During the 5th project-year of the Ludwig Boltzmann Cluster Oncology (LB-CO), a number of exciting new results and concepts have been generated. Individual projects in each project-line were conducted according to the aims and master-plan of the LB-CO. In these projects, several new stem cell markers and targets have been identified and characterized in myeloid, lymphatic, and mast cell leukemias. In addition, stem cell-niche interactions were examined. Some of the newly identified targets have been validated and the effects of various targeted drugs on leukemic stem cells have been characterized.

1. Overview of the LB-CO

1.1. Introduction and Aims

Over the past 15 years, several different studies have shown that most if not all leukemias are composed of two different fractions of neoplastic cells, a bulk-population of leukemic cells with limited proliferative potential, and a smaller cell population exhibiting the capacity of unlimited self-renewal, the so-called leukemic stem cells (LSC). After a time of debates, this hypothesis is now generally accepted and predicts that minimal residual disease, relapsing leukemia, and thus all relevant ‘disease components’ (subclones) contain LSC, and that any therapy is curative only when eliminating most or all LSC in a given leukemia. During the past few years, more and more data have shown that LSC fractions represent heterogeneous populations of cells, reflecting plasticity and genetic instability within these cell populations. So far, however, little is known about the expression of relevant targets in LSC (Figure 1). From these considerations it seems important to learn more about target expression profiles of LSC and about responses of these cells to targeted drugs, conventional drugs, and immunotherapy.

The aims of the LB-CO are to identify and characterize LSC in various human leukemias, to define target expression profiles in these cells, to validate molecular targets, and to examine the effects of various targeted drugs on growth and survival of LSC. Drugs and markers are selected based on our project-results (from previous years) and the available literature. The long-term goal of the project is to improve potentially curative therapies for human leukemias by applying drugs that recognize and eliminate LSC.
Figure 1
Expression of cell surface and cytoplasmic target antigens in leukemic cells. Many therapeutic targets have been identified in ‘bulk leukemic cells’. However, little is known about expression of molecular targets in leukemic stem cells (LSC).

General Aims in LB-CO Projects:

- Identification and phenotypic characterization of LSC in various leukemias

- Characterization of target expression profiles in LSC

- Delineation of stem cell plasticity and stem cell resistance: relationship to genetic instability and characterization of relevant LSC subclones

- Targeting of LSC using specific targeted drugs and drug combinations

- Evaluation of interactions between LSC and the so-called SC niche

- Evaluation of effects of endogenous immune-mediators (cytokines) and death-regulators on growth and survival of neoplastic stem cells
1.2. Budget of the LB-CO Cluster

The budget plan of 2012 was established in cooperation with the LB-Society. Budget was mainly used to employ personnel and to purchase consumables. Part of the budget was used to invite experts in the field or to send LB-society-employed LB-CO postdocs to conferences and stem cell meetings. The LB-CO budget scheduled for 2012 amounted to 823,000.-€. A detailed summary of the budget is provided in a separate file.

1.3. Partners and Internal Structure of the Cluster

Partners
As in previous years, two partner institutes were involved in the LB-CO in 2012, namely the Hanusch Hospital and the Medical University of Vienna.

Project Groups and Scientists
The LB-CO is based on 3 project lines (PL) that were maintained in 2012: a) myeloid neoplasms, b) lymphoid neoplasms, and c) mast cell neoplasms including mast cell leukemia (MCL). Group ‘a’ also worked on normal bone marrow stem cells and myelodysplastic syndromes (MDS). According to the Evaluation Report 2010 and the recommendations of the Scientific Advisory Board (SAB) all projects on solid tumors and melanomas were finalized in 2011. The following researchers were employed in 2012: Heidrun Karlic (MDS/sAML), Harald Herrmann (AML, CML) until February 2012, Gregor Eisenwort (AML, CML) from April 2012, Sabine Cerny-Reiterer (ALL, CML), Barbara Peter (mast cell leukemia=MCL) and Emir Hadzisufovic (MCL, CML/AML). The group organized weekly scientific meetings (1.5 hours), one week-start meeting (Monday, 10:00), and a Lecture Series (Friday 16:00). The group was also involved and participated actively in several stem cell workshops and other scientific meetings.

Administration
Administration was coordinated by the administrative head of the LB-CO, Prof. Dr. Thomas Grunt and our secretary, Sabine Sonnleitner, who handled technical, logistic, administrative and technical issues in the LB-CO in 2012.

Core Facility Groups
The core facility (CF) groups, established in cooperation with the Medical University of Vienna within the LB-CO, supported all LB-CO projects during the 5th project year. These CF-Platforms (PF) include a LSC-sorting PF (employing a high speed sorter), a gene chip PF (including proteomics, deep sequencing facilities, and bioinformatics), a NOD/SCID (NSG) mouse PF (in cooperation with the Veterinary University of Vienna), and a clinical PF (including a biobanking system and several disease-specific registries for data capturing and data calculations – coordinated by Prof. Dr. Wolfgang R. Sperr). All these CF PF were essential for the progress in our LB-CO projects in the year 2012.
1.4. Scientific Advisory Board (SAB)

Based on internal discussions, the recommendation of the Society, and the evaluation report, an external SAB was established. The first SAB-meeting was organized in March 2012. LB-CP projects were developed and adjusted according to the recommendations of this SAB. The LB-CO Cluster also maintained its local SAB and employed the same panel of experts that was in charge in the previous project years.

1.5. Personnel and Career Development

The following Scientists were employed via the LBG in the LB-CO Cluster in 2012:

Heidrun Karlic     Hanusch Hospital       01-12/2012
Harald Herrmann    Medical University of Vienna   01-02/2012
Gregor Eisenwort   Medical University of Vienna   04-12/2012
Sabine Cerny-Reiterer Medical University of Vienna   10-12/2012
Barbara Peter     Medical University of Vienna   06-12/2012
Emir Hadzijusufovic  Medical University of Vienna   10-12/2012
Asha Leisser      Medical University of Vienna   08-10/2012

Career development steps: Harald Herrmann has started a clinical career at the Medical University of Vienna in March 2012. The other LBG-employed Postdocs work on their scientific career in the LB-CO environment. Within the group of non-LBG-employed LB-CO colleagues, one new Associate Professorship in the group and one new application for an Associate Professorship should be mentioned.

