right graph) calculated with the Bio-Plex software. Fluorescence intensity plotted on the y-axis, concentration in pg/ml plotted on the x-axis.

In the cell lines we observed no differences at the different time points and again low fluorescence intensity around 17 and 5 in SDF-1alpha and I-TAC, respectively (Figure III-14). These data indicate that the investigated lymphoma cells do not produce CXCL12 and CXCL11.

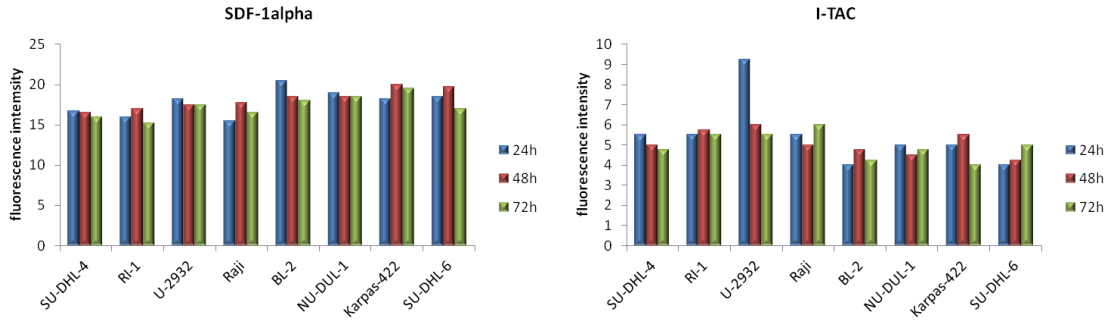

Figure III-14: Fluorescence intensity of SDF-1alpha and I-TAC in different cell lines

Fluorescence intensity of SDF-1alpha/CXCL12 (left graph) and I-TAC/CXCL11 (right graph). Each bar represents the mean values calculated with the Bio-Plex software.

CXCR4-Antagonists treatment

First, the BL-2 cell line exhibiting high *CXCR4* expression as shown above and by Beider *et al.* (Beider *et al.*, 2013), was used to test the inhibitory effects on CXCL12. Therefore, we incubated BL-2 cells with $CXCL12^{AF647}$ in the presence of increasing concentrations (0.005µM, 0.01µM, 0.05µM, 0.1µM, 0.5µM, 1µM, 5µM, and 10µM) of the CXCR4 antagonists AMD070 and WK1. The gradual decrease of $CXCL12^{AF647}$ binding in the presence of increasing concentrations of the CXCR4 antagonists is demonstrated in Figure III-15. By regression analysis we calculated the 50% inhibitory concentrations for the CXCR4 antagonists. The concentrations were 2µM for AMD070 and 1µM for WK1. $CXCL12^{AF647}$ binding was inhibited in both CXCR4 antagonists; however treated BL-2 cells (high *CXCR4* expression) demonstrated a better inhibitory effect with AMD070 with low concentration.

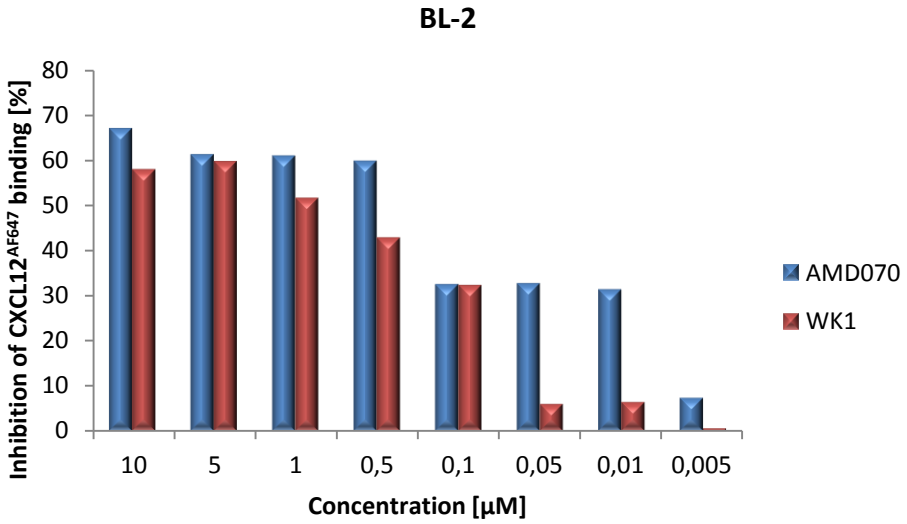

Figure III-15: The inhibitory effect of CXCR4 antagonists on the binding of $CXCL12^{AF647}$ in BL-2

