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# Characterization of dendritic cells generated from acute myeloid leukemia blasts as a potential cancer vaccine

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# Abbreviations

| | |
|---|---|
| AML | acute myeloid leukemia |
| APCs | antigen presenting cells |
| BAGE | B melanoma antigen |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt |
| bp | base pairs |
| °C | temperature in centigrade |
| CA9/G250 | carboanhydrase 9 |
| CEBPA | CCAAT/enhancer binding protein alpha |
| CD | cluster of differentiation |
| cDNA | complementary deoxyribonucleic acid |
| CEA | carcinoembryonic antigen |
| CML | chronic myeloid leukemia |
| CMV | cytomegalovirus |
| CTL | cytotoxic T lymphocyte |
| DCs | dendritic cells |
| DLI | donor lymphocyte infusion |
| DNA | deoxyribonucleic acid |

| | |
|---|---|
| dNTP | deoxynucleoside triphosphate |
| DW | distilled Water |
| EBV | epstein-barr-virus |
| EDTA | ethylenediamine tetraacetic acid |
| ELISPOT | enzyme-linked immunosorbent spot |
| FACS | fluorescence-activated cell sorting |
| g | gram |
| g | acceleration of gravity |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GVHD | graft versus host disease |
| Gy | Gray |
| HCV | hepatitis C virus |
| HER | human epidermal growth factor receptor |
| HCl | hydrogen chloride |
| HLA | human leukocyte antigens |
| HSJ2 | heat shock protein |
| hTERT | human telomerase catalytic subunit |
| HV | healthy volunteer |
| IFNγ | interferon gamma |
| Ig | immunoglobulin |

| | |
|---|---|
| IL | interleukin |
| IMP | influenza matrix protein |
| kDa | kilo Dalton |
| mAb | monoclonal antibody |
| MAZ | myc-associated zinc-finger protein |
| LAA | leukemia associated antigen |
| $MgCl_2$ | magnesium chloride |
| µg | microgram |
| MHC | major histocompatibility complex |
| µl | microliter |
| MLPC | mixed lymphocyte peptide culture |
| µM | micromolar |
| ml | milliliter |
| MLPC | mixed lymphocyte peptide culture |
| min | minute |
| mM | millimolar |
| MPP11/MIDA | M-phase phosphoprotein 11 |
| MUC/EMA | epithelial membrane antigen |
| NaCl | sodium chloride |
| NBT | nitroblue tetrazolium |

| | |
|---|---|
| NK | natural killer |
| NY-Ren60 | renal cell cancer antigen |
| OFAiLRP | oncofetal antigen immature laminin-receptor protein |
| PBMC | peripheral blood mononuclear cells |
| PCR | polymerase chain reaction |
| PINCH | particularly interesting new Cys-His protein |
| PRAME | preferentially expressed antigen in melanoma |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| RHAMM | receptor for hyaluronic acid mediated motility |
| RT-PCR | reverse transcription-polymerase chain reaction |
| RNA | ribonucleic acid |
| SEREX | serological analysis of recombinant cDNA expression libraries |
| SDS | sodium dodecyl sulfate |
| sec | second |
| TAA | tumor associated antigen |
| TAP | transporter associated with antigen processing |
| TAE | Tris acetate EDTA buffer |

| | |
|---|---|
| TBP | TATA-Box binding protein |
| Taq | thermophilus aquaticus |
| TBE | Tris borate EDTA buffer |
| TCR | T cell receptor |
| TNF | tumor necrosis factor |
| Tris | Tris (hydroxymethyl) aminomethane |
| WT-1 | Wilms’ tumor antigen 1 |

# 1 Introduction

## 1.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a hematological disease which is characterized by the clonal proliferation of undifferentiated myeloid progenitor cells. Most of the patients with AML achieve a complete hematological remission by chemo-therapeutical regimes. However, the long time prognosis for all AML patients is rather poor with a 5 year overall survival of only 20-25% depending on the individual risk profile and the chosen treatment option (Stockerl-Goldstein et al. 1999).

AML represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and overproliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts. While the specific cause for this biological abnormality in any individual patient is usually unknown, the burgeoning understanding of the genetic underpinnings of leukemia is beginning to lead to a wide array of so-called targeted therapies, many of which are in clinical development.

### 1.1.1 Cytogenetic analysis

Cytogenetic analysis provides some of the strongest prognostic information available, predicting the outcome of both remission induction and postremission therapy (Slovak et al. 2000). Cytogenetic abnormalities that indicate a good prognosis include t(8;21), inv(16), and t(15;17). Normal cytogenetics are found in average-risk AMLs. Patients with an AML that is characterized by deletions of the long arms or monosomies of chromosomes 5 or 7, by translocations or inversions of chromosome 3, by t(6;9) or t(9;22), or by abnormalities of chromosome 11q23

have a particularly poor prognosis with chemotherapy. These cytogenetic subgroups predict clinical outcome in elderly patients with AML as well as in younger patients (Grimwade et al. 2001). The fusion genes formed in t(8;21) and inv(16) can be detected by reverse transcriptase–polymerase chain reaction (RT–PCR), which will indicate the presence of these genetic alterations in some patients in whom standard cytogenetics was technically inadequate. Recently, It was reported that mutant CCAAT/enhancer binding protein alpha (CEBPA) analysed by RT-PCR predicts favorable prognosis and that this prognostic factor may improve risk stratification in AML patients with normal cytogenetics (Fröhling S et al. 2004).

### 1.1.2 Classification of AML

The classification of AML has been revised by a group of pathologists and clinicians under the auspices of the World Health Organization (WHO; Brunning et al. 2001). While elements of the French-American-British classification have been retained (i.e. morphology, immunophenotype, cytogenetics and clinical features), the WHO classification incorporates more recent discoveries regarding the genetics and clinical features of AML in an attempt to define entities that are biologically homogeneous and that have prognostic and therapeutic relevance (Brunning et al. 2001, Bennett et al. 1976, Cheson et al. 1990). Each criterion has prognostic and treatment implications but, for practical purposes, anti-leukemic therapy is similar for all subtypes except from M3.

FAB classification of acute myeloid leukemia

**M0** Acute myeloid leukemia with minimal evidence of myeloid differentiation
**M1** Acute myeloblastic leukemia without maturation
**M2** Acute myeloblastic leukemia with maturation

**M3** Acute promyelocytic leukemia (APL)

**M4** Acute myelomonocytic leukemia

**M5** Acute monocytic/monoblastic leukemia

**M6** Acute erythroleukemia

**M7** Acute megakaryoblastic leukemia

These terms describe the exact type of cell that is being overproduced and the stage of development (maturation) of the leukemia cells.

The blood and bone marrow samples will be checked by hematologists and pathologists to find out which type of leukemia it is. They will look to see exactly which type of cell has become leukemic and at which stage of their development. The cells may also be tested with antibodies for specific protein on the surface.

### 1.1.3 Novel therapeutical agents for AML

The increased understanding of the pathophysiology of AML has led to the development of a host of new so-called targeted therapies. The success of imatinib in the treatment of chronic myeloid leukemia (CML) and other diseases pathophysiologically based on the constitutive activation of a tyrosine kinase that is inhibited by the drug has spurred the search for similarly effective agents in AML. Table 1 lists some of the more recent therapies according to their proposed mechanism of action (Stone et al. 2004). Whether any of these will prove to alter the natural history of AML when used either alone or in combination with each other or with standard chemotherapy remains to be elucidated. That there are so many therapies in development is certainly positive; the challenge posed by this surfeit in a relatively rare disease for which fairly effective therapy already exists remains daunting. Immunological therapies in AML are based on the

enhancement of the host immunity or elegant strategies of making AML cells "visible" to the immune system

**Table 1. Categories of novel therapies for acute myeloid leukemia (AML).**

| | |
|---|---|
| Drug-resistance modifiers | (e.g. cyclosporine A) |
| Proteasome inhibitors | (e.g. PS－341) |
| Pro-apoptotic approaches | (e.g. G3139, a BCL-2 antisense oligo-nucleotide) |
| Signal transduction inhibitors | |
| "RAS"-targeted | (e.g., farnesyl transferase inhibitors) |
| Tyrosine kinase targeted | (e.g., FLT3 inhibitors, PKC-412, MLN-518) |
| Downstream signal inhibitors | |
| Immunotherapeutic approaches | |
| Antigens known | |
| anti-CD33 | (e.g. gemtuzumab ozogamicin , Mylotarg) |
| anti-CD45 | |
| Antigens unknown | |
| stimulation of the immune system (IL-2, GM-CSF) | |
| effective presentation of tumor antigens by administration of dendritic cells | |
| transfer of hematopoietic growth factor genes | |

## 1.2 Dendritic cells (DCs)

Dendritic cells are the most professional antigen presenting cells with the unique property to prime naive T cells (Sallusto et al. 1994). Hence, DCs are considered to be important elements also in the induction of specific anti-tumor immune responses (Banchereau et al. 2000, Banchereau et al. 2001, Sallusto et al. 1994). By harnessing the capacity of DCs to present tumor antigens to T cells, DCs may serve as the centerpiece of an immunotherapeutic approach to cancer. The ability of the immune system to recognize and attack tumors has been demonstrated unequivocally. While both humoral and cellular effector mechanisms can contribute to tumor lysis, the latter are felt to be responsible for tumor regression in the majority of cases. CD8+ cytotoxic T lymphocytes (CTL), in particular, have been demonstrated to recognize and kill cancer cells in various tumor models. The ability of DCs to prime T cells capable of recognizing and killing tumor cells in an antigen-specific fashion has also been demonstrated in various animal models (Fong et al. 2000). Moreover, DCs-based immunization can lead to immunologic memory with protection against subsequent tumor challenges (Mayordomo et al. 1995).

