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Immunogenicity of leukemia stem cells in acute myeloid leukemia

Dissertation

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1. List of abbreviations

ABC	ATP binding cassette
allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
BM	Bone marrow
CML	Chronic myeloid leukemia
CD	Cluster of Differentiation
CEBPA	CCAAT/enhancer-binding protein alpha
CR	Complete remission
CTLs	Cytotoxic T lymphocytes
DC	Dentritic cells
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FLT3	FMS-like tyrosine kinase 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVL	Graft-versus-leukemia
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
HVs	Healthy volunteers
LAAs	Leukemia-associated antigens
LSCs	Leukemia stem cells
MACS	Magnetic-activated cell sorting
MDS	Myelodysplastic Syndromes
MHC	Major histocompatibility complex

MM	Multiple myeloma
MRD	Minimal residual disease
NK	Normal karyotype
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NPM1	nucleophosmin member 1
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
WHO	World health organization

2. Introduction

2.1 Acute myeloid leukemia

2.1.1 Overview

Acute myeloid leukemia (AML) is a malignant disease of myeloid cells in which myeloid hematopoietic precursors are arrested in an early stage of development and proliferate abnormally. Those abnormal myeloid blasts accumulate quickly in the bone marrow and then released to the peripheral blood. Meanwhile the normal hematopoiesis is suppressed and consequently leads to corresponding symptoms like fatigue, frequent infection and easy bleeding. In addition, symptoms of extramedullary infiltration may be present in some patients like hepatomegaly or splenomegaly (Estey et al. 2006, Löwenberg et al. 1999, O'Donnell et al. 2011).

AML had been classified according to morphology, cytochemistry, immuno phenotype, as well as clinical features. During the last decades genetic abnormalities were found to be very significant in AML and taken into consideration for classification. The 2008 WHO classification of AML (Vardiman et al. 2009) which integrated genetic alterations have been widely accepted and adopted (table 1).

Table 1. 2008 WHO classification, AML. (Vardiman et al. 2009)
*: subclasses aren't listed.

Acute Myeloid Leukemia and Related Neoplasms
• AML with recurrent genetic abnormalities*
• AML with myelodysplasia-related changes
• Therapy-related myeloid neoplasms
• Acute myeloid leukemia, not otherwise specified*
• Myeloid sarcoma
• Myeloid proliferations related to Down syndrome*
• Blastic plasmacytoid dendritic cell neoplasm

2.1.2 Treatment and prognosis

Treatment of AML had been divided into induction therapy and consolidation therapy. Standard induction therapy achieves complete remission (CR) in 60-80% of younger adult patients and 20-30% of older patients (Estey et al. 2006, Appelbaum et al. 2006, Döhner et al. 2010). Consolidation therapeutic options include intensive chemotherapy, prolonged maintenance treatment, and high-dose therapy followed by autologous or allogeneic hematopoietic stem cell transplantation (HSCT). Strategies should be individualized based on the potential risk of relapse, with higher risk patients receiving more aggressive therapy (Schlenk et al. 2003, Döhner et al. 2010).

Aberrant karyotype is detectable in about 55% of patients with AML and is the most significant prognostic indicator (Döhner et al. 2008). Younger adult patients are commonly categorized into three risk groups, favorable, intermediate, or adverse based on karyotype, as shown in figure 1 (Byrd et al. 2002, Grimwade et al. 2010). Nevertheless patients with normal karyotype (NK) are heterogeneous with regard to prognosis. In recent years a series of gene mutations like NPM1 mutation, CEBPA mutation and FLT3-ITD, have been identified to predict favorable or unfavorable prognosis significantly within this group of AML patients (Schlenk et al. 2008, Schlenk et al.2009). Moreover, those gene mutations could further categorize NK AML patients and have been integrated in 2008 WHO classification of AML (Vardiman et al. 2009).

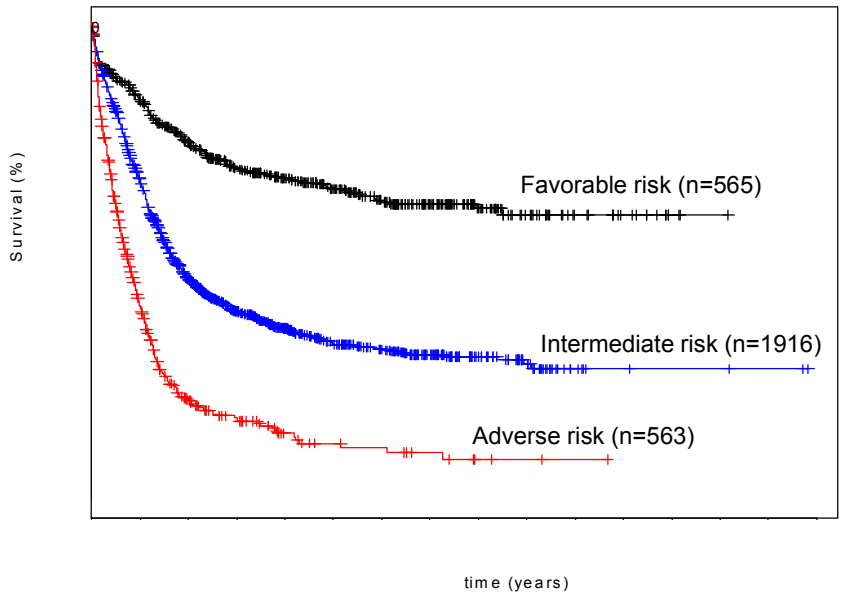


Figure 1. Survival rate of different risk groups of AML (from AMLSG database).

Table 2. Standardized reporting for correlation of cytogenetic and molecular genetic data in AML with clinical data. (Döhner et al. 2010)

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3-MLL* Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *MLL* rearranged -5 or del(5q); -7; abnl(17p); complex karyotype

Although standard induction therapy achieves CR in the majority of younger adult patients, overall survival (OS) remains only at about 50% for those patients who have a CR, or 20-30% overall (Shipley et al. 2009). Relapse, which is commonly regarded as a result from residing of leukemia stem cells (LSCs), is one of the main obstacles against more favorable outcome (Luo et al. 2006).

2.2 Leukemia stem cells

2.2.1 Definition

Leukemia stem cells (LSCs) have been biologically defined as a rare subpopulation within leukemia blasts that exhibits biological characteristics similar to those of nonmalignant stem cells and initiates and sustains leukemic proliferation. This concept was first demonstrated by John Dick and colleagues, who showed that cells with the ability to transfer human AML to nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice are frequently found exclusively in the CD34+CD38- compartment (Lapidot et al. 1994). Furthermore, they established a new model that AML is organized as a hierarchy that originates from LSCs, mimicking their normal counterpart (Bonnet et al. 1997). In this model, LSCs are in the position of self-renewal and to give rise to more differentiated progeny which includes every (malignant) cell type within the blasts (Figure 2). Since then, an ever expanding list of solid tumors has been reported to contain a cancer stem cell subpopulation, including breast cancer, ovarian cancer, pancreatic cancer, colon cancer, prostate cancer, hepatocellular carcinoma, melanoma, lung cancer, glioblastoma, other brain tumors, and neuroblastoma.

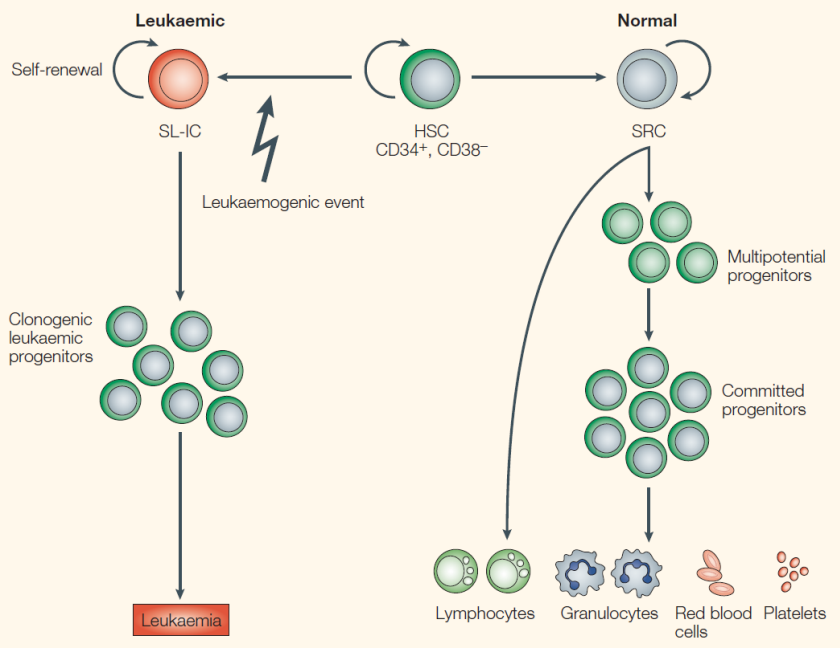


Figure 2. **AML is organized as a hierarchy with leukemia stem cells in the root.** Leukemia stem cells and hematopoietic stem cells share some surface markers. Leukemogenesis is driven by Leukemia stem cells, as hematopoiesis is driven by normal hematopoietic stem cells.
(Huntly et al. 2005)

2.2.2 LSCs and relapse

Acute myeloid leukemia stem cells and normal hematopoietic stem cells (HSCs) share many surface molecules, use common self-renewal mechanisms and are predominantly in a quiescent state. This quiescent state protects LSCs from excessive DNA damage and exhaustion, increasing their self-renewal capacity and survival, and makes LSCs insensitive to standard chemotherapy, or at least no more susceptible than normal HSCs. Meanwhile, several members of the ATP binding cassette (ABC) transporter family, have been found to be significantly higher expressed in AML LSCs (de Grouw et al. 2006, Raaijmakers et al. 2005), which can pump a wide variety of endogenous and exogenous compounds out of cells, thus adding to drug resistance. During selection by chemotherapy, CD34+ cells were significantly enriched in AML blasts (Kornblau et al. 2006), which also demonstrating the resistance of LSCs

to chemotherapy.