The cells were incubated with $CXCL12^{AF647}$ in the presence of increasing concentrations of CXCR4 inhibitors, AMD070 (blue bars) or WK1 (red bars). The bars represent the percentages of inhibition of $CXCL12^{AF647}$ binding calculated as described by Hatse *et al.*, 2004 in the presence of the inhibitor relative to the positive control, where the cells were exposed $CXCL12^{AF647}$ alone.

To investigate the effects of AMD070 and WK1 on lymphoma cells, we treated Raji, RI-1, U-2932, and SU-DHL-4 with both CXCR4 antagonists in different concentrations (20µM, 10µM, 5µM, and 1µM) and performed a MTS assay to estimate proliferation. DMSO treated cells served as control. AMD070 and WK1 inhibited in concentration-dependent manner the cell growth of all investigated lymphoma cell lines (Figure III-16).

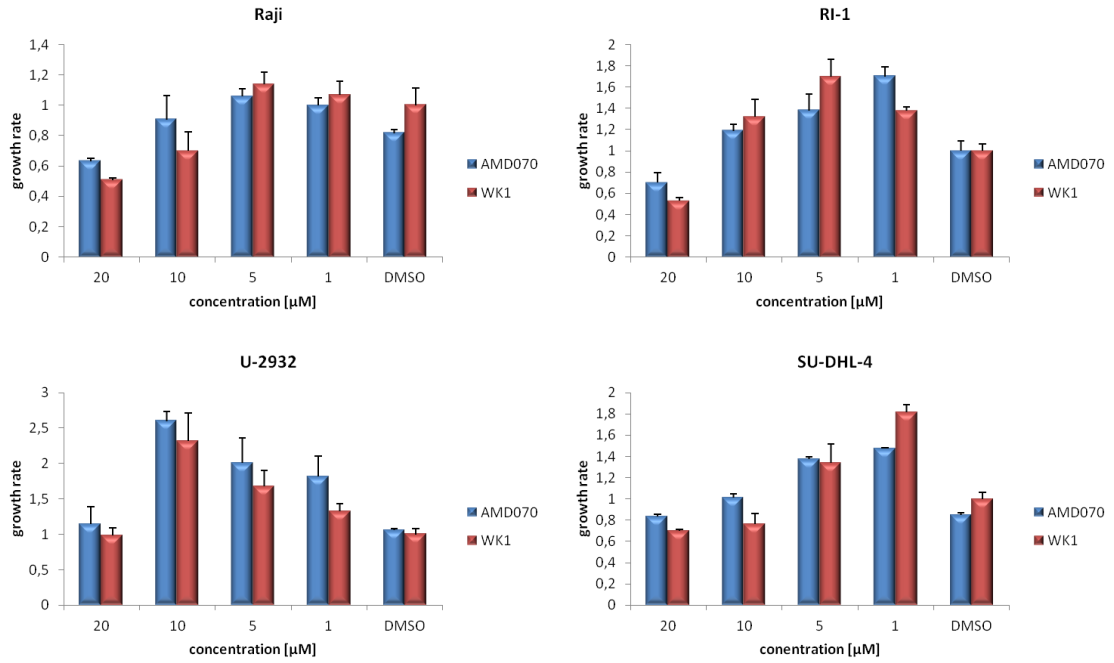

Figure III-16: Cell growth assay in AMD070 and WK1 treated cells

The cells (Raji, RI-1, U-2932, and SU-DHL-4) were treated with different concentration (20µM, 10µM, 5µM, and 1µM) of the CXCR4 antagonists, AMD070 (blue bars) or WK1 (red bars). Each bar represents the mean values of growth rate ± standard deviation (SD).