Several mechanisms are known to be used by leukemic cells to escape to the immune reaction: (a) the low expression of costimulatory or adhesion molecules (Hirano et al. 1996 , Notter et al. 2001); (b) Fas ligand expression responsible for activated T-lymphocyte apoptosis (Buzyn et al. 1999); and (c) secretion of cytokines inhibiting the development of efficient immune responses (Buggins et al. 1999). Thus manipulations of the immune system might represent an additional treatment option because recent observations indicate the crucial anti-tumor role of T lymphocytes in tumor diseases (Fong et al. 2000). DCs are currently promising adjuvants for cancer immunotherapy because they are the most

efficient APCs, and they induce both primary and secondary immune responses (Banchereau et al. 1998). DCs are located at the interface of potential pathogen entry sites as immature DCs and take up antigens. After antigen uptake, they move into secondary lymphoid organs, mature, and activate both helper and cytotoxic T cells (Banchereau et al. 2000). The first pilot clinical trials showed the feasibility of DCs administration in vivo and the objective clinical responses induced by tumor antigen-loaded DCs (Hsu et al. 1996, Nestle et al. 1998, Kugler et al. 2000, Reichardt et al. 1999). Several systems of tumor antigen delivery to DCs have been used, including defined peptides of known sequences (Mayordomo et al. 1997), retroviral or adenoviral vectors (Song et al. 1997, Specht et al. 1997), tumor cell-derived RNA (Boczkowski et al. 2000), and fusion of DCs with tumor cells.

Recently several groups showed that DCs can be generated from the leukemic blasts of patients with AML or CML (Cignetti et al. 1999, Kohler et al. 2000). These leukemic-derived DCs were shown in some cases to have a potent capacity to induce T cell proliferation, while still retaining the leukemic chromosomal abnormality of the original blasts. These observations constitute a unique model where the antigen-presenting cell (APCs) necessary for tumor antigen presentation and the tumor cells themselves correspond to the same cell. Therefore, DCs generated from AML patients can facilitate an immune response that might help in the induction of effective antileukemic T cells responses. Hence, DCs are considered to be important elements also in the induction of specific anti-tumor immune responses (Banchereau et al. 2000, Banchereau et al. 2001, Sallusto et al. 1994). Up to now, myeloid leukemias (AML/CML) and acute lymphoblastic leukemia (ALL) are the only malignancies in which DCs can be generated directly from the cancer cells (Choudhury et al. 1997, Choudhury et al. 1998, Choudhury BA et al. 1999, Cignetti et al. 1999).

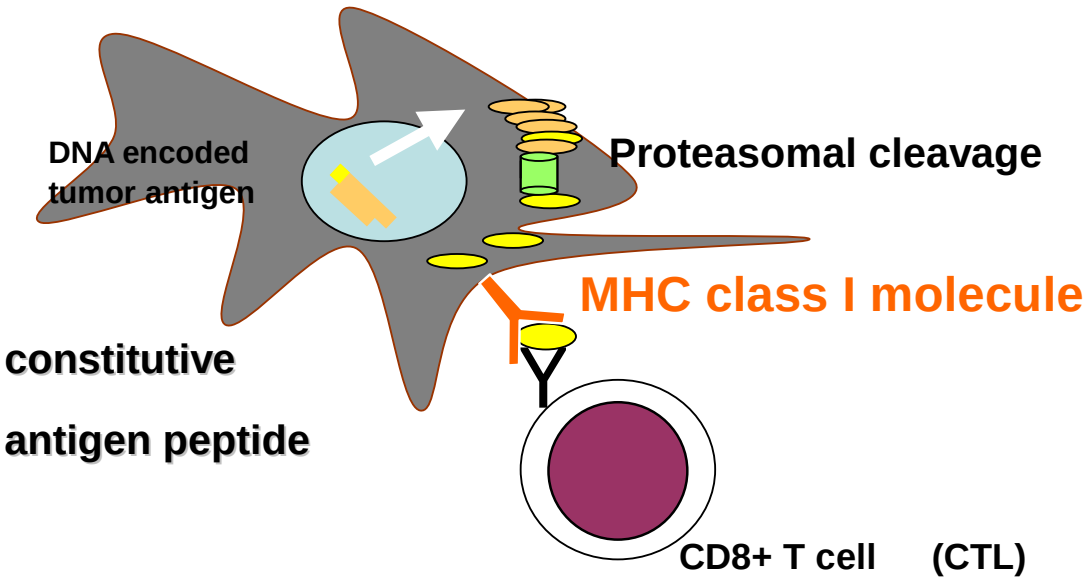



**Figure 1. MHC class I processing pathway in DCs and T cell recognition.** The DNA of a given TAA is translated into a protein. In the proteasome this particular antigen protein is cleaved into peptides which are processed in a MHC class I manner, such peptides mount on a MHC class I molecular on the surface of the APC. Eventually, the antigen peptide is recognized by a specific TCR of a CD8+ CTL.

## 1.3 Tumor associated antigens (TAAs)

### 1.3.1 Definition of TAAs

Tumor associated antigens (TAAs) are molecules preferentially expressed on tumor cells. These antigens are proteins that could serve as distinctive molecular markers of the disease as well as possible specific targets for treatment. Monoclonal antibodies directed against these structures and presensitized T cell

clones could act selectively against tumor cells. The anti-leukemic effect obtained by graft vs. leukemia (GvL) reaction after allogeneic stem cell transplantation or after by donor lymphocyte infusions (DLIs) suggests the existence of immunogenic antigens in leukemias (Marks et al. 2002, Ritgen et al. 2004).

### 1.3.2 Classification of TAAs

TAAs can be classified into five categories.

1) Cancer/testis (CT) antigens, such as PRAME (Ikeda et al. 1997), MAGE (Bruggen et al. 1991), GAGE (Eynde et al. 1995), NY-ESO-1 (Jäger et al. 1998), and SSX-1/-2 (Türeci et al. 1998) are members of a family of tumor genes expressed exclusively in cancer and in testis cells. The TAAs PRAME (preferentially expressed antigen of melanoma) also is expressed at a very low level in some other tissues such as the adrenals, endometrium, and ovary (Ikeda et al. 1997). These antigens are considered to be good candidate antigens to future immunotherapies because of their expression on tumor cells and on normal germ cells but not in other normal somatic tissues.

2) The other type of TAAs is represented by antigens that are expressed in normal tissues but overexpressed in tumor cells. The use of these TAAs as targets is not clear because of possible autoimmune reactions. There are suggestions that a log difference of expression of TAAs on normal cells versus tumor cells should be required for safe immunotherapy. Overexpressed genes are known to elicit

immune responses by overriding thresholds critical for the maintenance of tolerance (Viola et al. 1996). HER-2/neu and carbonic anhydrase 9 (CA-9/G250) belong to this group (Brossart et al. 1998, Slamon et al. 1998).

3) Antigens encoded by mutated genes, e.g. bcr-abl in CML is a result of the t(9;22) translocation and appears in the presence of the so called Philadelphia chromosome. This gene rearrangement results in the production of a novel oncoprotein, bcr/abl, a constitutively active tyrosine kinase.

4) Differentiation antigens show lineage-specific expression in tumors, e.g. tyrosinase is antigenic in melanomas, but it also is expressed in melanocytes (Wölfel et al. 1994).

5) Viruses are important co-factors in causing some malignancies. Viral genome are detected in tumour cells and code viral antigens. In principle these antigens are attractive targets for immunotherapeutic attack because T cells directed against these stucters should not be effectively removed from the T cell repertoire by central tolerance-inducing mechanisms. The success of therapy directed at Epstein-Barr virus (EBV) or Cytomegalovirus (CMV) antigens in transplant patients suggests that this type of response could be of clinical relevance (Rooney et al. 2001).

**Table 2. Types of tumor associated antigens.**

(after Benoit van den Eynde and Pierre van der Bruggen – www.cancerimmunity.org )

| Cancer-Testis Cancer/Germline Antigens | Overexpressed Antigens | Mutated Antigens | Differentiational Antigens | Viral Antigens |
|---|---|---|---|---|
| MAGE | MAZ | MUC1 | tyrosinase | HPV |
| BAGE | PRAME | HLA-A2 | PSA | EBV |
| NY-ESO 1 | Survivin | Bcr-abl | | HCV |
| SSX2 | proteinase 3 | p53 | | |
| | NewRen60 | | | |
| | WT1 | | | |
| | G250 | | | |
| | Survivin | | | |
| | hTERT | | | |
| | PINCH | | | |
| | HSJ2 | | | |
| | Her/Neu | | | |
| | CEA | | | |

### 1.3.3 Serological analysis of autologous tumor antigens by recombinant cDNA expression cloning (SEREX)

A powerful method to seach for new immunogenic antigens is SEREX, the serological analysis of autologous tumor antigens by recombinant cDNA expression cloning. It is based on a weak but detectable humoral immune

response against tumor cells in patients with malignancies. Briefly, the clones from a cDNA expression library reactive with patients' sera are selected. DNA is isolated and positive clones are sequenced. To identify the antigens, gene databases are screened for homologies. At the end, epidemiologiacal data i.e. the frequency of antigen expression in patients versus HVs are collected. The SEREX technology is schematically depicted in Figure 2.