1.6. Infrastructure

The infrastructure in 2012 included three labs (3x30 m$^2$) dedicated to LSC research at the Medical University of Vienna (MUV), several other labs of participating scientists at the MUV, and a lab at the Hanusch Hospital. In addition, one office for our secretary (Sabine Sonnleitner) as well as core facility rooms were made available and were used to run LB-CO projects. All in all, the labs and infrastructure shared by partners as well as the scientific environment provided optimal conditions for our LB-CO projects in 2012.

1.7. Scientific Highlights

Among several scientific highlights obtained in 2012, the following should be mentioned:
1. the identification of hepatocyte growth factor (HGF) and its receptor, c-Met as potential targets in CML, 2. the identification of BRD4 as potential new therapeutic target in LSC in myeloid leukemias, 3. the identification of CD52 as stem cell target in ALL, MDS, AML, and MCL, 4. the detection and partial characterization of a NSG-repopulating LSC in MCL, and 5. the presentation of a novel LSC concept that discriminates between pre-malignant and malignant (leukemic) neoplastic stem cells in various leukemias and other neoplasms (published in Nat Rev Cancer 2012).
1.8. Public Relation

The mission of the LB-CO cluster is to increase awareness and to gain knowledge in the field of neoplastic stem cells in leukemias and other blood cell malignancies, to establish pathogenetic and targeting concepts in these neoplasms, and to establish LSC-eradicating treatment concepts, with the ultimate aim to improve anti-leukemic therapy. The LB-CO team was able to transfer its mission-intention to the public in 2012 through publications and participation in meetings organized by the LB society. In addition, members of the LB-CO were involved in the organization of the 10-Year Jubilee Meeting of the Vienna Cancer Stem Cell Club (VCSSC) and a related stem cell meeting in 2012.

2. Results obtained in the LB-CO Cluster in 2012

2.1. Results obtained in Individual Projects

2.1.1. Role of Dipeptidylpeptidase IV (CD26) in the Mobilization and Re-Distribution of LSC in Chronic Myeloid Leukemia (CML)

The biology of CML is characterized by uncontrolled proliferation and expansion of immature clonal (Ph+) myeloid cells in the bone marrow (BM) and other (extramedullary) organs such as the spleen. The extramedullary redistribution of immature myeloid (stem) cells is a typical feature in this leukemia. However, so far, only little is known about the mechanisms that contribute to the expansion and redistribution of LSC in CML. During the past few years, members of the LB-CO were able to show that CD26 is a specific marker of LSC in Ph+ CML. In addition, the LB-CO described that CD26 exhibits dipeptidylpeptidase IV (DPPIV) activity and thereby degrades the niche-related cytokine ‘stroma cell-derived factor-1´ (SDF-1). Based on these observations, the hypothesis was raised that CD26 mediates the re-distribution and spread of neoplastic stem and progenitor cells in CML. To test this hypothesis, a number of experiments were performed. In a first step, we compared engraftment patterns produced by highly enriched CD26+ (leukemic) and CD26- (presumably non-leukemic, normal residual) stem cells obtained (sorted) from individual CML patients. Both cell populations were found to engraft NSG mice. However, as expected, BCR/ABL+ cells were only found in the BM of mice injected with CD26+ cells. In addition, the engraftment pattern was quite different when comparing the two stem cell fractions. Whereas the CD26+ (BCR/ABL+) stem cells produced diffuse engraftment in the BM of NSG mice, the CD26- (BCR/ABL- and thus normal) stem cells produced a multilineage para-endosteal and thus ‘regular´ engraftment in these mice (Figure 2). These data suggest that neoplastic stem cells in CML may redistribute more effectively from the bone marrow stem cell niche compared to normal stem cells, which may explain extramedullary LSC expansion in this disease.
Figure 2
CD34+/Lin-/CD26- cells (A,C) and CD34+/Lin-/CD26+ cells (B,D) from the same CML patients were injected into the blood of NSG mice. After 16 weeks, mice were sacrificed and BM sections stained with an antibody against CD45. Lineage+ cells (sorted CD14+/CD15+) served as negative-control (E) and CD3-depleted cells served as positive-control (not shown). Splenic tissue was also analyzed in these experiments (F). As visible, CD26+ LSC produced a diffuse engraftment in the BM, whereas normal CD26- SC produced regular myeloid engraftment that typically expands at the endosteal surface (endosteal BM niche).

In consecutive experiments, we asked whether the inhibition of DPPIV (CD26) on CML LSC would lead to a suppression of redistribution and of leukemic expansion in NSG mice. To address this questions, we injected CML LSC that had been pre-incubated with control medium or vildagliptin (10 µM), into NSG mice. As visible in Figure 3, vildagliptin-exposure resulted in a reduced engraftment of LSC.

Figure 3
Engraftment of CD26+ CML LSC after preincubation with control medium (-) or vildagliptin, 10 µM (+) at 37°C for 30 minutes. Engraftment in NSG mice (3 mice per group) was determined by measuring the percentage of human CD45+ cells in the bone marrow (after 18 weeks) by flow cytometry. Results represent the mean±S.D. of 4 mice per group.
In a next step, we asked whether treatment of CML patients with a CD26-blocking gliptin would result in a reduced expansion of CML cells. To address this question, we screened for CML patients who were treated with nilotinib and had received a gliptin because of progressive diabetes mellitus (sometimes seen in nilotinib-treated CML patients). We were able to follow two such patients. In both of them, BCR/ABL transcript levels decreased substantially during treatment with saxagliptin or sitagliptin (Figure 4).