Furthermore, we treated the different cell lines, Raji, RI-1, U-2932, and SU-DHL-4, with various concentrations (20µM, 10µM, 5µM, and 1µM) and performed AnnexinV/7AAD assay to examine the cytotoxic effects of the antagonists, AMD070 and WK1. After 48 hours the cells were stained with Annexin V and 7AAD and analysed by using flow cytometry. DMSO cells were included as vehicle controls. The cell lines, Raji and SU-DHL-4 showed a marked reduction of cell viability especially for 20µM and 10µM with AMD070 and WK1 (Figure III-17). RI-1 cells exhibited significantly reduced cell viability only in the WK1 treated cells. The U-2932 cell viability was slightly reduced in cells treated only with WK1. Difference between the cell viability of AMD070 and WK1 combining with the *CXCR4* expression on cell surface, suggested that WK1 possesses higher cytotoxic effects on the investigated cell lines.

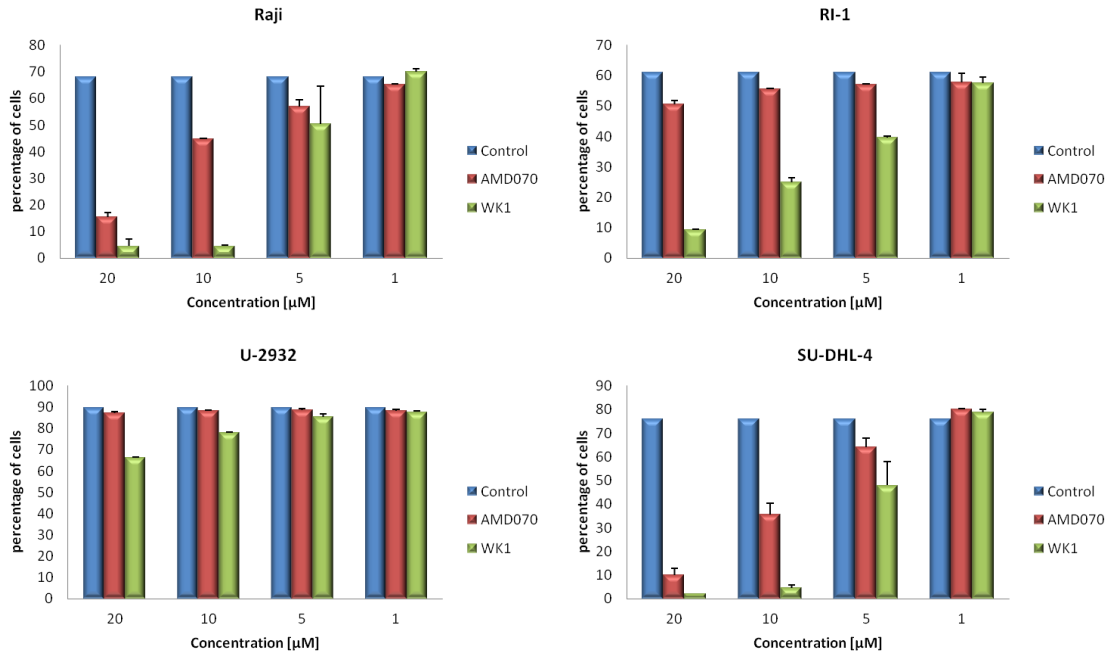

Figure III-17: Percentage of viable cells after 48 hours of treatment with AMD070 and WK1 in four lymphoma cell lines

Cell viability of Raji, RI-1, U-2932, and SU-DHL-4 treated with different concentrations (20µM, 10µM, 5µM, and 1µM) of AMD070 (red bars) and WK1 (green bars). DMSO served as control. Each bar represents the mean cell viability ± standard deviation (SD). Percentage of double negative cells was taken to determine viability.

Another apoptosis assay, using caspase 3/7 activity assay, showed no significant increase in apoptosis rate in the cell lines Raji and U-2932 (Figure III-18). The cell lines RI-1 and SU-DHL-4 showed an increased relative activity of the chemical modified WK1 compared to the control after 24 hours, which is in contrast to the observed data.