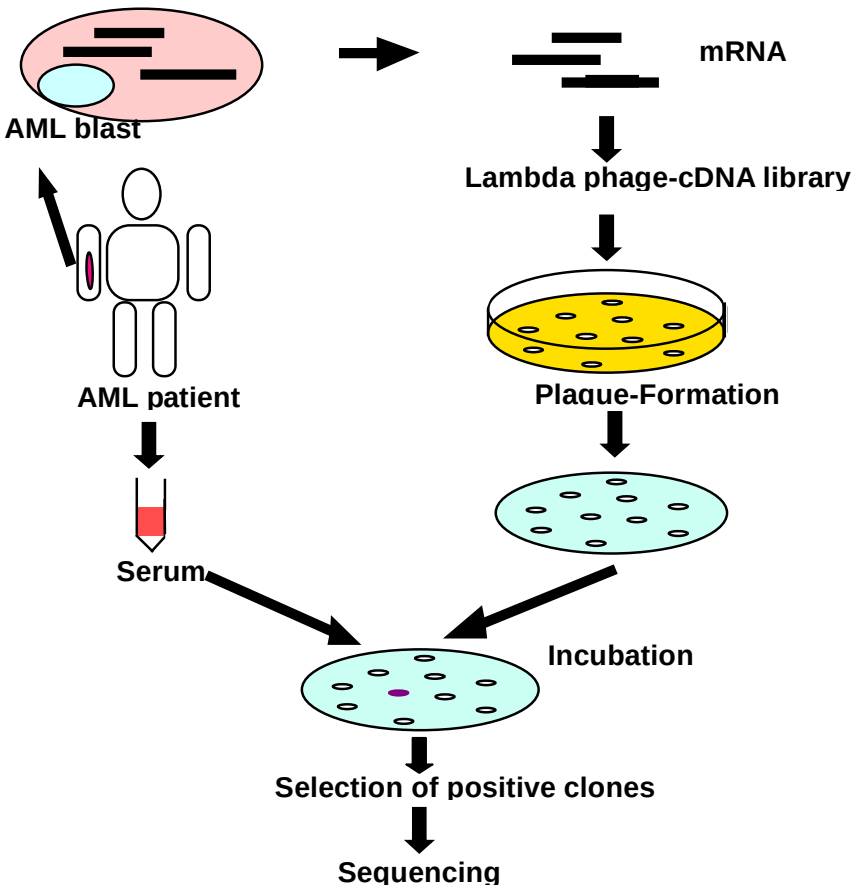



**Figure 2. Serological expression cloning (SEREX) approach for the definition human tumor antigens.**

## 1.4 The aim of this study

Specific immunotherapies with LAAs might be an option to enhance an anti-leukemic effect after chemotherapy and or hematopoeitic stem cell transplantation. Dendritic cells (DCs) are the most professional antigen presenting cells of the immune system. AML is one of the few malignancies where DCs can be generated directly from the tumor cell (AML-DCs), thus maintaining the expression of TAAs/LAAs. For the efficient presentation of such TAAs/LAAs, the expression of HLA and the costimulatory molecules CD40, CD80 and CD86 would be essential.

To evaluate the immunogenic potential of such AML-DCs for the stimulation of a LAAs specific T-cell response, we investigated in this study both their immunophenotype in terms of antigen presenting HLA molecules and costimulatory molecules by flow cytometry (Li et al. 2003) and their mRNA expression of LAAs by quantitative PCR. Immunocytochemistry for RHAMM was performed as well as chromium-51 release assays as T cell assays for a PRAME derived epitope peptide.

# 2 Material and Methods

## 2.1 Cell samples

Peripheral blood samples were taken from eight untreated patients with newly diagnosed AML and from seven patients at the time of relapse when patients were screened for an experimental therapy with autologous DCs. Informed consent to the use of their blood for scientific purposes was obtained from AML patients who were treated at our institution according to clinical study protocols approved by the local Ethics committee. Control samples were prepared from fifteen healthy blood donors at our institution after their informed consent was obtained. Table 1 displays the characteristics of the AML patients.

**Table 3. Patients' characteristics.**

| | AML (FAB) | Age (yrs) | Sex | WBC (x10^9) | Blasts (%) | Karyotype |
|---|---|---|---|---|---|---|
| 1 | M0 | 70 | m | 2.6 | 60 | 46, X0 |
| 2 | M1 | 63 | f | 4 | 50 | t (8;21) |
| 3 | Sec AML | 64 | m | 1.6 | 80 | normal |
| 4 | M2 | 85 | f | 26.8 | 52 | Inv (16) |
| 5 | M1 | 49 | m | 251 | 99 | Add(19)(p13) |
| 6 | Sec AML | 60 | m | 9.5 | 70 | normal |
| 7 | M5a | 45 | f | 150 | 90 | normal |
| 8 | M3 | 44 | f | 113 | 97 | 46, XY del(20)(q11);<br>46, XY del (20)(q11) add(22)(p11);<br>47, XY del (20)(q11), +21 |
| 9 | Sec AML | 75 | m | 6.4 | 80 | normal |
| 10 | M0 | 68 | f | 153 | 99 | 46, XY add8p |
| 11 | M2 | 68 | m | 4 | 60 | normal |
| 12 | M4 | 51 | m | 23 | 52 | normal |
| 13 | M4 | 64 | f | 84 | 80 | normal |
| 14 | M5b | 54 | f | 113 | 92 | normal |
| 15 | M5 | 49 | m | 86 | 89 | normal |

Sec. AML = secondary AML

## 2.2 Generation of DCs

Generation of DCs from peripheral blood cells was performed as described previously (Choudhury et al. 1997, Choudhury et al. 1998, Choudhury BA et al. 1999). Briefly, peripheral blood mononuclear cells (PBMC) were isolated using Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation of EDTA (Delta-Pharma, Pfullingen, Germany) anticoagulated blood buffy coat preparations from healthy volunteers or from EDTA anticoagulated blood from AML patients. PBMC were seeded ($1\times10^6$ cells/$cm^2$) into culture flasks (Nunc, Roslild, Denmark) in RPMI1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2% human AB serum (German Red Cross Blood Center, Ulm, Germany), 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. After 2 h of incubation at 37°C, non-adherent cells were removed and the adherent CD14+ blood monocytes (purity >80% by FACS analysis) were cultured in RPMI1640 medium supplemented with 100 ng/ml human GM-CSF (Leukomax, Novartis, Basel Switzerland), 1,000 IU/ml interleukin-4 (Strathmann, Hannover, Germany) and 10% human AB serum. For maturation of the cells, medium change was performed on day 6. The new medium contained GM-CSF and IL-4 in the concentrations mentioned above and additionally 50 ng/ml TNF-$\alpha$ (Strathmann, Hannover, Germany). The DCs cultures were fed with fresh medium and cytokines every three days and cell differentiation was monitored using inverse light microscopy. The expression of cell surface molecules on the DCs was analyzed using flow cytometry after eight days of culture.

## 2.3 Cell viability and morphologic studies

The viability of the DCs harvested after eight days of culture was assessed by trypan blue staining using a Neubauer counting chamber, and the morphology of

the cells was analyzed by light and scanning electron microscopy. For light microscopy, DC suspensions were spinned down and stained with May-Gruenewald solution (Merck, Darmstadt, Germany). For scanning electron microscopy, standard protocols were followed. In brief, samples were fixed 1:1 with 5% glutaraldehyde in 0.2 M phosphate buffer and 2% sucrose. 200 µl of the fixed cell suspension containing $10^5$ cells were transferred to poly-lysine coated slides, sedimented for 1 hr in a humidified chamber, washed with PBS and slides were cut to the appropriate size. Dehydration with a propanol series of increasing concentrations (30%, 50%, 70%, 90%, 100%) was followed by a critical point drying. Samples were coated with gold/palladium particles of 20 nm diameter. Visualization was performed using a scanning electron microscope DSM 962 (Zeiss, Oberkochen, Germany) at 15 kV.

## 2.4 Reverse transcriptase polymerase chain reaction (RT-PCR) for tumor/leukemia-associated antigens (TAAs/LAAs)

Based on our previous studies on TAAs/LAAs (Greiner et al. 2000, Greiner et al. 2002, Greiner et al. 2003), we tested both AML blasts and AML-DCs for their mRNA expression of the three TAAs/LAAs PRAME, RHAMM/CD168 and WT-1 by RT-PCR as described.

Briefly, polyA+ mRNA was isolated directly from 1x $10^7$ PBMC using the µMACS mRNA Isolation Kit (Miltenyi, Bergisch Gladbach, Germany). 200 ng of each sample was subjected to cDNA synthesis (1st strand cDNA synthesis kit for RT-PCR, Roche). The sequences of the primers for RT-PCR were as follows: TBP: forward: 5' TTAACTTCGCTTCCGCTGGC 3', reverse: 5' GAAACCCTTGCGCTGGAACT 3'; (accession number: NM_003194.2). PRAME: forward: 5' GTC CTG AGG CCA GCC TAA GT 3', reverse: 5' GGA GAG GAG

GAG TCT ACG CA 3' (accession number: NM_006115). RHAMM/CD168: forward: 5' CAG GTC ACC CAA AGG AGT CTC G 3', reverse: 5' CAA GCT CAT CCA GTG TTT GC 3' (accession number: NM_012484.1). WT-1: forward: 5' ATG AGG ATC CCA TGG GCC AGC A 3', reverse: 5' CCT GGG ACA CTG AAC GGT CCC CGA 3' (accession number: NM_000378.2).