Consequently, drug regimens designed to kill AML blasts may not be effective for destruction of the LSCs population. LSCs are pivotal in the development and pathogenesis of leukemic disease, so failure to target this population is a possible cause of relapse. This hypothesis is supported by the following facts: overall survival is significantly different between NOD/SCID mice engrafting and nonengrafting AMLs, nonengrafting ones survive longer (Pearce et al. 2006, Monaco et al. 2004); and high LSCs frequency in AML at diagnosis correlated with shorter overall survival, disease-free survival and relapse-free survival (van Rhenen et al. 2005), or lower 5 years event-free survival rate (Witte et al. 2011). Hence, here emerges a new challenge: how to accumulate more information about LSCs and identify optimal targets to eradicate LSCs in patients with AML.

2.3 Leukemia-associated antigens

2.3.1 Definition

Leukemia cells express unique antigens or overexpress normal cellular antigens, which are called leukemia-associated antigens (LAAs). During the last decade, several LAAs have been identified in AML patients like BAGE, BCL-2, OFA-iLRP, FLT3-ITD, G250, human telomerase reverse transcriptase (hTERT), preferentially expressed antigen of melanoma (PRAME), the receptor for hyaluronic acid mediated motility (RHAMM), proteinase 3, survivin, and Wilms' tumor 1 (WT-1). All those LAAs listed above can elicit specific T-cell responses in AML patients. RHAMM and WT-1 induce both serological and cellular immune reactions (Elisseeva et al. 2002, Greiner et al. 2002, Greiner et al. 2006).

2.3.2 LAAs as therapeutic targets

Due to their unique expression pattern, these antigens could be used as a marker to distinguish leukemia cells from normal cells, to help diagnosis and detect minimal residual diseases (MRD). Furthermore, they could serve as ideal targets for immunotherapies. Some of these LAAs, e.g. G250, PRAME, proteinase 3 are associated with an improved clinical outcome in AML, chronic myeloid leukemia (CML) or solid tumors (Zhang et al. 2011, Greiner et al. 2006, Steinbach et al. 2002, Bui et al. 2003, Yong et al. 2006). Post-transplant graft-versus-leukemia (GVL) effect is associated with cytotoxic CD8+ T cells against WT-1 (Rezvani et al. 2007). These data indicate that LAAs may elicit protective immune responses. In experimental settings, several epitopes in these LAAs have been shown to induce cell lysis of autologous leukemic blasts by specific cytotoxic T cells (Greiner et al. 2008). PR-1 (a peptide from PR3) specific cytotoxic T lymphocytes (CTLs) transfusion could reduce leukemia burden in a mouse AML xenograft model (Ma et al. 2010). The LAAs RHAMM, proteinase 3, and WT-1 were tested in clinical peptide vaccination trials. In these clinical trials, immunologic and clinical responses could be detected in patients with different hematological malignancies (Rezvani et al. 2008, Oka et al.2004, Rezvani et al. 2008, Schmitt et al. 2008, Greiner et al. 2010). It should be noticed that most LAAs are critically involved in mechanisms responsible for tumor growth, such as proliferation, inhibition of apoptosis, differentiation and demethylation (Greiner et al.2008). Thus, targeting these LAAs may not only elicit cytotoxic immune responses but also interfere with those critical functions, thereby adding to anti-leukemia effects.

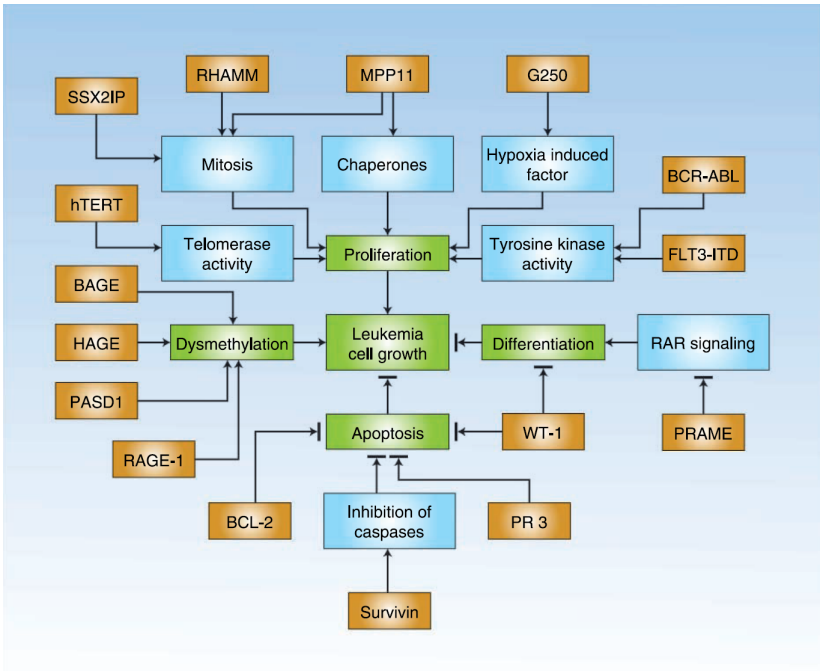


Figure 3. Functional role of leukemia-associated antigens. Leukemia-associated antigens are involved in several crucial cell activities and promote tumorigenesis. (Greiner et al. 2008)

2.4 Immunotherapy in AML

Immunotherapy for AML includes allo-HSCT, adoptive transfusion of allogeneic or autologous T cells or NK cells (donor lymphocyte infusion, DLI), vaccination with leukemia cells, dendritic cells, cell lysates, peptides and DNA vaccines and treatment with cytokines, antibodies and immunomodulatory agents (Figure 4).

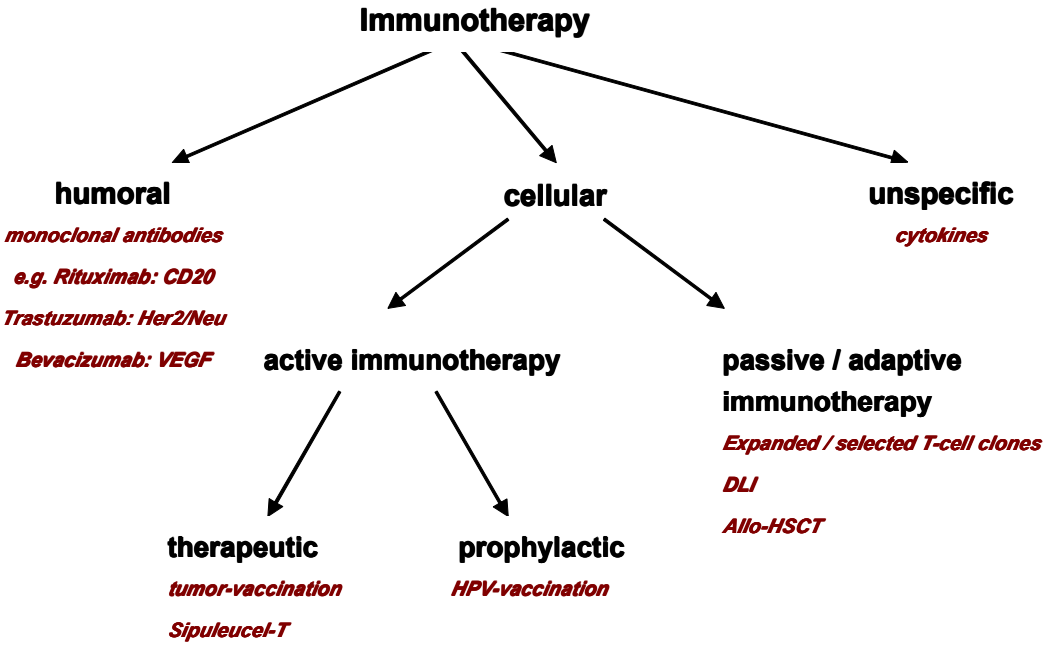


Figure 4. Overview of possible treatment strategies in immunotherapies: This figure describes the possibilities of treatment involving immunotherapy. (The picture was supplied by Prof. Jochen Greiner)

2.4.1 Allogeneic hematopoietic stem cell transplantation and donor lymphocyte infusion

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) and donor lymphocyte infusion (DLI) are able to induce GVL effect, and thus were established as curative therapies for patients with CML or AML. Since 1995, six cooperative group trials have examined the role of allo-HSCT in AML in first remission (Zittoun et al.1995, Harousseau et al.1997, Burnett et al.1998, Cassileth et al.1998, Suciu et al.2003, Cornelissen et al). They demonstrated lower relapse rates in patients undergoing allo-HSCT. Risk stratified analysis showed superior overall survival for patients with poor prognostic features who underwent allo-HSCT compared to auto-HSCT or intensive consolidation chemotherapy (Hamadani et al. 2008). For the approximately 30% of patients with AML who fail to achieve CR with standard induction chemotherapy, allo-HSCT is the only curative option (Song et al. 2005).