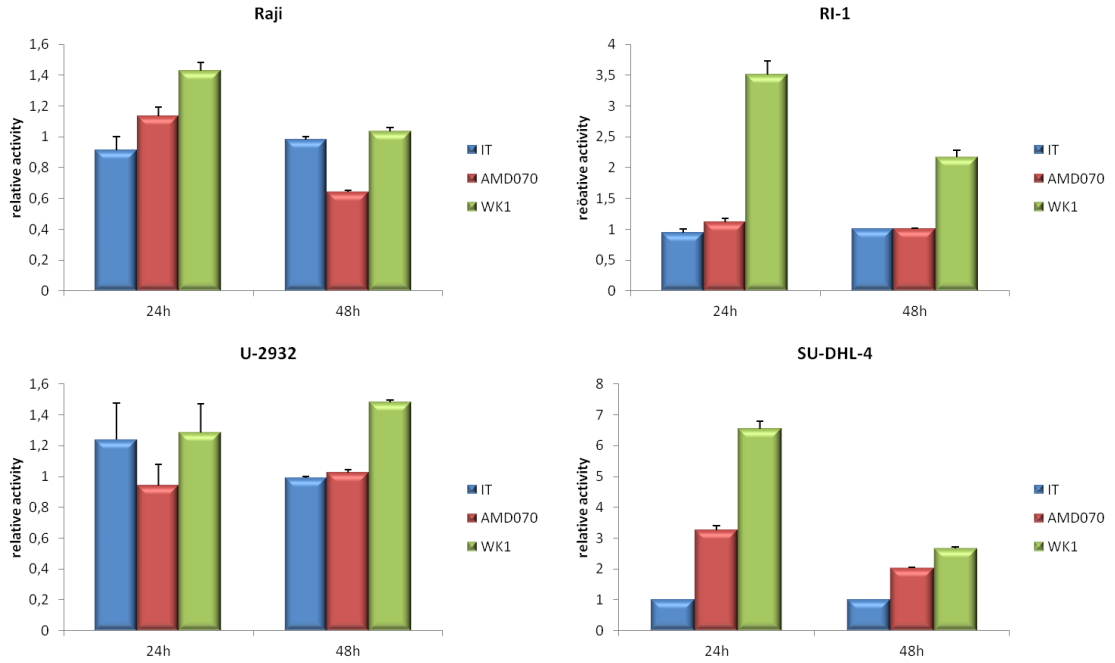

Figure III-18: Caspase 3/7 activity in four cell lines treated with AMD070 and WK1.

Relative activity of Raji, RI-1, U-2932, and SU-DHL-4 treated with the CXCR4 antagonists, AMD070 (red bars) and WK1 (green bars). DMSO served as control (blue bar). Each bar represents the mean values of relative activity ± standard deviation (SD).

A BrdU cell cycle analysis was performed using flow cytometry to determine the different stages of cell differentiation in the following cell lines, Raji, RI-1, U-2932, and SU-DHL-4 treated with the CXCR4 antagonists, AMD070 and WK1 and untreated cells as control. For SU-DHL-4 we observed no evaluable data with cells treated with WK1 because all cell died. WK1 treatment in Raji resulted in a significant higher G0/1- and a lower S-phase cell proportion compared to the IT control, suggesting a G0/1 arrest. No significant differences were observed in the three treated cell lines, RI-1, U-2932, and SU-DHL-4 (Figure III-19).