Temperatures of denaturation, annealing and elongation, the cycle number and the $MgCl_2$ concentration were as follows: PRAME and WT-1: 94°C, 64°C, 72°C; RHAMM/CD168 and TBP: 95°C, 60°C, 72°C; 35 cycles and 1.5 mM $MgCl_2$ for all PCR reactions.

### 2.5 Flow cytometry analysis

Harvested cells were washed in FACS medium (PBS containing 1% BSA) and stained at 4°C for 20 min by antibodies directly conjugated with FITC or PE. Thereafter cells were washed three times with PBS and analyzed by FACScan (Becton Dickinson, Heidelberg, Germany) using the CellQuest software (Becton Dickinson). Antibodies were the following: FITC–labeled anti-mouse IgG, anti-human HLA-DR, CD33, CD40, CD45 and CD83, as well as PE–labeled anti-mouse IgG, anti-human HLA-ABC, CD1a, CD11c, CD34, CD54, CD 80 and CD86 (Becton Dickinson). Double staining was performed using pairs of PE- and FITC-labeled antibodies. Results were obtained in the dot plot format and transferred from the MAC to the PC format.

### 2.6 Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for TAAs/LAAs

qRT-PCR was carried out using standard amplification conditions. The

LightCycler FastStart DNA SYBR Green I Kit (Roche Diagnostics Mannheim, Germany) was used according to the manufacturer's protocol. To quantify the mRNA expression of PRAME, RHAMM/CD168, WT-1 and proteinase 3, standard curves were established for RHAMM/CD168, WT-1 and proteinase 3 by a serial dilution of the products from conventional RT-PCR. A PRAME standard curve was established by a serial dilution of the pcDNA3.1-PRAME vector. The expression of PRAME, RHAMM/CD168 and WT-1 was evaluated against the standard, and in a second step it was correlated with the mRNA expression of the housekeeping gene TATA-box binding protein (TBP). Primers were designed to produce transcript specific bands for LAAs. PRAME: forward: GGA AGG TGC CTG TGA TGA AT, reverse: AAG GTG GGT AGC TTC CAG GT (accession number: NM_006115). RHAMM/CD168: forward: 5´ GCT GAA AGG CTG GTC AAG CAA 3´, reverse: 5´ TTC CTG CAA AAG CAG GGT GG 3´ (accession number: NM_012484.1). WT-1: forward: 5' ATG AGG ATC CCA TGG GCC AGC A 3', reverse: 5' CCT GGG ACA CTG AAC GGT CCC CGA 3' (accession number: NM_000378.2). proteinase 3: forward: 5´ AAC ATT TGC ACT TTC GTC CC 3', reverse: 5´ GCG TGA AGA AGT CAG GGA AA 3' (accession number:NM_002777). TBP: forward: 5' TTA ACT TCG CTT CCG CTG GC 3', reverse: 5' GAA ACC CTT GCG CTG GAA CT 3' (accession number: NM_003194.2). PCR was performed under the following conditions: PRAME: 95°C denaturation for 10 sec, 64°C annealing for 10 sec, 72°C elongation for 20 sec, 40 cycles, 0.5 µM each primer and 2.5 mM $MgCl_2$. RHAMM/CD168: 95°C denaturation for 10 sec, 62°C annealing for 15 sec, 72°C elongation for 20 sec, 45 cycles, 0.5 µM each primer and 3 mM $MgCl_2$. WT-1: 95°C denaturation for 10 sec, 62°C annealing for 15 sec, 72°C elongation for 20 sec, 45 cycles, 0.4 µM each primer and 2.25 mM $MgCl_2$. proteinase 3: 95°C 10 sec, 60°C 10 sec, 72°C 20 sec, 40 cycles. 0.4 µM each primer and 2 mM $MgCl_2$. TBP: 95°C 10 sec, 60°C

15 sec, 72°C 20 sec, 40 cycles. 0.4 µM each primer and 2 mM $MgCl_2$.

The formula for the calculation of copy numbers is as follows:

Copy number (copies/µl) = $6\times10^{23}$ (copies/mol) × concentration (g/µl) /molecular weight (g/mol), while $6\times10^{23}$ is the Avogadro factor. The expression ratio was calculated as the quotient copy number of LAAs/copy number of TBP, TBP being the housekeeping gene for normalization.

## 2.7 Immunocytology for RHAMM /CD168

The method has been described elsewhere (Barth et al. 2002). Briefly, cytospins of PBMC from HV and DCs generated thereof (HV-DCs), as well as AML blasts and DCs generated thereof (AML-DCs) were incubated with an monoclonal anti-CD168 antibody (clone 2D6, Novocastra, Newcastle upon Tyne, UK) at a 1:100 dilution. A polyclonal biotinylated Fab anti-body to mouse IgG reactive with all mouse isotypes and a streptavidin-biotinylated peroxidase complex (all obtained from Amersham, High Wycombe, UK) served as a detection system for the primary antibody. 3-Amino-9-ethylcarbazole (Sigma, St. Louis, MO) was used as substrate for the enzyme. The cytospin preparations were faintly counterstained with Harris` hematoxylin. Negative controls were performed without primary antibody.

## 2.8 Mixed lymphocyte peptide culture (MLPC)

PBMC from a healthy volunteer negative for RHAMM/CD168 were separated by Ficoll and subsequently selected by magnetic beads through a MACS column.

CD8- antigen presenting cells (APCs) were irradiated with 30 Gy and pulsed with the PRAME derived epitope decamer peptide ALYVDSLFFL (14; synthesized at a purity >95% by ThermElectron, Ulm, Germany) at a concentration of 20 µg/ml for 2 hrs. After co-incubation with CD8+ T lymphocytes over night, the MLPC was supplemented with 10U/ml IL-2 and 20ng/ml IL-7 on day +1. On day +7, the medium was changed and new irradiated and peptide-pulsed CD8- APCs were added as one week before. Again, the MLPC was supplemented with IL-2 and IL-7. After totally 14 days of culture, T cells were harvested and evaluated for their specific cytotoxicity in a standard Cr-51 release assay against T2 cells pulsed with the PRAME peptide or without peptide, as described earlier (Schmitt et al. 1997). K562, a cell line established from a CML patient in blast crisis, was used as a control for T cell specificity as K562 expresses PRAME (Greiner et al. 2002), but not HLA class I molecules, thus being a target to natural killer (NK) cells, but not to T lymphocytes. The percentage of specific lysis was calculated as Cr-51 release in the test well minus spontaneous Cr-51 release/maximum Cr-51 release minus spontaneous Cr-51 release.

## 2.9 Statistical analysis

The statistical evaluation was performed by the statistical software “SAS-Analyst” - a tool of SAS-System V.8 (SAS-Institute, Cary, NC, USA). The expression of HLA and costimulatory molecules in healthy volunteers and in patients with AML was compared using the two-sided Wilcoxon test for parallel groups. The significance level for all comparisons was set at the common standard of 0.05.

In case the calculated error probability was below the level of 0.05, the zero hypothesis “There are no significant differences in the expression of CD antigens and HLA-molecules between the two groups” was rejected.

# 3 Results

## 3.1 Morphology of AML- and HV-DCs

After 8 days of culture as described in the material and methods section, floating and semi-adherent cells generated from AML blasts (AML-DCs) and monocytes from HV (HV-DCs) showed a typical DCs morphology with characteristic veils, a kidney-shaped nucleus and a diameter of 15 to 20 µm. AML-DCs detected by both light and scanning electron microscopy are shown in Figures 3 and 4. The scanning electron microphotograph of an AML-DCs (Figure 4) demonstrates that AML-DCs can interact with lymphocytes. Viability of AML- and HV-DCs preparations was over 95% in the trypan blue staining.

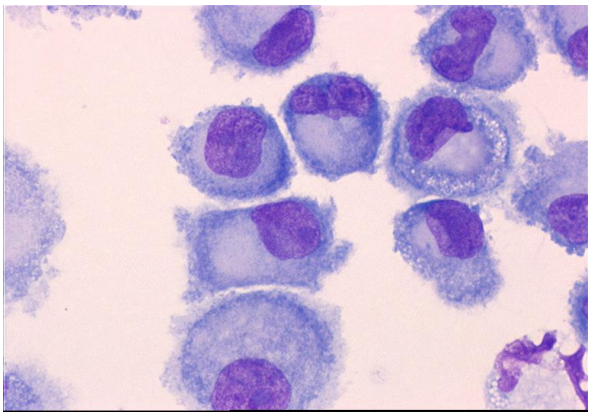


**Figure 3. Dendritic cells generated from AML blasts in light microscopy (x 400) [Li et al. 2003].**

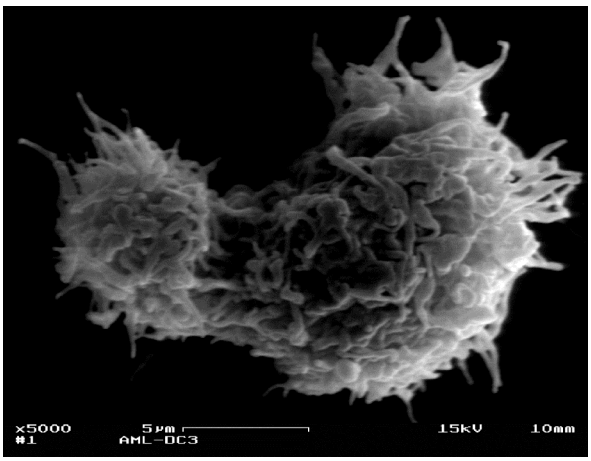



**Figure 4. A dendritic cell (right) generated from an AML blast interacting with a T cell (left) in scanning electron microscopy (x 5.000) [Li et al. 2003].**

### 3.2 RT-PCR for TAAs/LAAs

PBMC from AML patients were subjected to RT-PCR for the three LAAs PRAME, RHAMM/CD168 and WT-1. All 15 AML patients expressed at least one of these LAAs. Figure 5 shows representative results of an agarose gel for two exemplary patients and one healthy volunteer (HV). After DCs culture, also the generated DCs samples were subjected to RT-PCR. Expression of at least one of the three LAAs tested was detected in all 15 AML-DCs preparations. In the majority of the cases PCR signals for LAAs were stronger in AML-DCs than in AML blasts.