The best responses to DLI arise in patients with CML, but this therapy is also helpful for patients with AML. A retrospective analysis of 399 patients with AML in first haematological relapse following transplantation showed superior survival in patients receiving DLI (Schmid et al. 2007). DLI in a prophylactic setting after non-myeloablative transplantation or T-cell-depleted myelo ablative transplantation might be more effective (Soiffer et al. 2008).

2.4.2 Vaccination

Tumor vaccines are supported by encouraging outcomes from clinical trials (Palucka et al. 2011). Our group identified a HLA-I restricted peptide derived from RHAMM, which is named R3. Recently we performed 2 clinical trials in which patients with AML, MDS or MM were vaccinated with R3 peptide. Elicitation of immune response and reduction of leukemia blasts was observed in bone marrow of AML patient after vaccination (Schmitt et al. 2008, Greiner et al. 2010). Vaccination with dentritic cells (DC), both leukemia-derived and monocyte- or bone marrow–derived, can elicit or enhance specific T cells responses (Smits et al. 2009).

The susceptibility of AML cells to T cell therapy is attributed to two reasons：AML cells express both major histocompatibility complex (MHC) class I and class II, co-stimulatory molecules are also preserved on AML cells, constituting the prerequisites for a specific T cells response (Vollmer et al. 2003). Several LAAs were found to be able to elicit specific T cell responses and corresponding epitopes were identified, thus locating the targets for T cell based immunotherapy (Greiner et al. 2006).

2.5 Rationale of the study

With intensive induction therapy, 60 - 80% of younger patients (≤60 years) with AML achieve CR (Döhner et al. 2010). However, more than 50% of these patients will relapse, leading to an overall survival rate of only 30% to 40%

after 5 years. Results are more unfavorable in patients older than 60 years (Giles FJ et al. 2002). Relapse after chemotherapy is one of the major obstacles impeding radical treatment of AML. The outcome of AML will be improved remarkably with the decrease of the relapse rate. There is an urgent need for novel therapies which could reduce the risk of relapse after chemotherapy.

Unlike leukemia blasts, AML LSCs are found to stay in a quiescent state. Those AML LSCs express several transporters in a significantly high level. Therefore they are refractory to standard chemotherapy and can survive, even if CR is acquired. Afterwards, these LSCs are renewing themselves, they proliferate and differentiate (abnormally), and finally regenerate the AML disease, which results in clinical relapse. The correlation between high LSCs frequency at diagnosis and shorter relapse-free survival or lower event-free survival rate has been reported in both adult AML patients and childhood AML patients (van Rhenen et al. 2005, Witte et al. 2011). Eradication of LSCs must be included as an essential part in AML treatment. LSCs have become an important target for AML treatment (ten Cate B et al. 2010).

On the other hand, allo-HSCT and DLI are able to reduce relapse rate, due to the GVL effect, although the underlying mechanisms are not fully understood. Nonmyeloablative conditions followed by HSCT can cure AML patients, just like traditional myeloablative conditions. Allo-HSCT results in lower risk of relapse than syngeneic-HSCT. In patients who received T-cell depleted Allo-HSCT, the relapse risk is increased to the same level as for recipient of syngeneic-HSCT (Bruserud et al. 2001). Based on those reports, GVL is asserted to be mediated mostly by T lymphocytes.

Taken together, those facts gave us a suggestion that immunotherapy is able to eradicate LSCs. However, this speculation needs to be demonstrated by evidence. Here, we investigated the immunogenicity of LSCs, to find out whether the immune system can target LSCs or not, to improve our understanding about mechanism of immunotherapy, and to provide more

evidence for developing novel therapeutics.

2.6 The aim of the study

Since AML has a poor prognosis, as discussed extensively before, immunotherapy might be an option to target the disease in the state of minimal residual disease (MRD). LSCs are of pivotal importance in leukemogenesis and disease relapse, and thus a critical target for therapies. This study was initiated to investigate the immunogenicity of LSCs in AML patients and thus establish targeted therapies against AML LSCs.

Specific immune response between CTLs and LSCs requires two signals: (a) the first signal resulting from recognition of an antigen peptide presented by an HLA-ABC molecule to the TCR, and (b) the second signal resulting from binding of a co-stimulatory molecule, such as CD80 or CD86, to the molecule CD28 (Leitner et al. 2010). In this study we evaluated the prerequisite of immune response between CTLs and LSCs, with following aims:

- Establishment of an efficient separation assay for AML LSCs
- Identification of LAAs specifically expressed in AML LSCs
- Expression of HLA-ABC on AML LSCs
- Expression of co-stimulatory molecules (CD80, CD86 and CD40) on AML LSCs

To accomplish those aims, we separated LSCs with two different assays and compared the efficiency. CD34+CD38- cells, which contain enriched LSCs, were sorted from AML samples with a high blast count. Additionally, we isolated CD34+CD38+, CD34-CD38- as well as CD34-CD38+ subpopulations in order to make a comparison of the expression pattern. Correspondingly four subpopulations were isolated from HVs. DNA microarray was performed to determine gene expression profile (including LAAs) of LSCs and other three

subpopulations, as well as their normal counterpart. LAAs expression was compared between LSCs and the other three subpopulations, and between LSCs and HSCs, in order to identify LAAs which are expressed specifically in LSCs. Expression of HLA-ABC and co-stimulatory molecules on LSCs was evaluated by multi-color FACS examination.

3. Materials and Methods

3.1 Materials

3.1.1 Samples

Buffy coats of healthy volunteers (HVs) were obtained from the German Red Cross (DRK, Ulm, Germany). All patient samples were taken from AML patients treated at the department of Internal Medicine III at the University Clinic of Ulm. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. All patient samples consist of leukemia blasts more than 90% which was determined by routinely clinical lab test.

3.1.2 Reagents

AB-Serum	German red cross, Ulm
Aqua destillata (1000 ml)	Braun, Melsungen
Dimethylformaldehyde	Sigma-Aldrich, Germany
Encore Biotin Module	NuGEN, USA
FcR blocking reagent	Miltenyi Biotec, Bergisch Gladbach,Germany
Fetal Calf Serum	Gibco BRL, Eggenstein, Germany
Ficoll Seperating Solution	Seromed-Biochrom AG, Berlin, Germany
GeneChip Hybridization, Wash, and Stain Kit	Affymetrix, USA
Hybridization controls	Affymetrix, USA
L-Glutamin	Seromed-Biochrom AG Berlin, Germany

Ovation RNA Amplification System V2	NuGEN, USA
PBS 10x	Gibco, Invitrogen, USA
Penicillin	Gibco BRL, Eggenstein, Germany
QIAquick PCR purification kit	QIAGEN, Germany
RPMI 1640 medium	Biochrom AG
Streptomycin	Gibco BRL, Eggenstein, Germany
TRIzol reagent	Invitrogen, USA
Trypanblue 0.4%	Sigma-Aldrich, USA

3.1.3 Antibodies

APC mouse anti-human CD 40	Becton-Dickison, USA
APC mouse IgG1 Isotype control	Becton-Dickison, USA
Anti-Biotin magnetic beads	Miltenyi Biotec, Bergisch Gladbach,Germany
Anti-CD34 magnetic beads	Miltenyi Biotec, Bergisch Gladbach,Germany
Anti-CD38 Biotin	Miltenyi Biotec, Bergisch Gladbach,Germany
FITC mouse anti-human CD34	Becton-Dickison, USA
FITC mouse anti-human CD8	Becton-Dickison, USA
FITC mouse anti-human HLA-A2	Becton-Dickison, USA
FITC mouse anti-human HLA-ABC	Becton-Dickison, USA

FITC mouse IgG1 Isotype control	Becton-Dickison, USA
FITC mouse IgG2b Isotype control	Becton-Dickison, USA
PE mouse anti-human CD38	Becton-Dickison, USA
PE mouse anti-human CD80	Becton-Dickison, USA
PE mouse anti-human CD86	Biolegend, USA
PE mouse IgG1 Isotype control	Becton-Dickison, USA
PE mouse IgG2b Isotype control	Biolegends, USA
PE-Cy7 mouse anti-human CD38	Becton-Dickison, USA
PE-Cy7 mouse IgG1 Isotype control	Becton-Dickison, USA
PerCP mouse anti-human CD4	Becton-Dickison, USA
PerCP mouse anti-human CD34	Becton-Dickison, USA
PerCP mouse IgG1 Isotype control	Becton-Dickison, USA

4.1.4 Solutions and buffers

BD FACS Flow	BD Biosciences, Heidelberg, Germany
BD FACS Clean	BD Biosciences, Heidelberg, Germany
BD FACS Rinse	BD Biosciences, Heidelberg, Germany
Dilution-buffer	1x PBS 10% FCSe