![Figure 4](image)

**Figure 4**
Two patients with imatinib-resistant CML who received nilotinib (left panel-patient: 200 mg per day; right panel-patient: 800 mg/day) and developed progressive uncontrolled diabetes mellitus, were treated with a gliptin, namely saxagliptin, 5 mg daily per os (left panel) or sitagliptin, 50 mg daily per os (right panel). BCR/ABL transcript levels were quantified by qPCR according to the international scale (IS). The patient shown in the left panel received only 200 mg nilotinib per day because of drug-related toxicity. Despite this very low dose of nilotinib, the patient (unexpectedly) entered a major molecular remission (MMR) during drug-combination treatment.

### 2.1.2. Hepatocyte Growth Factor (HGF) and HGF Receptor c-MET as Novel Potential Targets in Ph+ CML

During the past few years, the LB-CO has screened for and has identified a number of different cytokine receptors on LSC in AML and CML. Several of these cytokine receptors turned out to serve as potential targets of therapy. During the 5th project-year, LB-CO members extended their studies to the microenvironment and the so-called stem cell niche, and asked what factors and cytokines could be most relevant to LSC-niche interactions in CML. During these studies, the LB-CO team was able to identify HGF as an interesting new target-molecule. HGF was found to be expressed abundantly in purified CML basophils and in the basophil-committed CML line KU812, whereas all other cell types examined expressed only trace amounts of HGF or no HGF. Interleukin-3, a major regulator of human basophils, was found to promote HGF expression in CML basophils. By contrast, BCR-ABL failed to induce HGF synthesis, and imatinib failed to inhibit expression of HGF in CML cells. Recombinant HGF as well as basophil-derived HGF induced endothelial cell migration in a scratch-wound assay, and these HGF effects were reverted by an anti-HGF antibody as well as by pharmacologic c-Met inhibitors.
These data suggest that HGF is a basophil-derived regulator of the BM microenvironment and of BM angiogenesis in Ph+ CML. In a next step, we asked whether HGF would also exert autocrine effects in CML cells. In order to address this question, several different experiments were performed. First, we were able to show that highly purified CML LSC express c-Met (HGF-receptor) mRNA (Figure 5).

**Figure 5**
Expression of c-Met mRNA in highly enriched (sorted) CD34+/CD38-/Lin- stem cells (LSC) and CD34+/CD38+ progenitor cells obtained from 3 patients with CML. Sorted cells and control mononuclear cells (MNC) were subjected to RNA isolation, cDNA synthesis and qPCR, using primers specific for c-Met and ABL. Results show c-Met mRNA levels as percent of ABL mRNA and represent the mean±S.D. of 3 independent experiments (3 patients).

In a next set of experiments, we were able to show that recombinant HGF induces growth of CML cells, and that an anti-HGF antibody as well as c-Met-targeting drugs interfere with HGF-dependent and spontaneous growth of various CML cell lines (Figure 6).

**Figure 6**
Left panels: KU812 and K562 cells were incubated in control medium (CO) or in various concentrations of the c-Met inhibitors PF-2341066 (upper panels) or SU11274 (lower panels) at 37°C for 48 hours. After incubation, 3H-thymidine uptake was measured. Results are expressed as percent of control (CO) and represent the mean±S.D. of 3 independent experiments. Right panels: BM MNC and PB MNC (CML patients) were incubated in control medium (CO) or in various concentrations of the c-Met inhibitors PF-2341066 (upper panels) or SU11274 (lower panels) for 48 hours. After incubation, 3H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. of 3 independent experiments (3 patients). Asterisk: p<0.05 compared to control (CO).
Together, these data suggest that HGF is an autocrine growth-regulator in CML. In addition, the LB-CO team was able to show that basophils are a unique source of HGF in these patients and play a more active role in disease-progression than has so far been assumed. Our data also suggest that HGF and c-Met are potential new targets in CML.

2.1.3. Additional New Targets detected in Myeloid Leukemias

During the past few years, the LB-CO has examined a large number of samples from patients with CML and acute myeloid leukemia (AML) for expression of various target antigens. This screen included CD34+ cells, CD34+/CD38-/Lin-/CD123+ stem cells (LSC), and more mature CD34+/CD38+ progenitor cells. In the year 2012, these analyses have been extended in order to detect a number of additional target antigens, and several of these targets have been examined in detail, the highlighting examples being BRD4 and Campath-1 (CD52). The LB-CO has already reported that AML LSC express BRD4 and that the BRD4-targeting drug JQ1 induces apoptosis in AML LSC. In the year 2012, these studies have been extended and have been published. We were also able to show that BRD4 is expressed in the nuclear and cytoplasmic compartment of AML blasts and AML LSC, whereas in normal BM, BRD4 is mostly expressed in the nuclei of stem and progenitor cells. In consecutive experiments, we were able to show that CML cells and CML LSC also express BRD4. Currently, the effects of JQ1 on CML LSC are examined.

Another interesting target identified in AML LSC and MDS LSC is the Campath-1 antigen (CD52). Notably, the anti-CD52 antibody alemtuzumab induces major responses in a group of patients with MDS, but the mechanism(s) underlying this drug-effect remain(s) unknown. The LB-CO has identified this surface target on AML LSC in 2011. In 2012, we have extended these studies to patients with MDS and other myeloid neoplasms, and analyzed the effects of the CD52-targeting antibody alemtuzumab:

As assessed by flow cytometry, CD52 was expressed on neoplastic stem cells (NSC) in 7/10 patients with MDS and isolated 5q-. In most other patients with MDS, CD52 was weakly expressed or not detectable on NSC. In AML, CD34+/CD38− LSC displayed CD52 in 23/62 patients, namely 4 with complex karyotype including 5q-, one with 5q- and t(1;17;X), 2 with inv(3), 2 with t(8;21), one with inv(16), one with isolated del(13q), 3 with trisomy 8, one with monosomy 7, and 8 with normal karyotype. An example of CD52 expression on AML LSC is shown in Figure 6. In highly enriched NSC/LSC of 5q- patients, qPCR confirmed expression of CD52 mRNA, and clonality was confirmed by FISH. Consecutive studies revealed that CD52-expression is specifically induced by mutated RAS and correlates with EVI1 mRNA levels. In a next step, we examined the effects of alemtuzumab, a humanized antibody directed against CD52 that can induce remission in advanced chronic lymphocytic leukemia and in a group of patients with MDS. In our studies, alemtuzumab induced complement-dependent lysis of CD34+/CD38−/CD52+ LSC (Figure 7), but did not induce lysis in CD52− LSC or in control BM cells. We were also able to show that alemtuzumab inhibits AML-formation in vivo in NSG mice. In conclusion, the target-antigen CD52 is expressed abundantly in NSC/LSC in a group of patients with MDS and AML, including 5q- patients, which may have clinical implications and may explain clinical effects seen with alemtuzumab.
An important new target in myeloid neoplasms appears to be STAT5. The LB-CO has identified cytoplasmic STAT5 as a potential therapeutic target in neoplastic cells in JAK2-V617F+ MPN in previous years. In the year 2012, these studies were extended to AML and CML. In both disease models, STAT5 may promote disease progression and mutation rates, and may serve as a molecular target of therapy. We were also able to show that during progression of CML, the total levels of STAT5 (per leukemic cell) increase. Two STAT5-inhibitors, piceatannol and pimoizide, were tested, and both were found to suppress the growth and survival of leukemic cells in CML. The effects of STAT5 and hypoxia on BCR/ABL mutations were also analyzed. Both, hypoxia and STAT5 upregulate ROS-production in CML cells, and both may contribute to the mutation of BCR/ABL during disease-progression. Part of these studies were performed in collaboration with Veronika Sexl (University of Veterinary Medicine Vienna).

2.1.4. New Targets identified in Acute Lymphoblastic Leukemia (ALL)

During the past few years, the LB-CO has screened a series of ALL samples for expression of target antigens on CD34+/Lin-/CD38- LSC and CD34+/CD38- progenitor cells. In these studies a number of interesting new markers and targets have been identified. In several instances, these markers were found to be specific for ALL LSC, and thus helpful to detect these cells and to confirm their clonality. In 2012, we have extended these studies and have started to validate these targets by examining the effects of various targeted drugs. One interesting observation was that LSC in Ph+ ALL exhibiting the p210-form of BCR/ABL express CD25 and CD26. In Ph+ ALL exhibiting the p190-form of BCR/ABL, LSC expressed CD25 in most cases, but did not express CD26. Currently, the LB-CO team is examining the effects of various targeted drugs directed against CD25 and CD26 on ALL LSC. Additional targets identified on ALL LSC were CD33, CD44, CD52, and CD123. In 2012, the cluster-team validated CD52 as a potential target in ALL. In a first step, we were able to show that highly purified...
CD34+/CD38-/Lin- LSC in Ph+ ALL and Ph- ALL express CD52 mRNA in all patients. Next, the CD52-targeted drug alemtuzumab (MabCampath) was applied. As shown in Figure 8, alemtuzumab induced rapid and dose-dependent lysis of LSC in patients with Ph+ ALL. Since ALL LSC may sometimes also reside in the CD38+ fraction of the clone, we also examined the effects of alemtuzumab on CD34+/CD38+ cells. Again, alemtuzumab was found to eliminate these CD34+/CD38+ cells in patients with Ph+ ALL (Figure 8). Finally, we were able to show that alemtuzumab also kills leukemic stem and progenitor cells obtained from patients with Ph- ALL (not shown). All in all, these data show that CD52 is an interesting new target in ALL, and that the CD52-targeting antibody alemtuzumab is capable of eliminating LSC in this type of leukemia.

![Figure 8](image)

**Figure 8**

Dose-dependent effects of alemtuzumab on survival of ALL LSC

CD34+/CD38- LSC and CD34+/CD38+ stem/progenitor cells obtained from patients with Ph+ ALL (n=4) were incubated in control medium (Co) or in medium containing various concentrations of alemtuzumab at 37°C. After 1 hour, the numbers of viable cells were measured. Results show cell numbers and represent the mean±S.D. of 4 independent experiments (4 donors). Asterisk: p<0.05.

Currently, the LB-CO team is examining the effects of various combinations of targeted drugs on ALL cells and ALL LSC. These combinations include alemtuzumab and various BCR/ABL TKI as well as alemtuzumab and bendamustin. Moreover, the LB-CO team will examine the effects of alemtuzumab on engraftment of LSC obtained from Ph+ ALL patients in an NSG mouse model.

### 2.1.5. Expression of Niche-relevant antigens in ALL LSC and other BM cells

During the 5th project-year, the LB-CO was interested to investigate potential interactions between LSC and the so-called stem cell niche. In these experiments, ALL LSC were examined for expression of niche-relevant antigens. The cluster team was able to show that ALL LSC in Ph+ ALL consistently express the SDF-1-degrading surface enzyme Dipeptidyl-Peptidase IV (DPPIV=CD26), the homing/invasion receptor CD44, and the SDF-1 receptor CXCR4 (CD184). Furthermore, ALL LSC were found to express several integrins and the ICAM-1 antigen (CD54). In a subset of patients with ALL, LSC also expressed the stem cell factor receptor KIT (CD117).

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In a next step, we examined the expression of various niche-related antigens in the BM of patients with Ph+ ALL and Ph- ALL. Normal BM (lymphoma staging or reactive BM) as well as BM obtained from patients with Ph+ CML served as control. Antigen-expression in BM cells was determined by indirect immunohistochemistry. In these experiments, we were able to show that niche-related cells, including endosteal-lining cells (ELC) and vascular endothelial cells (VEC), express a distinct composition of cytokine-ligands and adhesion molecules in ALL and CML. BM VEC were found to co-express CD31 and CD34 (positive control) as well as HGF, VEGF, oncostatin-M (OSM), SDF-1, and CD26. Endosteal cells (ELC) were found to stain positive for CD56 and osteocalcin (positive control) but did not stain positive for CD31 or CD34 (negative control). ELC also expressed VEGF, OSM, OSM-R and SDF-1 in all BM samples examined. Unexpectedly, these cells also expressed some CD26. Contrasting normal BM, endosteal cells in the BM of patients with ALL and CML did not express measurable levels of HGF (Table 1).