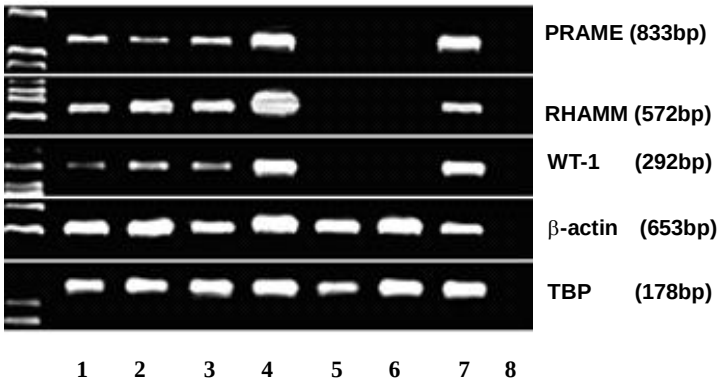



**Fig 5. RT-PCR for the leukemia-associated antigens PRAME, RHAMM/CD168 and WT-1.** PBMCs of AML patients (>80% blasts; shown for two patients on lanes 1 and 3) were evaluated for the mRNA expression of three LAAs that are not expressed in PBMCs from healthy volunteers (lane 5) against the housekeeping genes beta-actin and TATA-box binding protein (TBP). AML-DCs (lanes 2 and 4) generated from the PBMCs of the respective AML patients (lanes 1 and 3) maintained the mRNA expression of these genes, which clearly demonstrates that they are blast-derived DCs. PBMCs and DCs from healthy volunteers (lanes 5 and 6) do not express these genes. K562 (lane 7) served as a positive control and distilled water (lane 8) as a negative control [Li et al. 2003].

### 3.3 Immunophenotyping of PBMC from 15 healthy donors on day 0 and of 15 HV-DCs on day eight

Figure 6a shows representative flow cytometry results for the expression of HLA class I/II and costimulatory molecules on freshly prepared PBMC from

healthy volunteers in the monocyte gate. HLA-ABC and -DR were expressed at the highest level. The expression for CD86 was high, but very low for CD40. We failed to detect CD80 and CD83. Figure 6b demonstrates that for HV-DCs a higher expression of CD40, CD80, CD83 and CD86 could be observed on day 8. HLA-ABC and -DR expression remained unchanged at high levels. Maturation of DCs was substantiated particularly by the expression of CD83 on DCs ranging from 37% to 89% (median 79%) in contrast to 0.5-4% (median 1%) before culture. The data of the FACS analysis for PBMC before DCs culture (day 0) from 15 HV are displayed as box plot in Figure 7a. The FACS analysis (Figure 7b) after 8 days of HV-DCs culture demonstrates a higher level of CD40, CD80, CD83 and CD86 on all HV-DCs when compared with PBMC ($p < 0.0001$).