Freezing medium	RPMI1640
	20% human AB serum
	1% penicillin
	1% streptomycin
	2 mM L-glutamine
	10% DMSO
FACS buffer	500 ml 1x PBS
	5 g BSA
Hybridization solution A	Affymetrix, USA
Hybridization solution B	Affymetrix, USA
Plain medium	RPMI 1640
	1% L-glutamine
	1% Penicillin
	1% Streptomycin
Separation buffer	PBS
	2 mM EDTA
	0.5 % heat inactivated, FCS
Wash buffer A	Affymetrix, USA
Wash buffer B	Affymetrix, USA

3.1.5 Equipments and plastic ware

Autoclave	WEBECO GmbH, Germany
24-well tissue culture plate (flat bottom)	BD Biosciences, New Jersey, USA
-80°C ultra low temperature freezer	SANYO Electric Biomedical Co. Ltd, Japan

96-well tissue culture plate (flat bottom)	BD Biosciences, New Jersey, USA
Cell culture flasks	Nalge Nunc International, Roskilde, Denmark
Cryo TubeTM Vials (1.8 ml)	Nalge Nunc International, Roskilde, Denmark
CS-15R Centrifuge	Beckman Biotechnology, Germany
CS-6R Centrifuge	Beckman Biotechnology, Germany
FACSAria Flow Cytometer	BD Biosciences, New Jersey, USA
FACScan Flow Cytometer	BD Biosciences, New Jersey, USA
GeneChip Scanner 3000	Affymetrix, USA
Hemocytometer chamber	Optik Labor, Berlin, Germany
HG U133 Plus 2.0 microarray	Affymetrix, USA
Hybridization oven	Affymetrix, USA
Incubator	Heraeus, Hanau, Germany
Inverse microscope	Carl Zeiss, TELAVAC31, Germany
Light microscope	Carl-Zeiss, Germany

Liquid nitrogen tank	Model 8038 S/N 14830.59 Forma Scientific Inc, Germany
LD columns	Miltenyi Biotec, Bergisch Gladbach,Germany
LS columns	Miltenyi Biotec, Bergisch Gladbach,Germany
MS columns	Miltenyi Biotec, Bergisch Gladbach,Germany
MACS MultiStand	Miltenyi Biotec, Bergisch Gladbach,Germany
MACS Pre-Separation Filters	Miltenyi Biotec, Bergisch Gladbach,Germany
Microcentrifuge	Eppendorf, 5415D, Germany
Non pyrogenic serologic pipettes (2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Corning Incorporation, New York, USA
OctoMACSTM separator	Miltenyi Biotec, Bergisch Gladbach,Germany
Pipets (0.1-20 µl, 2-200 µl, 50-1000 µl)	Eppendorf AG, Hamburg, Germany
Pipette Boy (IBS Pipetboy accu)	Integra Biosciences AG, Chur, Switzerland
Polypropylene Conical Centrifuge Tubes (15 ml, 50 ml)	Becton and Dickinson Labware, NJ, USA

Refrigerator	Liebherr, Ochsenhausen, Gemany
Safe-Lock Tube (1,5 ml and 2 ml)	Eppendorf AG, Hamburg, Germany
Sterile bench	TYP. HS 18/2, Heraeus instruments, Germany
Thermal cycler	GeneAmp PCR System 9700, Applied Biosytstems, USA
Vortex-2TM genie	Model G-560E, Scientific Industries,Inc. Bohemia, NY, USA
Waterbath	GFL, Burgwedel, Germany

3.2 Methods

3.2.1 Ficoll density separation

All samples were taken from 5 healthy donors and 10 AML patients after their informed consent was obtained in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Biocoll Separation Solution density gradient centrifugation from Ethylen ediaminetetra acetic acid (EDTA) anticoagulated blood buffy coat preparations from healthy donors or from EDTA anticoagulated blood samples from patients. The viability of PBMCs obtained was >95%, as determined by trypan blue staining. The viable cells were quantified in a Neubauer chamber. For cellular assays, Ficoll separated PBMCs were tested freshly or cryopreserved in freezing medium and stored in liquid nitrogen until

further use. For recovery, cryopreserved cells were thawed at 37 °C quickly and washed once with plain medium for further use.

3.2.2 Methods for LSCs separation

3.2.2.1 Screening of AML samples

First of all, we performed FACS to screen primary AML samples by testing the expression of CD34 and CD38. Cryopreserved cells were thawed in a water bath at 37 °C, viability was determined by trypan blue staining and was always >95%. 5-10 x 10^5 cells were washed once with FACS buffer, then stained with respective antibodies as listed in table 3 for 30 min at 4 °C. Thereafter, cells were washed once with cold 1xPBS and resuspended in 400µl 1xPBS, then applied to the FACScan flow cytometer. Samples consisting of enough CD34+CD38- cells were used for LSCs separation.

Table 3. Antibodies used for FACS analysis and sorting.

Tube	Mouse IgG FITC	Mouse IgG PE	CD34 FITC	CD38 PE
Isocontrol	10µl	10µl	-	-
Samples for analysis	-	-	10µl	10µl
Samples for sorting	-	-	60-120µl	60-120µl

3.2.2.2 LSCs separation by FACS

A similar staining protocol was used for FACS sorting. 5 x 10^5 cells were stained with corresponding isotype control and 1-5 x 10^7 cells were stained with antibodies for sorting. A higher concentration of antibodies was used for staining because of higher cell numbers, as listed in table 3. After staining, cells for sorting were resuspended in 1ml cold PBS, then sorted with the FACSAria cytometer. All the four populations (CD34+CD38-, CD34+CD38+, CD34-CD38+ and CD34-CD38- population) were sorted, and then reanalyzed by FACS to evaluate the purity. Sorted cells were washed once with 1xPBS

and centrifuged. Pellets were frozen quickly in liquid nitrogen and then stored at -80 °C.

3.2.2.3 LSCs separation by MACS

Two rounds of MACS were performed to obtain CD34+CD38- population from PBMC: CD38 depletion and CD34 enrichment. In the first round, PBMC were washed once and resuspended in Separation buffer, then incubated with anti CD38 Biotin for 10min at 4 ℃. Thereafter, cells were washed and resuspended in Separation buffer, then incubated with anti-Biotin magnetic beads for 15 min at 4 ℃. Labeled cells were applied to MACS column which was put in a magnetic OctoMACS™ separator. The effluent consisted of the CD38- fraction of PBMC.

The CD38- fraction was used for the second separation round. The cells were incubated with anti CD34 magnetic beads and the FcR blocking reagent for 30 min at 4 ℃ for labeling. After incubation, cells were washed and resuspended in Separation buffer, and then applied to MACS column. The effluent consisted of the CD34-CD38- cells and CD34+CD38- cells remained in MACS column. Then the column was removed from the magnetic field and the CD34+CD38- cells were eluted with Separation buffer. CD34+CD38- cells were applied to a second MACS column for further purification. Finally, the CD34+CD38- subpopulation was achieved by repeated rounds of MACS separation.

The final populations obtained by FACS and MACS were counted and the purity was assessed by FACS. In order to evaluate separation efficiency in a standardized manner, the recovery rate was defined in the following manner:

$$\text{Recovery rate} = \frac{\text{CD34+CD38- cell number obtained}}{\text{total CD34+CD38- cell number}} \times 100\%$$

The total CD34+CD38- cell number was calculated according to FACS analysis before separation.

3.2.3 FACS analysis of co-stimulatory molecules

Multicolor FACS analysis was performed to detect co-stimulatory molecules on the surface of LSCs. PBMCs of AML patients were thawed and counted. Aliquots containing 1 x 10^6 cells were stained for 30 min at 4 °C. The antibodies panels are listed in table 4.

Table 4. Antibodies used for co-stimulatory molecules detection.

Tube	Antibodies
Isocontrol 1	Mouse IgG1 PerCP / Mouse IgG1 PE-Cy7 / Mouse IgG1 FITC / Mouse IgG1 APC / Mouse IgG1 PE
Sample 1	CD34 PerCP / CD38 PE-Cy7 / HLA-ABC FITC / CD40 APC/ CD80 PE
Isocontrol 2	Mouse IgG1 PerCP / Mouse IgG1 PE-Cy7 / Mouse IgG2b PE
Sample 2	CD34 PerCP / CD38 PE-Cy7 / CD86 PE

3.2.4 cDNA-based Microarray for LAAs expression

Total RNA was isolated from frozen pellets using TRIzol reagent according to the manufacturer's protocol. Briefly, cells were lysed by TRIzol reagent and RNA was then isolated by phase separation. Then the RNA was purified through precipitation and wash procedure. Quality and quantity of RNA was assessed by Spectrophotometric analysis. As LSCs is a rare population in most samples, the RNA concentration of some pellets was insufficient for microarray. Thus all RNA samples were subjected to reverse transcription and linear amplification with Ovation RNA Amplification System V2 (Figure 5).

Amplified cDNA was purified and quantified. 3.75 μg of cDNA was fragmented, generating cDNA fragments of 50-100 bases. Subsequently, cDNA fragments were labeled with biotin, and then hybridized to HG U133 Plus 2.0 microarray (Affymetrix). Microarray hybridization, staining and washing was performed

using the GeneChip Hybridization, Wash, and Stain Kit, following the GeneChip Expression Analysis Technical Manual (Affymetrix) and the GeneChip Expression Wash, Stain and Scan User Manual (Affymetrix).