Table 1
Expression of various cytokines and cytokine receptors in niche-related cells in the bone marrow (BM) of patients with ALL and CML

<table>
<thead>
<tr>
<th>Antigen</th>
<th>CD34</th>
<th>OSM</th>
<th>OSM-R</th>
<th>HGF</th>
<th>VEGF</th>
<th>CD26</th>
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<tr>
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<td>VEC</td>
<td>ELC</td>
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<tr>
<td>Ph+ CML</td>
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<td>+</td>
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<tr>
<td>Ph+ ALL</td>
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BM, bone marrow; ALL, acute lymphoblastic leukemia; VEC, vascular endothelial cells; ELC, endosteal-lining cells; OSM, oncostatin M; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; SDF-1, stroma cell-derived growth factor-1.

In consecutive studies, we attempted to explore the mechanisms underlying expression of niche-related antigens in ALL and CML LSC. In Ph+ ALL and CML, we examined the effects of BCR/ABL on expression of CD25 and CD26. However, unexpectedly, although STAT5 was found to upregulate expression of CD25 on LSC, BCR/ABL was not sufficient to induce expression of CD25 or CD26 in leukemic cells. In addition, exposure to imatinib was not followed by a downregulation of CD25 or CD26. In a next step, we applied various cytokines, cytokine-blocking antibodies (anti-G-CSF, anti-IL-3) and hypoxia. However, no cytokine or ‘condition’ regulating expression of CD25 or CD26 could be identified so far. Currently, we are applying additional cytokines (VEGF, OSM, HGF, SDF-1) and various signal-transduction inhibitors in order to define mechanisms underlying the expression of CD25 and CD26 in LSC in ALL and CML.
During the 5th project year, the LB-CO has also extended target screens in ALL by analyzing interactions between ALL cells and certain targeted drugs, including the TKI imatinib, nilotinib and dasatinib, the pan-Bcl-2-blocker obatoclax, and the PI3-Kinase/mTOR-blocker BEZ235. TKI effects on Ph- ALL and certain types of lymphoma cells have also been analyzed. These studies have in part been performed in collaboration with the Center for Molecular Medicine (CeMM; Dr. Uwe Rix and Prof. Dr. Giulio Superti-Furga) and the Ludwig Boltzmann Institute for Cancer Research (LBI-CR; Prof. Dr. Lukas Kenner and Prof. Dr. Richard Moriggl). Complete target expression profiles for all 3 TKI have been established in Ph+ ALL. Among many different targets, SRC, LYN and the PDGFR were identified as interesting new TKI-targets in malignant lymphatic cells. Finally, the LB-CO has tested the effects of various targeted drugs on growth (proliferation) and survival of various neoplastic lymphoid cells and putative LSC, including cells derived from patients with Ph+ ALL, Ph- ALL, or multiple myeloma (MM). Major growth-inhibitory effects were seen with the TKI nilotinib and dasatinib, the PI3-kinase/mTOR-blocker BEZ235, the pan-Bcl-2 inhibitor obatoclax, the Hsp90-inhibitor 17AAG, and the polo-like kinase-1 blocker BI2536. These agents produced growth inhibition in ALL cells and MM cells at pharmacologically reasonable concentrations (< 1 µM). We also applied these drugs in combination on ALL cell lines and MM cell lines, and several different combinations of drugs were found to produce synergistic effects on growth of ALL and/or MM cells. Table 2 shows a summary of drug combination effects on MM cell lines.

Table 2

<table>
<thead>
<tr>
<th>Drug combinations</th>
<th>MM1.S</th>
<th>NCI-H929</th>
<th>OPM-2</th>
<th>RPMI8266</th>
<th>U266</th>
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<tbody>
<tr>
<td>17AAG and BI2536</td>
<td>s</td>
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<tr>
<td>17AAG and BEZ235</td>
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<td>an</td>
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</tr>
<tr>
<td>BEZ235 and obatoclax</td>
<td>s</td>
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<td>an</td>
<td>s</td>
<td>an</td>
</tr>
</tbody>
</table>

s: synergistic, a: additive, an: antagonistic. Proliferation was determined by measuring \[^{3}\text{H}\]-thymidine uptake. Only drugs that had shown major effects as single agents (IC50 < 1 µM) were employed. Synergism was analyzed by determining combination index (CI) values using Calcusyn software.
2.1.6. Characterization of Neoplastic Stem Cells in Mast Cell Leukemia

During the past 2 years, members of the LB-CO have ambitiously tried to identify a neoplastic progenitor cell in patients with MCL. As assessed by flow cytometry, the CD34+/Lin- fraction of the clone often co-expresses KIT and CD123. However, in contrast to CML and Ph+ ALL, no aberrantly expressed marker could be identified on LSC in MCL so far. In 2012, we were able to analyze the CD34+ fraction of MCL in more detail. In a first step, we confirmed that in those patients in whom the MCL had replaced normal hematopoiesis completely (acute form of MCL – no remaining normal stem cells), the CD34+/Lin- fraction exhibited KIT D816V and high levels of KIT and CD52. In a next step, the CD34+/KIT+ stem cells and the KIT+/CD34- mast cells were sorted out separately, and were injected into NSG mice. In these pivotal experiments, we were able to show that MCL-initiating (NSG-repopulating) cells reside within the CD34+/KIT+ fraction of the clone, whereas the more mature KIT+/CD34- mast cells from the same patients did not produce MCL in NSG mice (Figure 9).