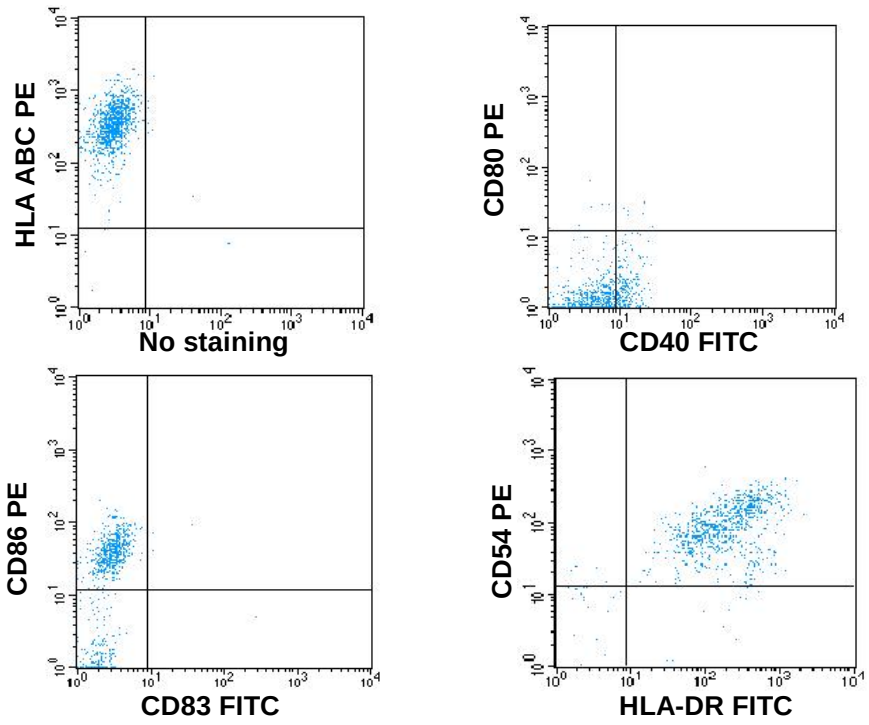



**Figure 6a. Phenotype of PBMC cells from healthy donors in the monocyte gate before culture (day 0).**

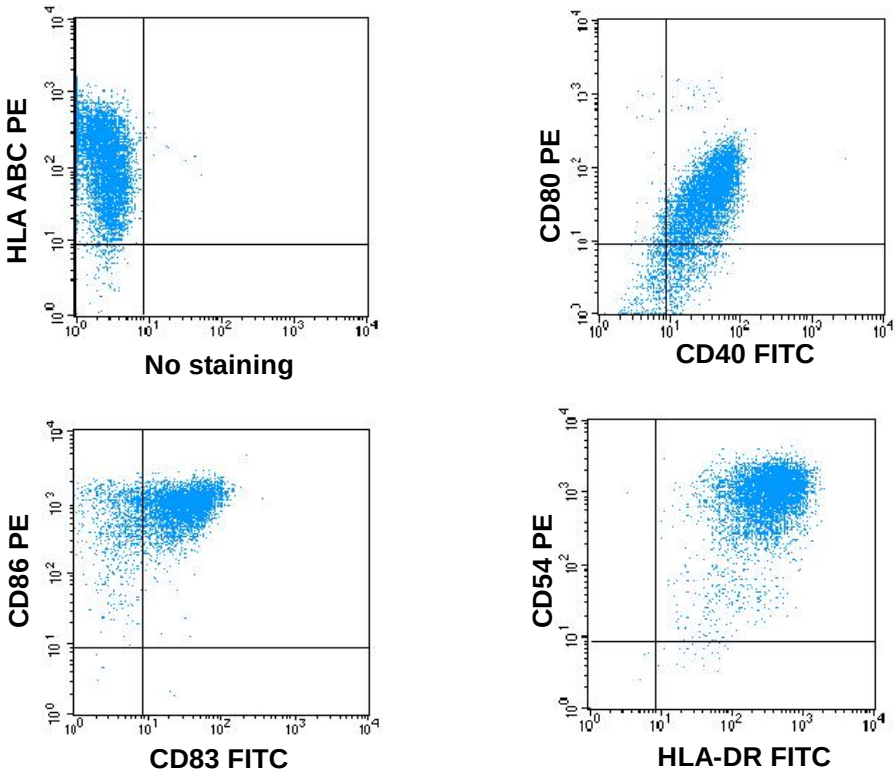



**Figure 6b. Phenotype of dendritic cells generated from monocytes of healthy donors after eight days of culture.**

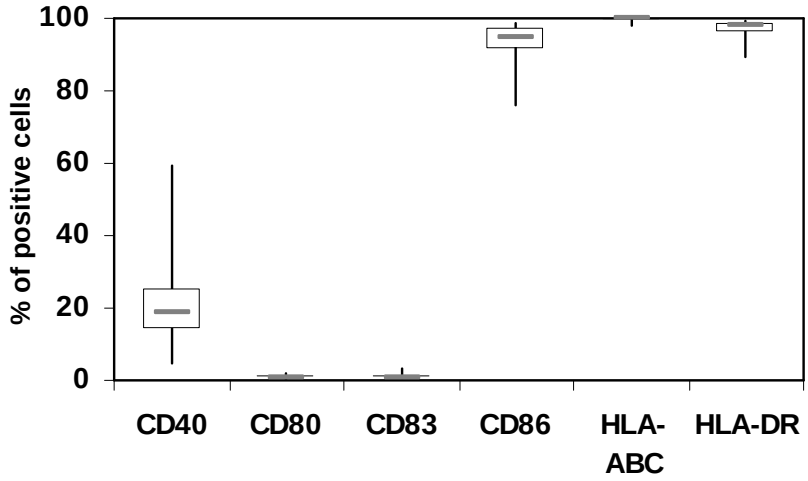



**Figure 7a. Phenotype of cells in the monocyte gate from healthy donors before culture (day 0) [Li et al. 2003].**

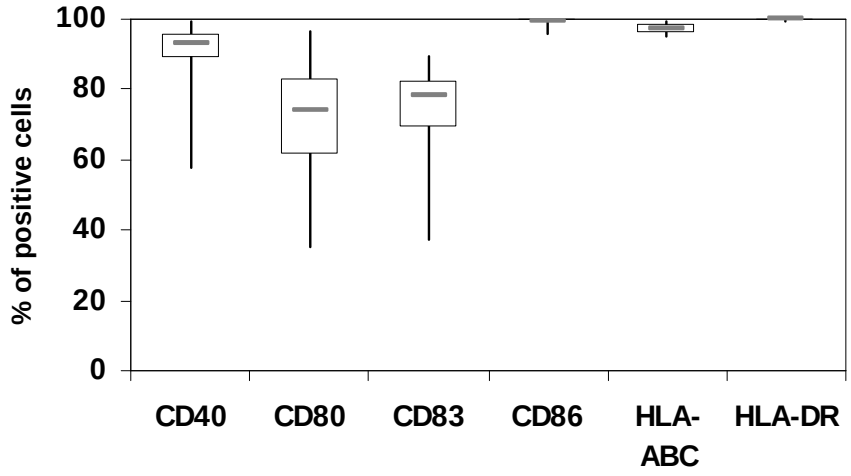



**Figure 7b. Phenotype of DCs from healthy donors after eight days of culture [Li et al. 2003].**

### 3.4 Immunophenotyping of AML blasts and AML-DCs

Figure 8a shows representative flow cytometry results for HLA class I/II and costimulatory molecules on AML blasts from patient #9 characterized by the co-expression of CD34, a marker for hematopoetic progenitor cells. HLA-ABC and -DR are present on most of the cells. The co-stimulatory molecule CD86 is high, but CD40, CD80 and CD83 are very low. Figure 8b demonstrates that HLA-ABC remained unchanged on AML-DCs at approximately 100%. The FACS analysis of CD34+ blasts from 15 AML patients is displayed in Figure 9a, and the FACS analysis of AML-DCs obtained after 8 days of culture in Figure 9b. The data demonstrate the upregulation of CD40, CD80, CD83, CD86 and HLA-DR on all AML-DCs when compared with AML blasts ($p < 0.001$ for HLA-DR and $p < 0.0001$ for all other markers).

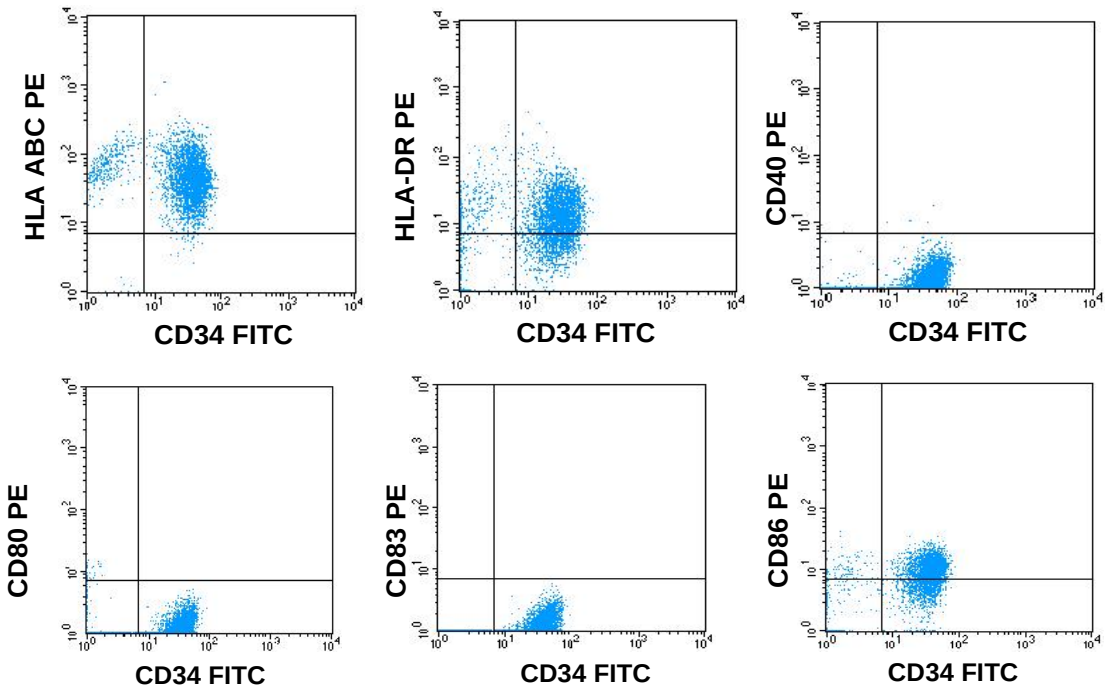



**Figure 8a. Phenotype of leukemic blasts from AML patient #9 in the monocyte gate before culture (day 0).**

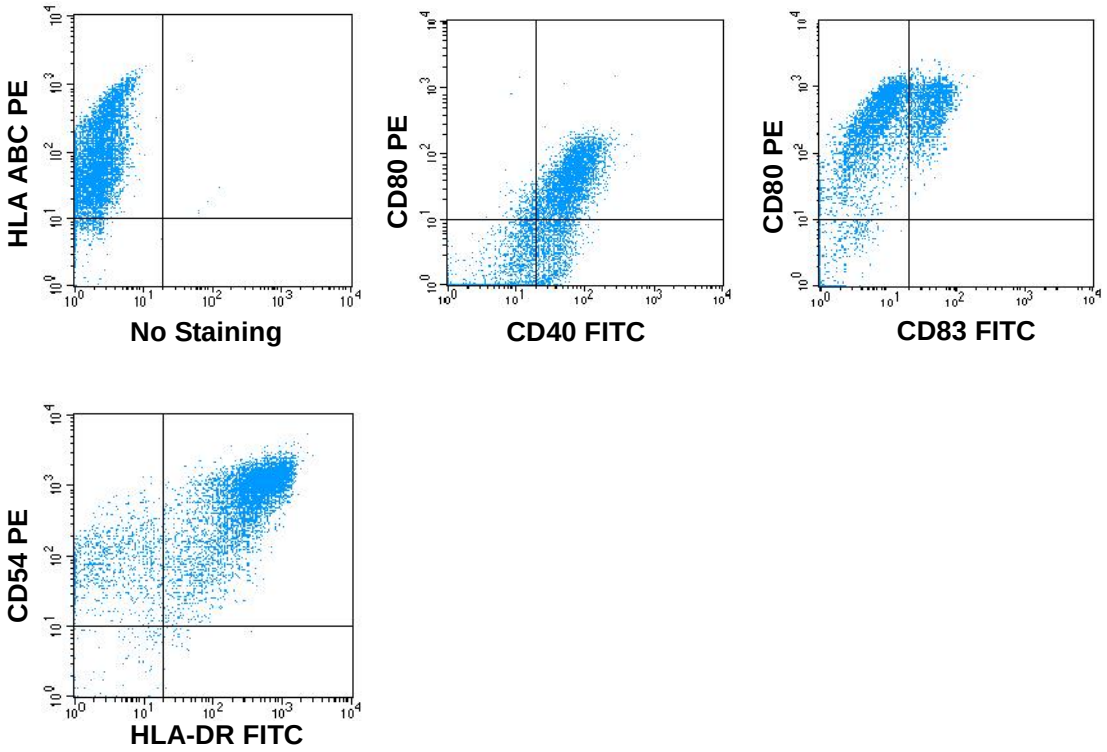



**Figure 8b. Phenotype of DCs generated from blasts of AML patient #9 after 8 days of culture.**

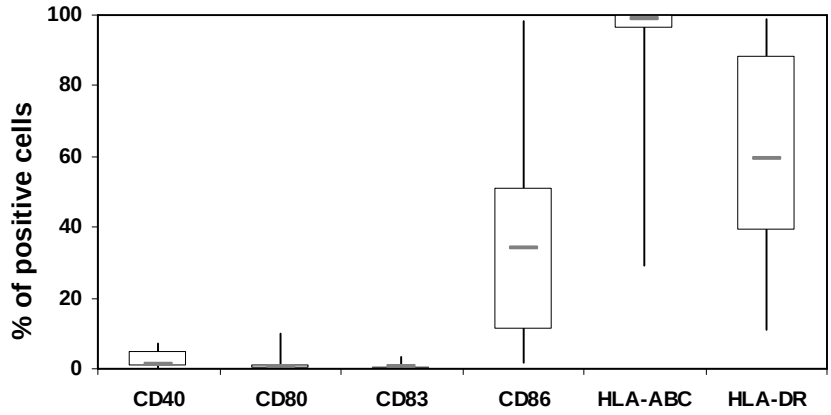



**Figure 9a. Phenotype of leukemic blasts from AML patients in the monocyte gate before DC culture (day 0) [Li et al. 2003].**