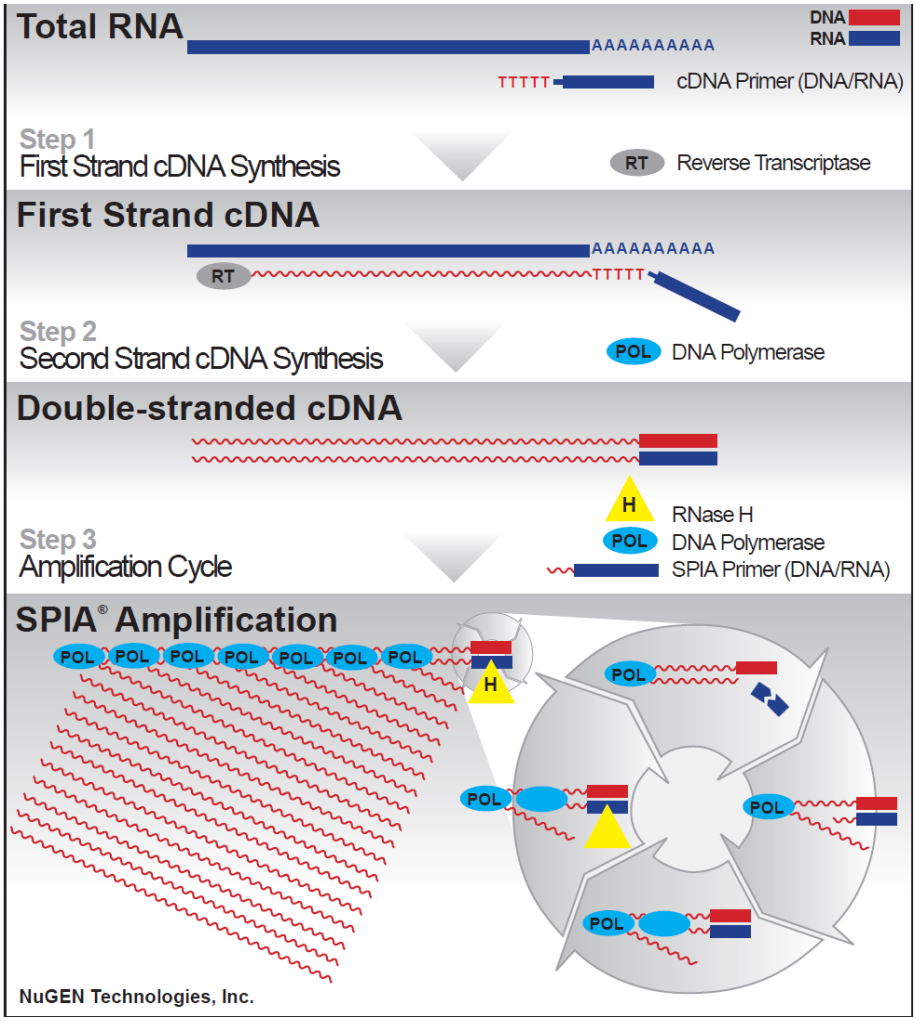


Figure 5. Illustration of reverse transcription and linear amplification. (Courtesy by NuGEN Technologies, Inc. http://www.nugeninc.com**)**

4. Results

4.1 Screening of patient samples based on expression of CD34 and CD38

Samples from 21 AML patients were screened by FACS. 10 patient samples were chosen for LSCs separation. Table 5 shows the characteristics of these patients. In this study AML were categorized according to WHO classification 2008 (Vardiman et al. 2009). However, some samples were collected before 2008 and cytogenetic information was unavailable or insufficient for WHO classification 2008 in these samples. Corresponding descriptions were also presented in Table 5.

Table 5. Characteristics of AML patients. All samples consist of leukemia blasts more than 90%.

*: These patient samples were used for separation.

No.	Sex	Age	Classification	HLA-A2	CD34+CD38-
1*	M	46	AML with recurrent genetic abnormalities, t(8;21)	neg	6.67%
2*	M	74	AML with myelodysplasia-related changes	pos	86.20%
3	M	17	AML with recurrent genetic abnormalities, inv(16)	neg	4.94%
4	M	72	AML, no cytogenetic analysis	pos	0.10%
5*	F	57	AML, t(6;11)	neg	86.10%
6	M	50	AML, normal karyotype	pos	0.01%
7*	F	46	AML, normal karyotype	pos	1%
8*	M	57	AML, XY, +8	pos	2.95%
9*	F	75	AML, therapy related	pos	9.27%
10	M	68	AML, therapy related	neg	0.86%
11	M	81	AML, no cytogenetic analysis	neg	0.04%
12*	M	64	AML, therapy related	pos	0.78%
13	M	65	AML, no cytogenetic analysis	pos	0.05%
14*	F	75	AML with *NPM1*-Mutation	pos	6.8%
15*	M	53	AML, normal karyotype, *NPM1* wild type	pos	39.1%
16	F	45	AML, no cytogenetic analysis	pos	10.4%
17	M	47	AML, no cytogenetic analysis	pos	0.09%
18	F	73	AML, therapy related	pos	0.22%
19	M	75	AML, normal karyotype, *NPM1* wild type	neg	2.88%
20*	F	69	AML, XX, +8	neg	0.63%
21	M	62	AML with recurrent genetic abnormalities, inv(16)	pos	0.02%

4.2 Efficiency for LSCs separation by MACS or FACS

In order to establish the most efficient assay for LSCs separation, we separated the CD34+CD38- population from AML PBMC samples with FACS and MACS, and compared the efficiency. This comparison was performed in 2 representative samples, in which the CD34+CD38- subpopulation was rather small (patient 20: 0.63%) or large (patient 5: 86.1%). In patient 20, MACS resulted in a recovery rate of 6.4% with a purity of 90.3%, which is inferior to the recovery rate of 17% and the corresponding purity of 92.1% by FACS. In patient 5, MACS resulted in a recovery rate of 0.4% with a purity of 98.8%, compared to the recovery rate of 11.6% with a purity of 98.1% by FACS. These data show the tendency that in samples with a low CD34+CD38- subpopulation, the recovery rate was higher but the purity decreased slightly. When comparing the 2 methods, it becomes clear that the purity is comparable in both methods, but the yield is much higher using FACS, and it makes no difference if the CD34+CD38- subpopulation is small or large. Thus, FACS sorting was the method we chose for LSCs separation and employed for next experiments.

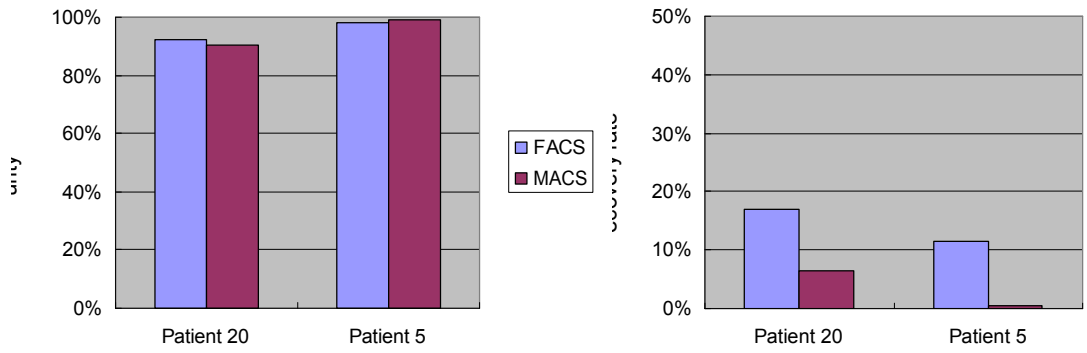


Figure 6. Comparison of separation efficiency of FACS and MACS, assessed by both purity and recovery rate. Patient 20 contained 0.63% of CD34+CD38- population, patient 5 contained 86.1% of CD34+CD38- population. These data show that both methods resulted in very pure products, but the recovery rate of the FACS was much superior regardless of the frequency of CD34+CD38- cells.

4.3 Results of FACS sorting

The frequency of CD34+CD38- cells before sorting was 0.63-86.2% in AML samples. 1-5 x 10^7 cells were used for separation, resulting in a yield of 0.2-36×10^5 cells for LSCs. For the 5 HV samples, 1-10×10^4 CD34+CD38- cells were obtained.

PBMC could be categorized into four populations according to expression of CD34 and CD38: CD34+CD38-, CD34+CD38+, CD34-CD38+ and CD34-CD38-. We sorted all the four populations from both patient and HV samples by FACS. The purity of all populations, which was assessed by reanalysis after sorting, was higher then 90%.