Figure 9
Detection of a NSG mouse-repopulating LSC in MCL
Primary BM cells obtained from a patient with acute MCL (>95% immature, rapidly proliferating mast cells in the PB and BM) were sorted into a KIT+/CD34+ fraction and KIT+/CD34- fraction. The total MNC (CD45+) served as control. Sorted cells were injected i.v. into NSG mice. After 16 weeks, mice were sacrificed and BM analyzed for the presence of human cells. Neoplastic human mast cells were found in NSG mice injected with CD34+ cells, but not in NSG mice injected with KIT+/CD34- mast cells. The left panel shows the percentage of engrafted human cells (by flow cytometry). Results represent the mean±S.D. from 4 mice each. The middle panel shows examples of engrafted human cells (red color) in mouse BM (blue colored dots represent mouse leukocytes) after injection of KIT+/CD34+ cells (upper panel) and KIT+/CD34- cells (lower panel) in NSG mice. Asterisk: p<0.05. The right panel shows a Giemsa stain of engrafted immature mast cells grown from KIT+/CD34+ stem cells in the BM of NSG mice.
These data show that LSC reside within the CD34+/KIT+ compartment of the MCL clone. Currently, the LB-CO team is comparing engraftment rates of CD34+/KIT+ cells and CD34+/KIT- cells in ASM and MCL. In addition, the LB-CO is screening for aberrantly expressed MCL LSC markers by gene chip analyses. Finally, deep sequencing is performed using MCL LSC in freshly diagnosed and relapsed patients, in order to learn what additional molecular lesions (apart from mutated KIT) are responsible for disease initiation, disease progression and the development of drug resistance. Notably, MCL is characterized by multidrug resistance and most patients have refractory disease and a very poor prognosis with median survival times of less than 1 year.

2.1.7. Evaluation and Exploitation of Novel Human Mast Cell Lines

During the past two years, the LB-CO has established a number of novel human mast cell lines. Most cell lines were generated in collaboration with other national or international partners. During the 5th project-year (2012), these cell lines were further characterized and employed as useful tools to study the biology of MCL and responses of malignant mast cells to various targeted drugs. MCPV clones represent extremely immature human mast cells, typically seen in patients with MCL. These cells express mutated RAS as well as several other oncogenes. Using this cell line model, we were able to show that expression of certain surface targets, such as CD30 (Ki-1 antigen) or CD52, is dependent on a functionally active RAS, and incubation of MCPV cells with downstream MEK inhibitors led to a decreased expression of these surface antigens. Figure 10 shows expression of CD30 on MCPV and HMC-1 cells after incubation with MEK-targeting drugs.

![Figure 10](image)

HMC-1 cells (grey bars) and MCPV1 cells (black bars) were examined for expression of CD30. Whereas MCPV1 cells expressed substantial amounts of CD30, HMC-1 cells stained negative for this antigen. After incubation with the MEK inhibitors PD032509 or RDEA119 (24 hours, 37°C), the levels of CD30 in MCPV1 cells decreased. The effects of both drugs were dose-dependent. Results show the relative expression of CD30 compared to an isotype-matched control antibody (Staining Index=1) and represent the mean±S.D. of three independent experiments.
2.1.8. Identification of Novel Molecular Targets on Neoplastic Mast Cells and LSC in MCL

During the past 2 years, the LB-CO team has identified a number of novel interesting targets in neoplastic mast cells, including several survival-related molecules and signal-transduction molecules. In the year 2012, the LB-CO examined several cell surface antigens for their potential role as molecular targets. Of these, the CD30 antigen (Ki-1 antigen) and the CD52 antigen (Campath-1) were identified as most interesting target structures. However, whereas CD52 was found to be expressed in both, the mast cells and CD34+ LSC in our MCL patients, the CD30 antigen was only detected on neoplastic mast cells, but not on neoplastic stem cells in MCL which may be clinically relevant. We next examined the effects of various targeted drugs on growth and survival of neoplastic mast cells. The CD30-targeting agent SGN-35 was found to suppress the growth of primary neoplastic mast cells and various human mast cell lines, including all MCPV clones tested. However, as mentioned CD30 is not expressed on LSC in MCL. Therefore, the LB-CO decided to focus more on the CD52-targeted antibody alemtuzumab. We found, the alemtuzumab induces dose-dependent growth-inhibition and apoptosis in primary MCL cells as well as in various human mast cell lines (Figure 11).

![Figure 11](image)

**Figure 11**
Effects of Alemtuzumab on survival of neoplastic mast cells
Left panel: Primary neoplastic mast cells obtained from a patient with MCL were incubated with alemtuzumab and 30% human serum for 60 minutes. Thereafter, cells were stained with antibodies against CD34, CD38, and CD117 (KIT), and the total numbers of viable cells (adjusted to counting beads) were assessed. Right panel: MCPV4 cells were cultured in the absence (Co) or presence of various concentrations of alemtuzumab at 37°C for 60 minutes. Then, cell viability was measured by Propidium Iodide (PI) staining. Results show the number of PI-positive cells and represent the mean±S.D. of three independent experiments. Asterisk: p<0.05.
Several cytoplasmic drug targets were also identified in neoplastic mast cells in 2012. Among these, the most interesting one may be the epigenetic reader BRD4. The LB-CO has already started to explore epigenetic mechanisms and related targets in MCL cells in previous project-years. In 2012, we were able to show that neoplastic mast cells in high-grade mastocytosis including MCL as well as various mast cell lines, express cytoplasmic BRD4, whereas in indolent systemic mastocytosis, BRD4 is usually expressed in the nuclei rather than in the cytoplasm of neoplastic mast cells. We therefore asked whether the BRD4-blocking agent JQ1 would inhibit growth and survival in neoplastic mast cells. In these experiments, we were indeed able to show that JQ1 induces dose-dependent apoptosis and growth-inhibition in various mast cell lines (Figure 12).

![Figure 12](image)

**Figure 12**
JQ1 induces caspase 3 activation and thus apoptosis in HMC-1 cells
HMC-1.1 cells lacking KIT D816V (black bars) and HMC-1.2 expressing KIT D816V were cultured in the absence (co) or presence of various concentrations of JQ1 (as indicated) for 48 hours. Then, apoptosis was measured by determining the levels of active caspase 3 by flow cytometry. Results show the percentage of apoptotic cells and represent the mean±S.D. of three independent experiments. Note the different responses in the two HMC-1 subclones.