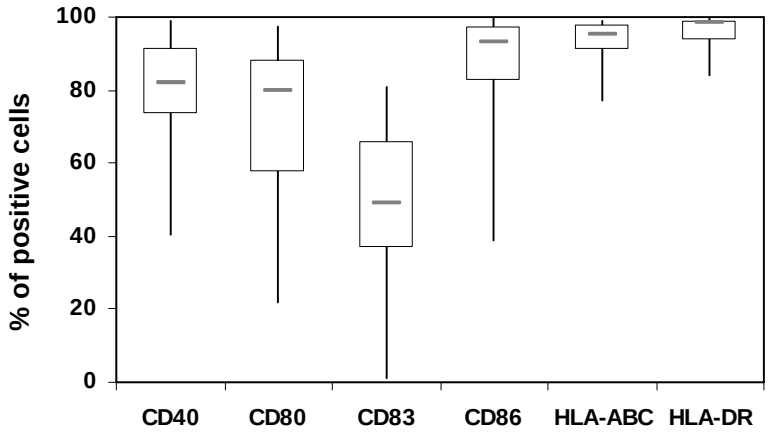



**Figure 9b. Phenotype of DC generated from AML patients after eight days of culture [Li et al. 2004].**

### 3.5 Comparison of the immunophenotype of PBMC from healthy volunteers versus primary AML blasts

To assess the difference of HV-DCs versus AML-DCs, we compared the phenotype of PBMC from 15 healthy volunteers in the monocyte gate with blasts from 15 AML patients before culture.

Figure 7a shows the immunophenotype of PBMC from 15 HV in the monocyte gate, in Figure 9a the immunophenotype of leukemic blasts from 15 AML patients. Expression of HLA-ABC on blasts was similar to that on monocytes of healthy volunteers, whereas HLA-DR and CD86 were expressed at a lower level. On monocytes from HV, CD 80 was absent; the median of the CD40 expression was 19%. No expression of CD40 and CD80 were detected in AML blasts. Expression of CD40, CD86 and HLA-DR was significantly higher on monocytes from HV than on blasts ($p<0.0001$). No significant difference was observed between PBMC and AML blasts for the expression of HLA-ABC ($p > 0.05$).

Both AML blasts and monocytes from HV tested negative for expression of CD3, CD16, CD19 and CD56 (data not shown).

### 3.6 Comparison of the immunophenotype of DCs from HV and AML patients on day eight

After 8 days of culture, both HV-DCs and AML-DCs showed the phenotypic characteristics of monocyte derived DCs with weak or absent CD14 expression (data not shown) and high levels of major histocompatibility complex (MHC) class II (HLA-DR) molecules. The data obtained by FACS analysis of 15 samples each from HV and AML patients are displayed in Figure 7b for HV, as well as in Figure 9b for AML patients. No significant differences were observed between HV-DCs and AML-DCs for the expression of HLA-ABC (median 98% in HV-DCs versus 95 % in AML-DCs), HLA-DR (median 100% in HV versus 99% in AML patients) and CD80 (median 74% in HV-DCs versus 80 % in AML-DCs) with p-values of 0.98 and 0.07 respectively. However, significant differences were observed for the expression of CD40, CD83, CD86, with p-values $< 0.05$. The presence of CD83 expression substantiates the DCs maturation.

### 3.7 qRT-PCR for PRAME, RHAMM/CD168 , WT-1 and proteinase 3

qRT-PCR protocols for the four LAAs (RAME, RHAMM/CD168, WT-1 and proteinase 3) using the Light cycler SYBR green method were optimized as described earlier for PRAME and demonstrated for RHAMM/CD168 in Figure 10. PCR conditions were chosen to obtain a clear and sharp single peak for the PCR products in the melting curve analysis presented in Figure 10. For a dilution of a conventional RHAMM/CD168 PCR product the light cycler procedure was performed an amplification for one representative analysis. Subsequently, the regression analysis of these curves was calculated as displayed in the insert of Figure 10.

Results of quantitative real-time PCR for AML-DCs versus AML blasts showed an up to one log alteration in mRNA expression of LAAs in AML-DCs. As displayed in Figure 11, a stronger PCR signal for PRAME was detected in 7/12 AML-DCs preparations, in 4/12 AML-DCs preparations the expression level was maintained and only reduced in 1/12 AML-DCs preparations. In 6/12 AML-DCs preparations a

significant upregulation of the PCR signal for RHAMM/CD168 could be observed. In 4/12 AML-DCs preparations, the RHAMM/CD168 mRNA expression was maintained and in 2/12 AML-DCs preparations it was reduced (Figure 12). A stronger PCR signal for WT-1 was observed in 2/12 AML-DCs preparation, 3/12 AML-DCs preparations maintained the level of WT-1 expression. In 7/12 cases, the WT-1 mRNA expression was downregulated in the AML-DCs (Figure 13). A stronger PCR signal for proteinase 3 was observed for only 1/12 AML-DCs preparation, 4/12 AML-DCs preparations maintained the level of proteinase 3 expression. In 7/12 cases, the proteinase 3 mRNA expression was downregulated (Figure 14). In PBMC from HV and in HV-DCs none of the four LAAs revealed to be expressed (all LAAs/TBP copy number ratios < 0.01; data not shown).

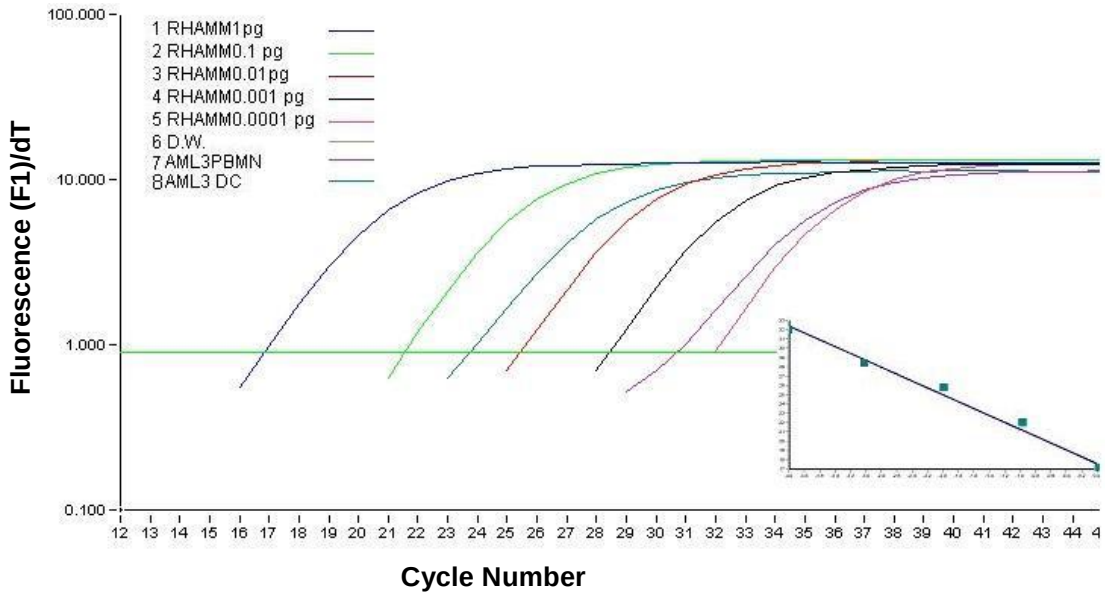



**Figure 10. Normalized amplification curves of different amounts of the antigen RHAMM/CD168 cDNA. The insert shows the regression analysis of these curves.**

### 3.8 Simultaneous expression of LAAs

Table 4 gives a summarized comparison of the LAAs expression data obtained in both AML blasts and AML-DCs. The majority of LAAs is upregulated in

most of the patients during AML-DCs generation. LAAs are upregulated (for example PRAME, RHAMM/CD168 and WT-1 in patient #1), while another LAAs is downregulated in the same patient (proteinase 3). In all patients at least one LAAs was maintained or even upregulated.

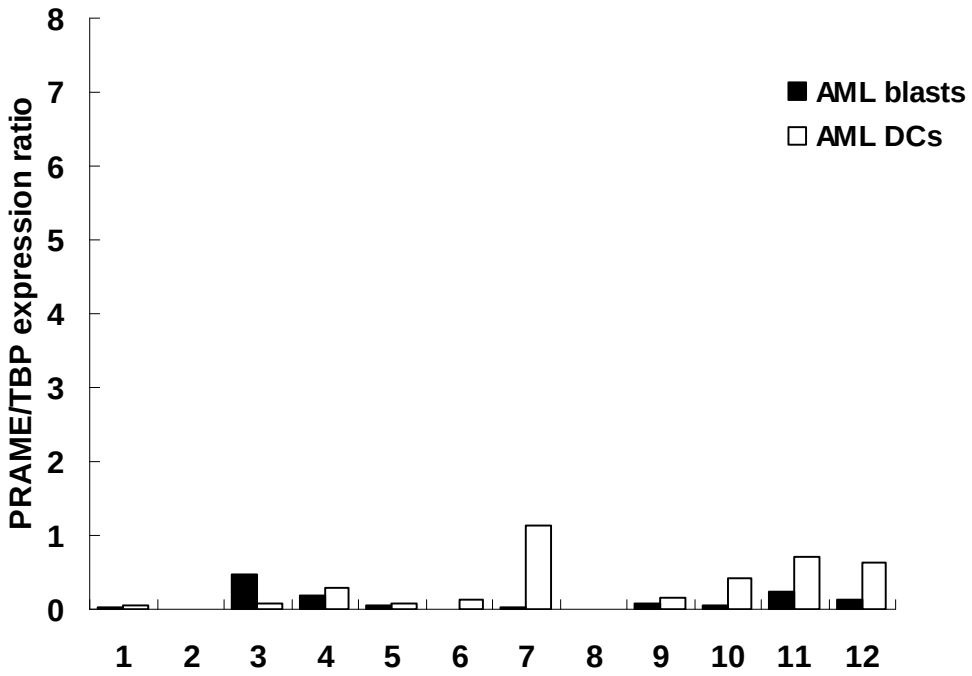



**Figure11. Quantitative real time PCR analysis of PRAME expression compared between AML blasts from 12 AML patients and AML-DCs generated thereof [Li et al. 2004].**