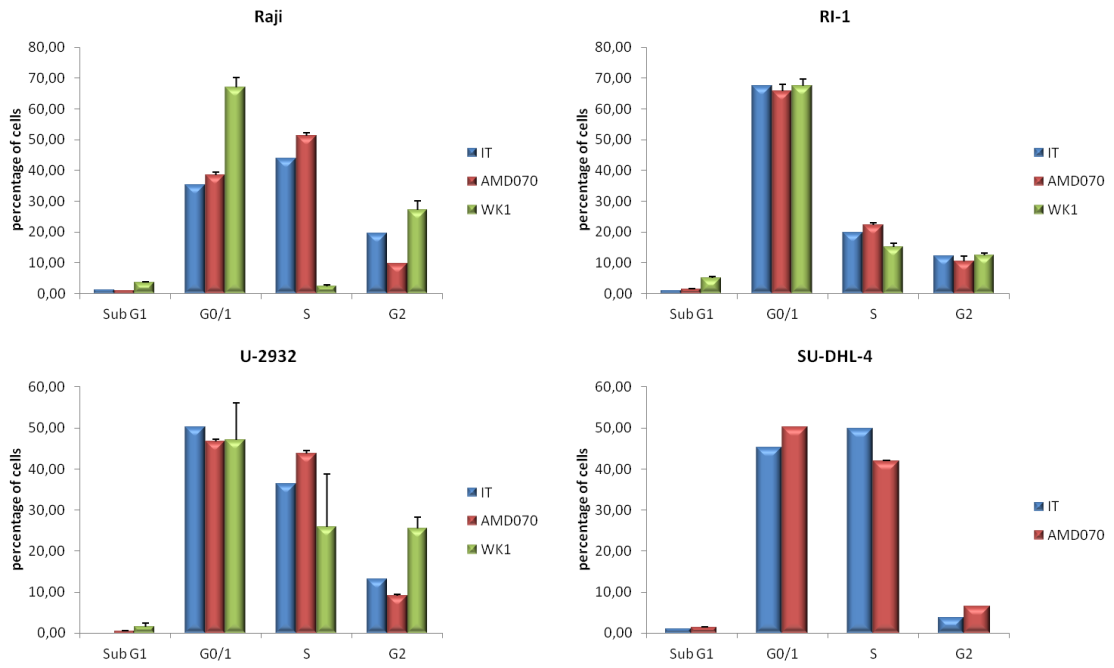

Figure III-19: Cell cycle analysis in four cell lines treated with AMD070 and WK1

Percentage of cells, Raji, RI-1, U-2932, and SU-DHL-4 treated with the CXCR4 antagonists, AMD070 (red bars) and WK1 (green bars) and untreated cells served as control (blue bar). Each bar represents the mean values ± standard deviation (SD).

IV. Discussion

The aim of this study was to elucidate the *CXCL12/CXCR4/CXCR7* chemokine axis in aggressive B cell lymphomas with respect to crucial consequences for bone marrow infiltration of lymphomas and implications of inhibitory antagonists, AMD070 and a chemically modified side chain derivative of AMD070 in lymphoma cell lines.

In the first part of this study we focused on investigations such as sequence analysis, immunohistochemical analysis on primary lymphomas and chemokine expression profiles of bone marrow biopsies of patients with aggressive lymphomas to confirm our previous findings of CXCR4 and CXCR7. We observed a higher expression of *CXCR4* and *CXCR7* in aggressive lymphomas in patients with an advanced stage (stage II-IV) compared to patients with stage I. Further, we detected a higher expression in aggressive lymphomas with bone marrow infiltration. Survival analysis from a data set of Lenz *et al.*, 2008 observed a worse clinical outcome for high expression of *CXCR4* (Lenz *et al.*, 2008). Against our hypothesis, the mutations we found in the coding sequences of both receptors CXCR4 and CXCR7 did not cause the high expression of *CXCR4* and *CXCR7* and the worse survival curves in aggressive lymphomas. The only described somatic *CXCR4* mutation was found in Waldenström macroglobulinemia (WM) and is similar to those found in WHIM-Syndrome (warts, hypogammaglobulinemia, infection, and myelokathexis syndrome) (Hunter *et al.*, 2014). Therefore, we hypothesize that CXCR4 and CXCR7 are rarely somatic mutated during the development of aggressive B cell lymphomas.

The IHC *CXCR4* surface expression results of lymphoma cells of this study did not correlate with our *CXCR4* mRNA expression profiles of primary lymphomas. For mRNA expression analysis macrodissected tissue primarily containing lymphoma but also a small fraction of non-neoplastic tissue (reactive tissues) was used whereas for immunohistochemical analysis only lymphoma cells were evaluated. That might be the reason for the differences between CXCR4 mRNA and protein expression. Another explanation might be that the binding of the antibody is unspecific and / or the translational regulation of CXCR4 differs.