### 2.1.9. A Novel Subclone-Concept of Neoplastic Stem Cells

Since their description and identification in leukemias and solid tumors, cancer/leukemic stem cells (CSC/LSC) have been the subject of intensive research in translational oncology. Important issues in CSC/LSC research are the characterization of SC-related markers and SC-augmenting oncogenic pathways, the identification of molecular targets in CSC/LSC, and the preclinical and clinical evaluation of the SC-eradicating potential of various targeted drugs. The LB-CO has also followed these aims in several projects in the past 5 years. However, although diverse LSC markers, targets, and target pathways have been identified, several questions remain, such as the origin and evolution of neoplastic SC, resistance against conventional or/and targeted drugs, and the mechanisms that underly extensive subclone formation. It also remains unclear whether subclone formation leads to completely independent subclones and whether each subclone contains comparable stem cell pools. Notably, various cell fractions with different combinations of
molecular aberrations may display CSC/LSC function in a given neoplasm, and subclone-formation and CSC/LSC plasticity may also contribute to acquired drug resistance. During the past few years, the LB-CO has developed a new concept as well as a classification of neoplastic stem cells that takes these considerations into account. The novel concept predicts that cancer development is a step-wise process that includes defined phases of SC evolution. Subclone-formation results from a continuous diversification of the cancer-genome characterized by multiple pro-oncogenic lesions and hits that accumulate in neoplastic stem cells over time. In an early phase of CSC/LSC evolution, neoplastic stem cells (and their progeny) are slowly-cycling cells, and the subclones remain small and cannot expand or replace the normal organ. However, over time, these clones slowly expand and later can replace the normal organ. In many instances, these cells are then still fully differentiating and functionally normal-appearing cells that are responsive to most or all physiologic regulators, so that no overt malignancy is detectable (can be diagnosed). In a final step, some of the neoplastic SC (in dominant clones) acquire invasive properties and expand to an overt malignancy (e.g. overt cancer or overt leukemia). Whereas all cells with stem cell properties in a given clone should be termed neoplastic SC (NSC), only those SC that can produce and maintain an overt malignancy (overt cancer or leukemia) should be termed cancer stem cells (CSC) or – in case of a leukemia - leukemic stem cells (LSC).

Table 3

Classification of Neoplastic Stem Cells (NSC) and Examples of related Conditions

<table>
<thead>
<tr>
<th>Stem Cell</th>
<th>Definition</th>
<th>Conditions – Examples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Stem Cell</td>
<td>No potential to produce a neoplastic process</td>
<td></td>
</tr>
<tr>
<td>Premalignant Neoplastic Stem Cell (pre-m-NSC)</td>
<td>Clonal persistent cells that can produce a premalignant neoplastic process and may or may not transform into overt CSC/LSC during further disease-evolution</td>
<td>Monoclonal hematopoietic cells with undetermined (unknown) clinical significance (e.g. MGUS), indolent neoplasms, early phase CML, persistent BCR/ABL in apparently healthy individuals, …</td>
</tr>
<tr>
<td>Malignant NSC (=CSC/LSC)</td>
<td>Clonal persistent cells that can produce and maintain an overt malignancy for unlimited time periods</td>
<td>Overt cancer or leukemia</td>
</tr>
</tbody>
</table>

*Most examples refer to clonal hematologic conditions, however, very similar conditions are also found in non-hematopoietic tissues. CSC, cancer stem cells; LSC, leukemic stem cells.
Those cells that can produce a premalignant state, such as a MDS or a very early chronic phase of CML (or even only BCR/ABL-positivity in a few cells) should be termed premalignant NSC or – in case of a leukemia – preleukemic NSC (Table 3). This newly proposed classification together with a general discussion about terminologies and assays, has been published in Nat Rev Cancer 2012. Figure 13 shows a scheme of CSC/LSC evolution, with premalignant (preleukemic) NSC and fully malignant NSC (=CSC/LSC).

![Figure 13](image_url)

**Figure 13**
Cancer/Leukemia and CSC/LSC evolution and classification of neoplastic stem cells

The development and evolution of a malignancy is usually a multi-step process, involving premalignant and malignant phases and stem cells. In an early phase of cancer evolution, neoplastic SC (NSC) are all premalignant and slowly-cycling cells and thus produce only small-sized sub-clones. However, during their evolution, these NSC acquire more and more lesions and hits, and at some point, one or more of these NSC-clones acquire malignant properties. In a final phase, an overt cancer or leukemia develops; note that at this time, still many other premalignant subclones and their NSC are present (coexist with CSC/LSC), and may often be more resistant cells because of their (relative) quiescence. Therefore, relapses may occur from either residual CSC/LSC (early relapses) or from NSC after these have again transformed into CSC/LSC (late/r relapses). Blue boxes: premalignant NSC; red boxes: CSC/LSC compartments. Stippled lines: normal organ architecture that is replaced and finally destroyed by the invasive malignancy. Modified from: Valent et al, Nat Rev Cancer 2012;12:767-775.
Strategic Aims reached in the project year 2012:

Several strategic aims formulated for the Cluster were reached in the year 2012. One important aim was to expand the number of NSG xenotransplantation models for human leukemias. In the year 2012, we were able to establish a xenotransplantation model for human mast cell leukemia. In addition, using this novel assay, we were able to characterize the NSG-repopulating MCL stem cell as a CD34+/KIT+ progenitor. We were also able to establish NSG facilities employing humanized membrane-bound SCF as a relevant niche factor in the year 2012. This model and other similar models (other humanized cytokines or KO models) will be used in the next few years in order to better define LSC-niche interactions and to increase the rates of LSC-engraftment in our mouse experiments. Another strategic aim was to select most promising candidate targets in human leukemias. This aim has also been reached. Two types of target-structures have been identified, namely i) cell surface antigens that may be exploited to eradicate even quiescent LSC, and ii) cytoplasmic signaling molecules and epigenetic targets that may be employed to attack proliferating LSC. Finally, we were able to start drug validation experiments. These experiments will be continued during the next project phase in our LB-CO. Finally, we have been able to establish a number of novel stem cell-like cell lines in order to study LSC biology and LSC responses to various targeted drugs. For the next project year of the LB-CO, an important strategic aim will be to further validate identified LSC markers and targets in human leukemias, and to further establish and validate LSC-niche interactions in the leukemic bone marrow. In addition, the LB-CO consortium will try to identify robust aberrant markers expressed on LSC in Ph- ALL and MCL.