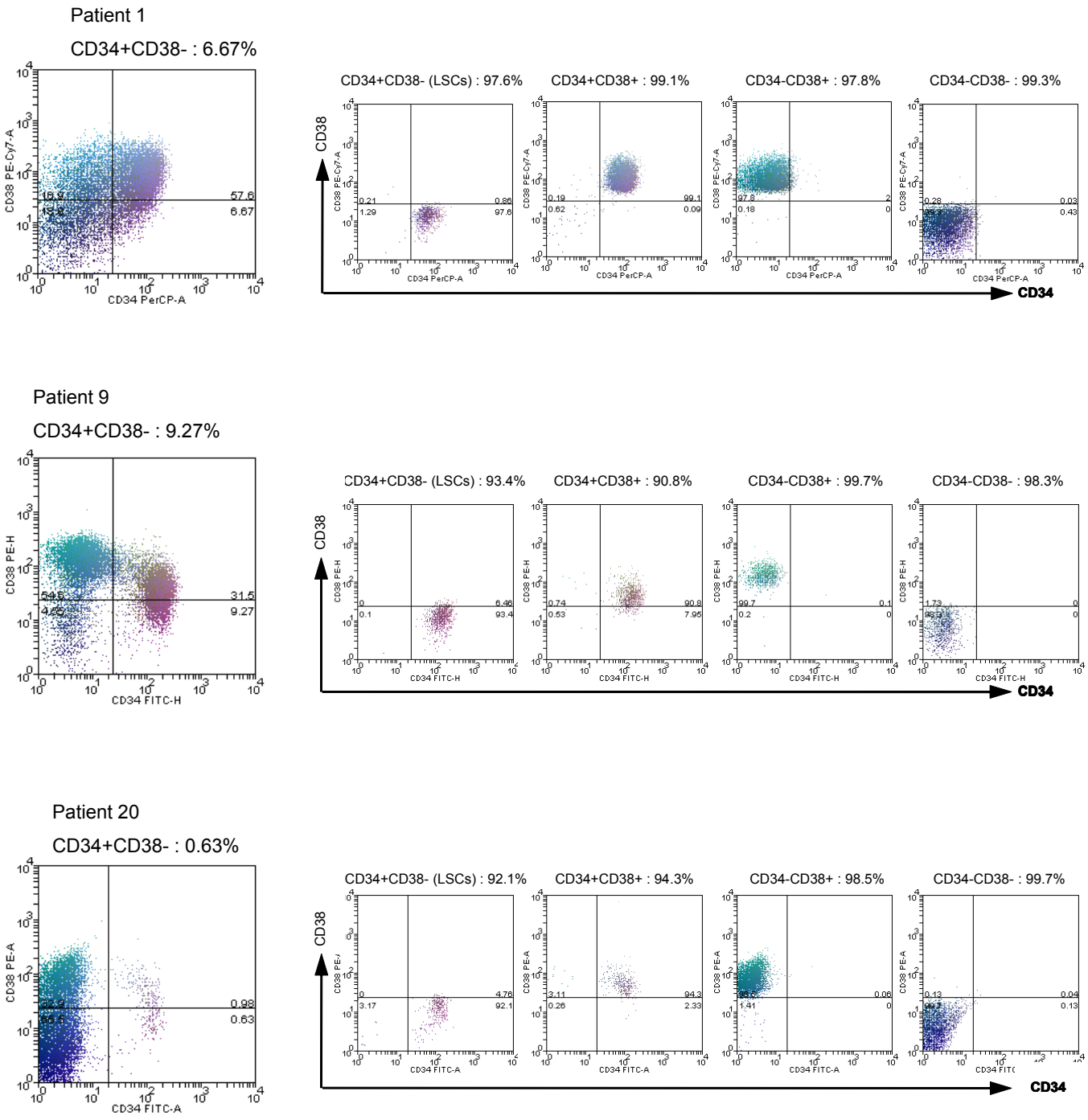


Figure 7. Purity of different populations after sorting. This figure shows FACS analysis before and after separation of three AML samples. The purity of all populations is over 90%.

4.4 Co-stimulatory molecules on LSCs

Multicolor FACS was performed in 10 AML samples to detect co-stimulatory molecules. LSCs were gated according to their specific immunophenotype: CD34+CD38-. HLA-ABC, CD40, CD80 and CD86 expression were investigated in this study. The gating strategy is shown in figure 8.

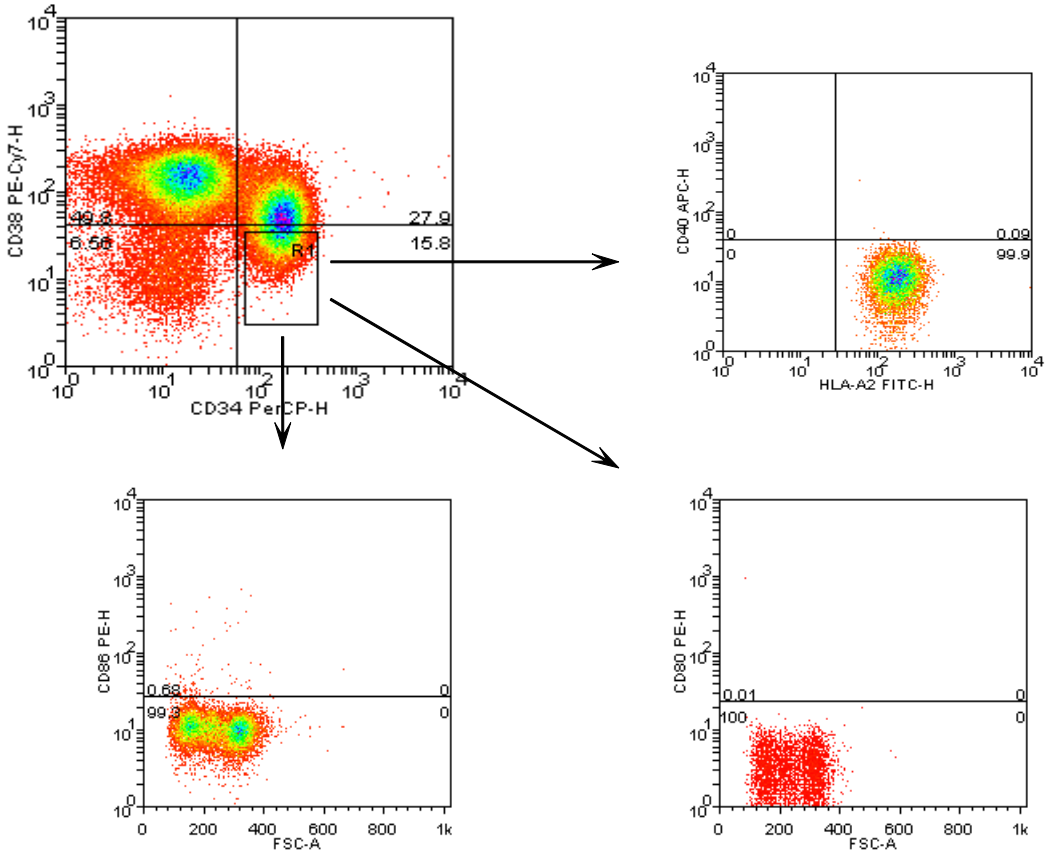


Figure 8. The gating strategy for leukemia stem cells. Leukemia stem cells, which are the CD34+CD38- population, were gated (R1 in this figure) and evaluated further as to HLA-I, CD40, CD80 and CD86 expression.

As shown in table 6, HLA-I was preserved on nearly all leukemic malignant cells, including LSCs, which makes it theoretically feasible to lyse LSCs by CTLs. CD40 was detectable on blasts of three patients but on LSCs of only two patients. CD80 was absent in all patients. CD86 was expressed on blasts of eight patients and on LSCs of 6/8 patients.

When comparing the expression of those molecules in LSCs and blasts, HLA-I

was equally highly expressed. In the patient who is CD40 positive, the positive rate is lower on LSCs than on blasts. As for CD86, the positive rate is also much lower on LSCs than blasts.

Table 6. Expression of HLA-I and CD40, CD80, CD86 on LSCs (leukemia stem cells) and leukemia blasts. n.t.: not tested.

Patient		HLA-I	CD40	CD80	CD86
1	Blasts	99.58%	7.18%	1.62%	54.6%
	LSCs	100%	4.03%	0.34%	7.88%
2	Blasts	n.t.	n.t.	n.t.	23.4%
	LSCs	99.98%	0.26%	0.08%	9.58%
3	Blasts	99.1%	0.11%	0.21%	0.54%
	LSCs	99.9%	0.11%	0.07%	0.16%
4	Blasts	98.42%	0.43%	0.03%	1.43%
	LSCs	100%	0.41%	0%	1.07%
5	Blasts	99.9%	0.35%	0.08%	38.9%
	LSCs	100%	0.09%	0.01%	0.68%
6	Blasts	99.8%	0.13%	0.6%	5.02%
	LSCs	100%	0.2%	0.36%	0.9%
7	Blasts	n.t.	n.t.	n.t.	4.8%
	LSCs	99.98%	0.58%	0.74%	5.69%
8	Blasts	98.98%	1.08%	0.72%	28.2%
	LSCs	99.47%	1.28%	0.72%	6.27%
9	Blasts	99.2%	2.36%	0.85%	42.1%
	LSCs	97.5%	0.79%	0.79%	30.4%
10	Blasts	98.1%	0.8%	0.3%	1.92%
	LSCs	100%	0.13%	0.04%	1.33%

4.5 Gene expression profile of LSCs

Microarrays were performed with GeneChip HG U133 Plus 2.0 from Affymetrix in 58 samples from 10 AML patients and 5 HVs. We applied class comparison to analyze differentially expressed genes between different groups of arrays. Comparison between LSCs and HSCs discovered 2617 differentially expressed genes ($P<0.05$): 1727 genes higher expressed in LSCs and 890 genes higher expressed in HSCs (Figure 9a). The expression of two LAAs, WT1 and SSX2IP, were found to be significantly higher in LSCs than in HSCs (Table 7).