To confirm our previous significant positive correlation with bone marrow infiltration in *CXCR4* expressed diffuse large B cell lymphomas (Deutsch *et al.*, 2013) we further investigated the mRNA expression levels of *CXCR4*, *CXCL11* and *CXCL12* from bone marrow biopsies. *CXCR4* and its ligand, *CXCL12*, are known as homing factors of hematopoietic stem cells to the bone marrow microenvironment (Taichman *et al.*, 2002; Hayakawa *et al.*, 2009; Mazo *et al.*, 2011). Feng *et al.* indicated that the chemokine CXCL11 is produced by bone marrow-derived mesenchymal stem cells (MSCs) (Feng *et al.*, 2014), therefore we supposed that *CXCL11* is downregulated by the displacement process of the normal bone marrow structure by the infiltrating lymphoma cells. Further, we observed a positive correlation of *CXCL12* expression with increasing bone marrow infiltration levels in bone marrow biopsies of DLBCL. Bone marrow involvement is associated with poor prognosis in DLBCL (Viswanatha and Foucar, 2003). In accordance with Mazur *et al.*, 2014, we found a significant decrease in *CXCR4* expression in bone marrow after chemotherapy (Mazur *et al.*, 2014). Therefore, *CXCR4* might be a good prognostic marker of DLBCL and represents a potential target for lymphoma malignancy therapies.

For *in vitro* characterization of CXCR4 we used eight different lymphoma cell lines exhibiting different *CXCR4* surface expression. We observed that CXCL12 treatment did not prevent lymphoma cells to undergo apoptosis under starvation. Furthermore, none of our investigated lymphoma cell lines produced detectable amounts of CXCL11 and CXCL12, which is in compliance with Dürr *et al.* who demonstrated that CXCL12 production is dependent on antigen representing cells (APC) such as fibroblasts and endothelial cells located in the lymphoma microenvironment (Dürr *et al.*, 2010).

For therapeutic applications several CXCR4 inhibitors are known. The most known specific small molecule CXCR4 antagonist is the bicyclam AMD3100 (Donzella *et al.*, 1998). The development of new oral CXCR4 inhibitors such as AMD070 plays an important role. Recent studies showed that AMD070 affects the proliferation, migration and survival of tumour cells (Wong *et al.*, 2008; O'Boyle *et al.*, 2013) and might be a good inhibitor in the CXCR4/CXCL12 interactions. Debnath *et al.* showed a variety of small molecule inhibitors for CXCR4, including AMD3100 and AMD070, and their derivatives (Debnath *et al.*, 2013). Nevertheless, Debnath *et al.* demonstrated non derivative of AMD070, which was chemical modified at the primary amine of the

AMD070 structure (Debnath *et al.*, 2013). Therefore, we derivatized AMD070 with nicotine acid on the primary amine, namely WK1 in this study and we investigated the blockade of CXCR4/CXCL12 with regard to therapeutic intervention. AMD070 and WK1 resulted in a *CXCR4* dependent induction of apoptosis in the lymphoma cells as demonstrated by increased Annexin V staining and enhanced cleaved caspase 3 activity of treated cells. In the caspase 3 deficient lymphoma cell line, Raji (Kawabata *et al.*, 1999) WK1 caused G0/1 cell cycle arrest. Interestingly, several studies demonstrated cell cycle arrests in Raji cells induced by diverse chemicals (Takase *et al.*, 1992; Long *et al.*, 2008; Yap *et al.*, 2012) These findings indicate that AMD070 and its derivatives WK1 possess cytotoxic effects on aggressive lymphoma cells.

Based on our results showing a higher *CXCR4* expression in lymphoma patients with an advanced stage (stage II-IV), *CXCR4* may be a target for therapy especially for lymphoma patients with a high clinical stage. As we demonstrated by higher cytotoxic effects of side chain modified AMD070, in our point of view AMD070 together with AMD3100 represent a new source for developing promising novel agents for anti-lymphoma therapies.

V. References

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