2.2. Publications and Reports – Overview

A number of original publications and review articles have been published in the project year 2012. Among these papers are several first- and/or senior authorships in Blood, Neoplasia, Allergy, Oncotarget, and other top journals. A novel stem cell concept has been published in Nat Rev Cancer. In addition, the LB-CO cluster was able to contribute substantially to publications in other high impact journals, such as Nat Med or Nat Chem Biol. In these cooperations, novel target structures and the effects of various targeted drugs have been investigated. It should be pointed out that several of these studies were conducted in collaboration with the LBI for Cancer Research (LBI-CR) and the CeMM. Finally, it should be mentioned that the LB-CO cluster was involved in several clinical studies in 2012, including the revised international prognostic scoring system (IPSS) for MDS. A list of publications generated in 2012 is provided in a separate file.

2.3. Patents

No patents were filed during the period 2012-2013 in our LB-CO consortium. However, the cluster is actively scouting for potentially `patentable` results.
2.4. Conferences

Members of our consortium attended several national and international conferences in the fields of Hematology and LSC Research. In addition, members of LB-CO were involved in the organization of national and international conferences and meetings dedicated to stem cell research. In October 2012, the Vienna Cancer Stem Cell Club (VCSCC) celebrated its ‘10-Year-Jubilee’ in Vienna (Billroth House). Members of the LB-CO participated and were actively involved in the organization of this workshop. Several top experts including Connie Eaves participated in this celebration event.

2.5. Lectures and Presentations

Members of the LB-CO consortium presented their data in a number of invited lectures and other presentations in national and international conferences and workshops in 2012. A list of all presentations is provided in a separate file (publications).

3. Additional Information

3.1. Scientific Cooperations

Scientific Cooperation within the Cluster LB-CO
During the past few years, several scientific cooperations have been established in the scientific environment of the MUV and LB-CO. These cooperations have been exploited and were extended in the 5th project year to strengthen our projects. We have especially extended our strong ongoing cooperation with our colleagues from the departments of laboratory medicine (mast cell leukemia models), pathology (stem cell niche studies), hematology (leukemias and myeloma) and radiation therapy (various leukemia models).

Scientific Cooperation with other Groups in Vienna
During the first 4 project years, the core cluster group has established new important cooperation or intensified pre-existing scientific cooperation with a number of groups working in the field of LSC research and translational hematology in Vienna. These cooperations were maintained and expanded in 2012. Most relevant cooperations to be mentioned are the ongoing cooperation with our colleagues at CeMM (Giulio Superti-Furga), the LBI for Cancer Research, LBI-CR (Richard Moriggl), and the VetMedVienna (Michael Willmann & Thomas Rülicke). We also started a collaboration (CD52 on MDS/AML LSC) with the LB Cluster for Translational Oncology (Klaus Geissler).

3.2. Organization of Conferences and Meetings

As mentioned above, members of the LB-CO consortium were actively involved in the organization of various national and international conferences and meetings, including the
‘10-Year-Jubilee’ Meeting of the Vienna Cancer Stem Cell Club in Vienna (October 5, 2012) and a stem cell session organized by the European Hematology Association, EHA.

3.3. Education and Ph.D. Program

The Ph.D. program of the MedUniWien is coordinated successfully by Brigitte Marian, a valuable member of the cluster LB-CO. Members of our LB-CO consortium were also involved in teaching and training of students and postdocs in 2012.

4. Aims for the Next Project-Year

Based on the master-plan of the LB-CO and results obtained in 2012 and in previous project years, the LB-CO project lines will continue to focus on novel markers and targets in neoplastic stem cells in myeloid, lymphatic and mast cell leukemias (MCL). In MCL and Ph- ALL, the major goal for the next project years will be to identify new aberrantly expressed markers through which (NSG-repopulating) LSC can be identified and separated from normal stem cells in the same way as in Ph+ CML and Ph+ ALL (as demonstrated by the LB-CO). In addition, several LSC markers, including CD52 and BRD4, will be validated in MCL and ALL. Another major aim will be to define novel LSC-niche interactions and related molecular targets in MCL and ALL. In CML and Ph+ ALL, major aims will be to validate CD26 as a therapeutic LSC-target. In addition, the role and function of CD25 on CML LSC will be examined. Finally, exome sequencing studies have been initiated in MCL and MDS/AML in order to identify somatic lesions responsible for the aberrant phenotype, behaviour and resistance of LSC. In these studies somatic aberration profiles will be compared in various phases of the disease and will be correlated with clinical parameters and end points. New targets and targeted drugs will be selected based on our previous data and the published literature. In addition, we will continue to study genomic diversification in our leukemia models in order to confirm our subclone-and-latency hypothesis of LSC evolution. Results will be confirmed by mutation specific quantitative PCR. The long-term goal in all LB-CO project is to develop new improved (potentially curative) therapies for leukemias by applying drug combinations that have the capacity to eliminate all NSC and LSC in these leukemias.

5. Publications

Original Manuscripts


Review Articles

1. Valent P, Bain BJ, Bennett JM, Wimazal F, Sperr WR, Mufti G, Horny HP. Idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of uncertain significance (IDUS), and their distinction from low risk MDS. Leuk Res. 2012;36:1-5. IF: 2.923


Oral Presentations / Lectures


2. Valent P. Therapie der CML mit Tyrosinikinase-Inhibitoren: Ist eine Heilung möglich?


Abstracts


18. Sperr WR, Pfeiffer T, Kundi M, Sillaber C, Herrndhofer S, Valent P. Serum Tryptase Is a Strong Predictive Biomarker That Improves Prognostication in Ph+ Chronic Myeloid Leukemia. 54th ASH Annual Meeting and Exposition, Atlanta, GA, USA, Dec. 9, 2012;632:2783