We also compared gene expression profile between LSCs and the other three subpopulations of blasts and discovered 2278 genes ($P<0.05$) differentially expressed between the four populations (Figure 9b). Then we compared these two groups of differentially expressed genes, and found that the expression of three genes were significantly higher in LSCs than in HSC or other three AML blasts populations: TFPI (tissue factor pathway inhibitor), ZNF43 (zinc finger protein 43) and C18orf54 (chromosome 18 open reading frame 54). However, these three genes are not LAAs.

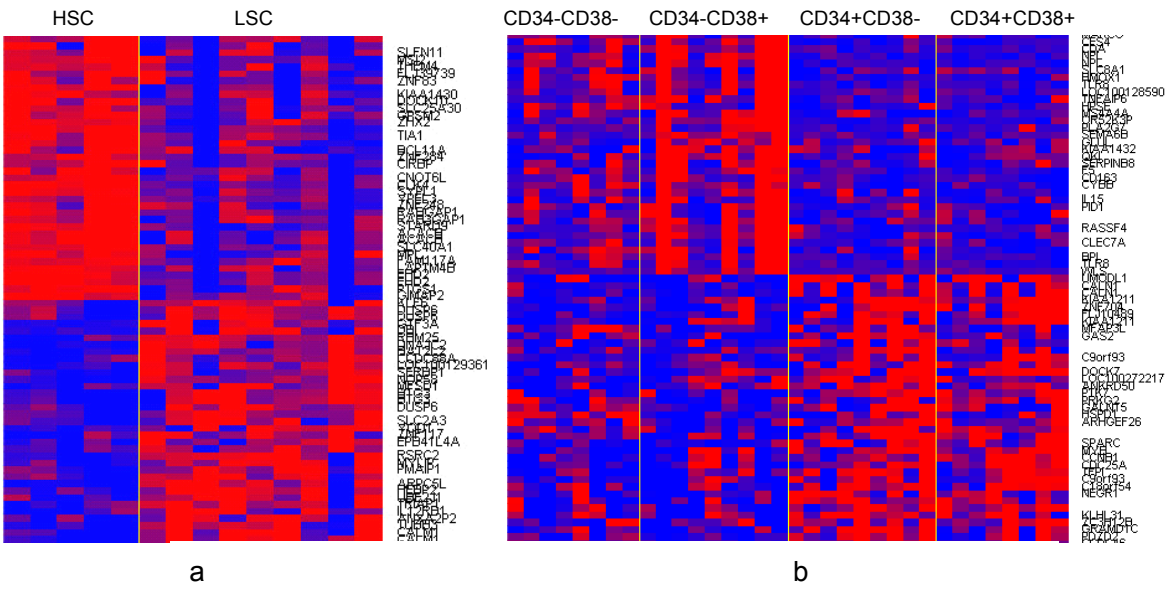


Figure 9. Results of class comparison analysis of microarray data. This figure shows part of the class comparison results of microarray data: a. HSCs (hematopoietic stem cells) *vs* LSCs (leukemia stem cells); b. four different subpopulations of AML blasts.

Table 7. Class comparison of microarray data showed that the expression of WT1 and SSX2IP was significantly higher in leukemia stem cells than in hematopoietic stem cells.

Symbol	p-value	Geom mean of intensities in **hematopoietic stem cells**	Geom mean of intensities in **leukemia stem cells**	Fold-change
WT1	0.0200826	27.65	162.16	0.17
SSX2IP	0.0383467	25.13	49.28	0.51

5. Discussion

5.1 Efficient separation of LSCs

Due to the crucial role of LSCs in sustaining and relapsing of AML, these have become a very important target for AML treatment. Since traditional chemotherapy often fails to kill LSCs, development of novel therapeutics is essential for eradication of LSCs. This study was designed to discover LAAs specifically expressed on AML LSCs, in order to identify target structure for specific immunotherapy, and in this way make LSCs vulnerable. Thus, efficient separation of LSCs from AML blasts is mandatory for this purpose.

MACS and FACS are two prevalent cell separation assays. In the MACS assay, cells are labeled with a specific antibody which is conjugated with magnetic beads. When the cell suspension runs through a strong magnetic field, positive cells stay in magnetic field because they bind magnetic beads and therefore are separated from negative cells. In the FACS assay, cells are labeled with specific antibody conjugated with fluorochrome, then applied to flow cytometer. Cells with certain fluorescence can be collected, according to operator's demand. In this study we compared these two methods as separation assay by evaluating purity and recovery rate. Because the frequency of CD34+CD38- cells in primary samples may affect separation efficiency, we chose 2 representative samples for this comparison. Patient 20 contained 0.63% of CD34+CD38- cells, which represented a low frequency. Patient 5 contained 86.1% of CD34+ CD38- cells, which represented a very high frequency. Our data showed that both methods resulted in very pure populations, from both samples. But when evaluating the recovery rate, the FACS method showed a much better recovery of cells, thus this could be a powerful tool to be employed when isolating rare populations.

We further compared other characteristics of these two methods, as shown in table 8. Other differences are: MACS can be performed using to 1 or 2

markers, resulting in the isolation of 2-3 populations, and this method is not as time consuming. On the other hand, FACS sorting can be performed with multi-parameters (depending on the number of channels in the FACS device employed). With this method up to four populations can be separated simultaneously. However, more time is needed for this method. Therefore, MACS is the best choice for separation of samples with a high cell number, with a single marker, FACS is recommended for the separation of rare populations, or several populations, or separation with multi-parameters.

Table 8. Comparison of MACS and FACS as separation assays.

Method	MACS	FACS
Markers	1 or 2 parameter	Multi-parameter
Purity	+++	+++
Recovery	+	+++
Subpopulations	2~3	Up to 4
Time consumption	+	+++

FACS sorting was then established as preferred separation method. This method then was employed for different subtypes of AML samples as well as HV samples, for BM and PBMCs and for either fresh or cryopreserved samples, with a varying size of the CD34+CD38- population and the other three populations. The subpopulations obtained were very pure, and the yield was sufficient for microarray analysis, although in some cases amplification had to be performed. Therefore, this study demonstrated that FACS sorting is a widely efficient method to separate LSCs from AML samples.

5.2 Co-stimulatory molecules on LSCs

Cellular immune response plays a major role in self-surveillance as well as clearance of malignant cells, which has been exemplified most sufficiently by

allo-HSCT and DLI in leukemia. Recently, successful employment of cellular immune response was applied to solid tumors. Sipuleucel-T is an active cellular immunotherapy, which consists of autologous PBMCs, pulsed *in vitro* with PA2024, a recombinant fusion protein consisting of a prostate antigen, prostatic acid phosphatase, and GM-CSF. Benefit from Sipuleucel-T was confirmed in patients with castration-resistant prostate cancer by a phase III randomized trial (Kantoff et al. 2010). Nevertheless, recognition of target cells and thus normal function of T lymphocytes requires the presence of both HLA and co-stimulatory molecules on the surface of target cells (Leitner et al. 2010), which are LSCs in this study. The presence of those molecules is a critical factor of immunogenicity of LSCs.

We performed multi-color FACS to investigate HLA-I, CD40, CD80 and CD86 on the surface of AML LSCs. The data show that HLA-I is expressed on all LSCs, making it possible for T lymohocytes to recognize LSCs. CD40 and CD80 were absent on LSCs in most patients but CD86 was preserved on LSCs, demonstrating that the prerequisites for antigen presentation on LSCs are still present.

Further comparison of expression of these molecules on LSCs and on leukemia blasts indicates a concordance. HLA-I was equally highly expressed while CD40 and CD80 were absent in most patients, on both LSCs and blasts. As for CD86, there seems to be some correlation between LSCs and blasts. In patients with higher CD86 ratio on LSCs, blasts also express higher CD86 ratio, which may reflect the hierarchy of AML. However, the positive rate of CD86 on LSCs was lower than on blasts, suggesting that LSCs might not be as immunogenic as blasts in AML patients. Nevertheless, the amount of samples tested in this study isn’t sufficient yet, a further project with more AML samples is being started in order to find out the if our conclusions we have reached until now can be further verified.

5.3 The use of Microarray for LAAs expression in LSCs

Microarray is an efficient assay for investigation of gene expression profile and was applied to LSCs and non LSCs subpopulations in this study. However, as LSCs is a rare population in most AML patients, the concentration of RNA isolated from LSCs was often too low for microarray. Thus RNA amplification was performed with all samples in order to resolve this problem.

As shown in figure 5, RNA was first reversely transcribed into cDNA, and then amplified in the presence of a DNA/RNA chimeric primer, as well as DNA polymerase and RNase H. This system is designed to amplify mRNA up to ten thousand-fold. After amplification, all samples generated more than 3.75 μg of cDNA product, which was the minimum quantity for microarray. Furthermore, microarrays were performed successfully, demonstrating that RNA amplification is a feasible complementation for normal microarrays, especially for samples with low quantity of RNA. In this study, microarray was performed successfully with some samples consisting of as few as 10,000 cells.

Our analysis of microarray data revealed that LAAs WT1 and SSX2IP were expressed at a significantly higher level in LSCs than in HSCs. WT1 can elicit both humoral and cellular immune response and might be responsible for inducing a protective GVL effect (Rezvani et al. 2007). The overexression of SSX2IP is associated with improved survival and the underlying mechanism was presumed to be resulting in the eradication of residual disease (Guinn et al. 2009). Thus in the state of MRD, immunotherapy against these 2 LAAs could target LSCs with the sparing of HSCs. However, in this study the comparison of gene expression profile between four blasts populations didn't discover LAAs whose expression was restricted to LSCs. Novel LAAs with LSCs restricted expression have to be identified.

5.4 Conclusions and outlook

Taken together, in this study we demonstrated that FACS sorting is an efficient method for separation of LSCs from paitents with different AML subtypes. With this method, we separated LSCs (CD34+CD34-) and other three populations (CD34+CD38+, CD34-CD38- and CD34-CD38+) from AML samples, as well as the corresponding counterparts in HVs. RNA amplification resulted in generation of adequate cDNA for microarray with little nanograms of total RNA, and microarrays were performed successfully, demonstrating that the combination of FACS sorting and RNA amplification as well as subsequent microarray is a feasible tool for studying LSCs, and also other rare cell populations. Now the ayalysis of microarray data is still ongoing. The results of LAAs expression showed that the LAAs WT1 and SSX2IP were expressed in LSCs and could be the target for immunotherapy. Since those microarrays supplied abundant of information, the complete analysis is very time consuming and cumbersome. So the complete spectrum of these results will have to be shown in a further study. Additionally, we are going to detect NPM1 mutation of those AML samples and further compare gene expression profile of NPM1 mutated and NPM1 wild type AML.

On the other hand, HLA-I and co-stimulatory molecules are preserved on LSCs, demonstrating that the prerequisites for antigen presentation on LSCs are still present. In future we are going to investigate the cytotoxic effect of allogeneic or even autologous CTLs against LSCs, identify the optimal target structure for this cytotoxic effect, and finally develop a novel immunotherapy for AML.

6. Summary

BACKGROUND: Leukemia stem cells (LSCs) are likely to be the source for leukemic disease self-renewal and the cause for relapse of acute myeloid leukemia (AML) after treatment, and thus, a critical target for therapeutic options. However, traditional chemotherapy often fails to kill LSCs. As a promising therapeutic option, immunotherapy based on T lymphocytes might eliminate LSCs.

AIM: Since LSCs may account for relapse and thus affect prognosis of AML, this study were designed to investigate the immunogenicity of LSCs and the feasibility of targeting LSCs by specific CTLs in the setting of AML. Therefore the first aim was to establish an efficient method to separate LSCs from AML samples and to separate LSCs. The immune response between CTLs and LSCs require the presence of LAAs, HLA-I and co-stimulatory molecules, thus in this study we tested HLA-I and co-stimulatory molecules expression on LSCs and investigated expression of LAAs in LSCs.

MATERIALS AND METHODS: LSCs were separated from peripheral blood mononuclear cells (PBMC) from AML patients using fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) according to their unique immunophenotype: CD34+CD38-. Efficiency of the two methods was compared to establish a preferred separation assay. Normal HSCs which are also CD34+CD38- were separated from healthy volunteers (HVs) as a control group. Meanwhile, the other three populations (CD34+CD38+, CD34-CD38+ and CD34-CD38-) were also separated from both AML and HV samples. MicroArrays were performed with GeneChip Human Genome U133 Plus 2.0 from Affymetrix to compare the gene expression of relevant immunological parameters, such as LAAs in these cell populations. Multicolcor FACS was performed to detect the expression of HLA-I and co-stimulatory molecules on LSCs.

RESULTS: By comparing purity and yield, we proved that FACS separation was more efficient than MACS. We separated CD34+CD38- cells and the other three populations from peripheral blood of 10 AML patients and 5 HVs with FACS. For the 10 AML samples, the ratio of the CD34+CD38- cells ranged from 0.63% to 86.2%; 1-5×10^7 PBMC were used for separation. After sorting, the purity of all populations including LSCs was more than 90% while 0.2-36×10^5 LSCs were obtained. HLA-I was expressed and CD86 was preserved on LSCs, however, expression of CD40 and CD80 was deficient. Microarray showed that the LAAs WT1 and SSX2IP were present in LSCs and their expression was significantly higher than in HSCs, thus they could be used as the target for immunotherapy against LSCs.

CONCLUSION: FACS sorting is a widely efficient method to separate LSCs from patients with different subtypes of AML. HLA-I and co-stimulatory molecules are detectable on LSCs. Microarrays were performed with good quality in different populations, including LSCs which is difficult to prepare due to rare materials. Microarray data showed that LAAs are expressed in LSCs. Therefore, AML LSCs is a feasible target of CTLs based immunotherapy.

Zusammenfassung

HINTERGRUND: Leukämie-Stammzellen (LSCs) sind die Quelle für die Selbsterneuerung der leukämischen Erkrankung und vermutlich die Ursache für ein Rezidiv bei Patienten mit einer akuten myeloischen Leukämie (AML) nach einer intensiven Chemotherapie. LSCs sind deshalb ein interessantes Ziel für therapeutische Ansätze. Die konventionelle intensive Chemotherapie kann LSCs oft nicht vollständig eradizieren, da einige dieser chemoresistent sind und als minimale Resterkrankung verbleiben. Immuntherapeutische Ansätze, die LSCs eliminieren können, wären somit eine vielversprechende therapeutische Option.

ZIELSETZUNG: In meiner Arbeit wurde die potentielle Immunogenität von LSCs untersucht und die Möglichkeit LSCs durch spezifische CD8-positive cytotoxische T-Lymphozyten (CTLs) anzugreifen. Voraussetzung hierzu ist, eine effiziente Methode zur Isolierung von LSCs aus AML Proben zu etablieren. Die Immunreaktion zwischen CTLs und LSCs erfordert die Expression von HLA-I und kostimulatorischen Molekülen. Deshalb wurde die Expression dieser Moleküle auf LSCs untersucht und auch die Expression von LAAs (leukämie-assoziierte Antigene) auf LSCs, da diese potentielle immunogene Targetstrukturen für eine spezifische Immuntherapie darstellen.

MATERIAL UND METHODEN: LSCs wurden aus peripheren mononukleären Blutzellen (PBMC) von AML Patienten mittels Durchflusszytometrie (FACS) und magnetischer Zellsortierung (MACS) entsprechend ihres spezifischen Immunphänotyps (CD34+ CD38-) isoliert. Die Effizienz der beiden Methoden wurde verglichen. Hämatopoetische Stammzellen (HSCs) von Probanden wurden als Kontrollgruppe isoliert und mit den CD34+CD38-, CD34+CD38+, CD34-CD38+ und CD34-CD38- Leukämiezellen der AML Patienten verglichen. Microarrays wurden mit dem GeneChip Human Genome U133 Plus 2.0 von Affymetrix durchgeführt, um die Genexpression relevanter immunologischer

Parameter wie LAAS in diesen Zellpopulationen zu vergleichen. Mehrfarben-FACS Messungen wurden durchgeführt, um die Expression von HLA-I und ko-stimulatorischen Molekülen auf LSCs zu untersuchen

ERGEBNISSE: Die Daten der verschiedenen Isolationsverfahren wurden in Bezug aus Reinheit und Ausbeute verglichen. Die FACS-Methode stellte sich als eindeutig bessere Technik heraus. LSCs wurden via FACS aus dem peripheren Blut von 10 Patienten mit AML und von 5 Probanden isoliert. Bei den 10 AML Proben lag das Verhältnis der CD34+CD38- Zellen zwischen 0,63% bis 86,2%, für die Isolierung wurden 1-5 x 10^7 PBMC verwendet. Nach dem Sortieren erhöhte sich die Reinheit dieser AML-Proben auf mehr als 90%, wobei die absolute Zellzahl bei 0,2 bis 36×10^5 Zellen lag. HLA-I ist auf LSCs exprimiert, ebenso CD86, hingegen CD40 und CD80 Expression nicht nachweisbar war. Die Expressionsanalysen mittels Microarrays zeigten, dass die LAAs WT1 und SSX2IP in LSCs exprimiert werden und das Signal signifikant höher ist als bei HSCs. Diese LAAs könnten somit gute Zielstrukturen für eine spezifische Immuntherapie sein.

FAZIT: Das FACS-Sorting ist eine effiziente Methode zur Isolierung von LSCs bei Patienten mit unterschiedlichen Subtypen der AML. HLA-I und ko-stimulatorische Moleküle sind auf LSCs nachweisbar als Vorraussetzung für eine spezifische Immuntherapie. Microarrays konnten von allen präparierten Zellpopulationen durchgeführt werden, auch von den zum Teil durchaus seltenen LSCs. Die Microarray-Daten zeigen, dass LAAs im LSC exprimiert werden. Daher sind LSCs potentielle Zielstrukturen für eine spezifische zelluläre Immuntherapie.

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