**Table 4. The distribution of LAAs expressed on AML-DCs compared with those on AML blasts.↑upregulation, ↓downregulation, ~maintenance of the LAAs expression; the expression ration for the respective LAAs in AML blasts is indicated in brackets below the respective symbol.**

| Patient No. | PRAME | RHAMM/CD168 | WT-1 | proteinase 3 |
|---|---|---|---|---|
| 1 | ↑ (0.020) | ↑ (0.938) | ↑ (0.950) | ↓ (0.069) |
| 2 | ~ (0.006) | ↑ (0.012) | ~ (0.267) | ↑ (0.043) |
| 3 | ↓ (0.483) | ↑ (0.000) | ↓ (7.990) | ~ (0.006) |
| 4 | ~ (0.176) | ~ (2.400) | ~ (3.550) | ↓ (0.675) |
| 5 | ~ (0.044) | ~ (0.961) | ↓ (2.190) | ~ (0.030) |
| 6 | ↑ (0.006) | ↑ (0.438) | ↓ (2.010) | ↓ (0.163) |
| 7 | ↑ (0.020) | ↓ (3.260) | ↓ (2.210) | ↓ (0.483) |
| 8 | ~ (0.009) | ~ (1.460) | ↓ (0.267) | ↓ (1.720) |
| 9 | ↑ (0.070) | ↑ (0.143) | ↑ (0.776) | ~ (0.021) |
| 10 | ↑ (0.059) | ↓ (3.960) | ↓ (3.960) | ↓ (0.745) |
| 11 | ↑ (0.231) | ↑ (1.400) | ~ (3.870) | ↓ (3.210) |
| 12 | ↑ (0.128) | ~ (0.395) | ↓ (0.600) | ~ (0.138) |

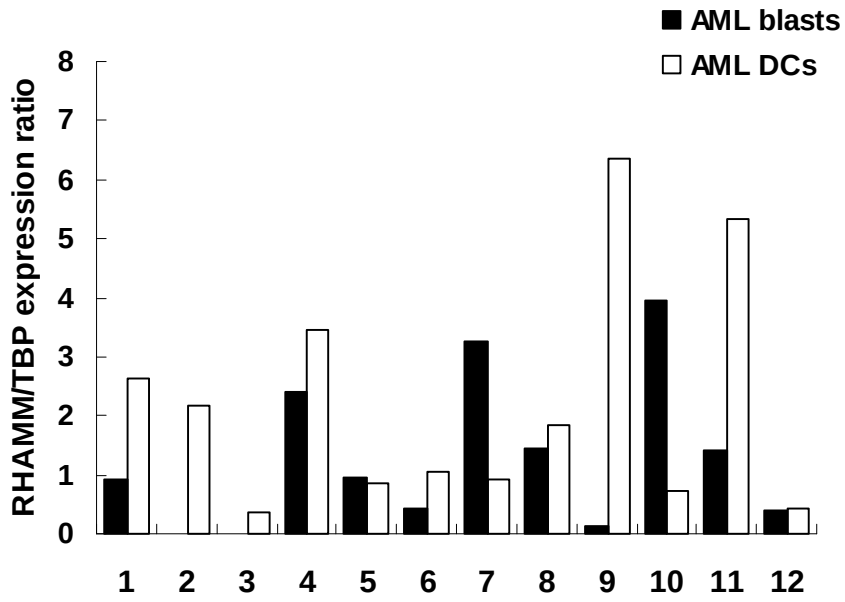



**Figure 12. Quantitative real time PCR analysis of RHAMM expression compared between AML blasts from 12 AML patients and AML-DCs generated thereof [Li et al. 2004].**

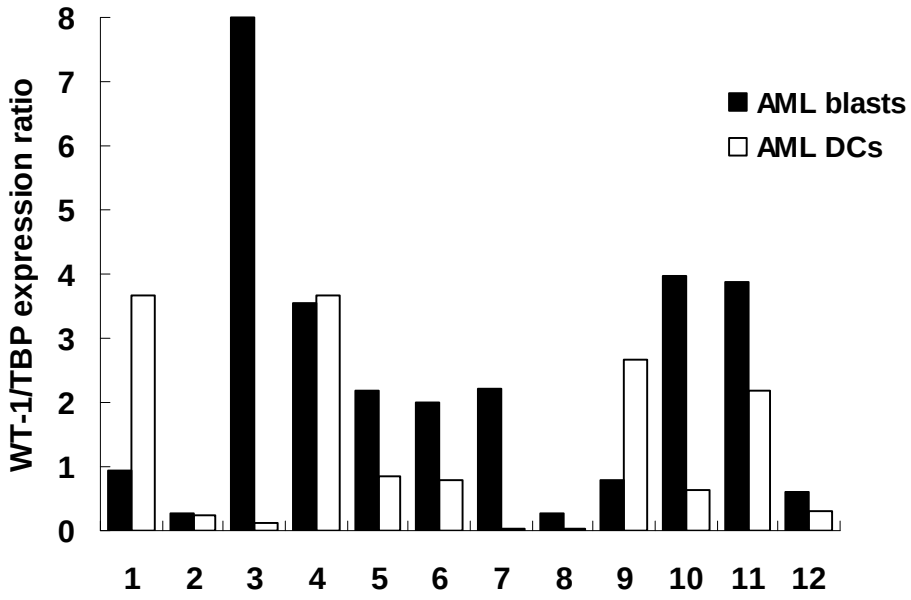



**Figure 13. Quantitative real time PCR analysis of WT-1 expression compared between blasts from 12 AML patients and AML-DCs generated thereof [Li et al. 2004].**

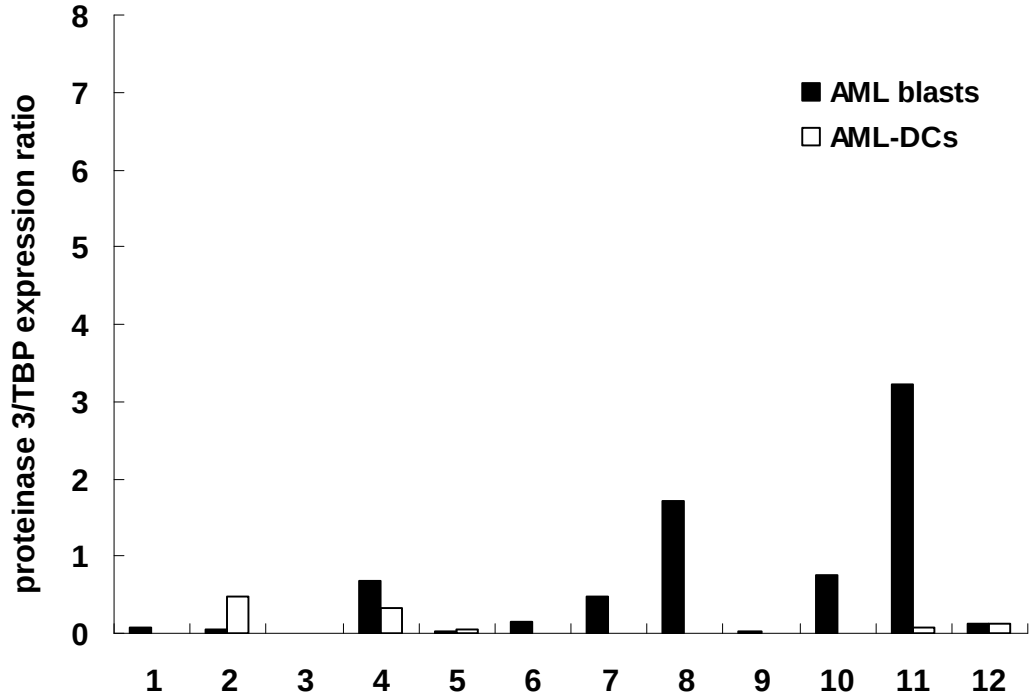



**Figure 14. Quantitative real time PCR analysis of proteinase 3 expression compared between blasts from 12 AML patients and AML-DCs generated thereof [Li et al. 2004].**

### 3.9 Immunocytology for RHAMM/CD168

AML blasts staining positive for RHAMM/CD168 were detected in the Ficoll preparation of the peripheral blood from AML patients (Figure 15; left). AML blasts identified by morphology showed staining positive for RHAMM/CD168 at different levels of intensity maybe reflecting a different stage of maturation or a different stage of cell cycle. Furthermore, we detected CD168 positive DCs generated from AML blasts (Figure 15; right). False positive reactions due to intrinsic peroxidase activity was excluded by negative controls without primary antibody. PBMC from HV and DCs generated thereof tested negative for RHAMM/CD168 (data not shown).

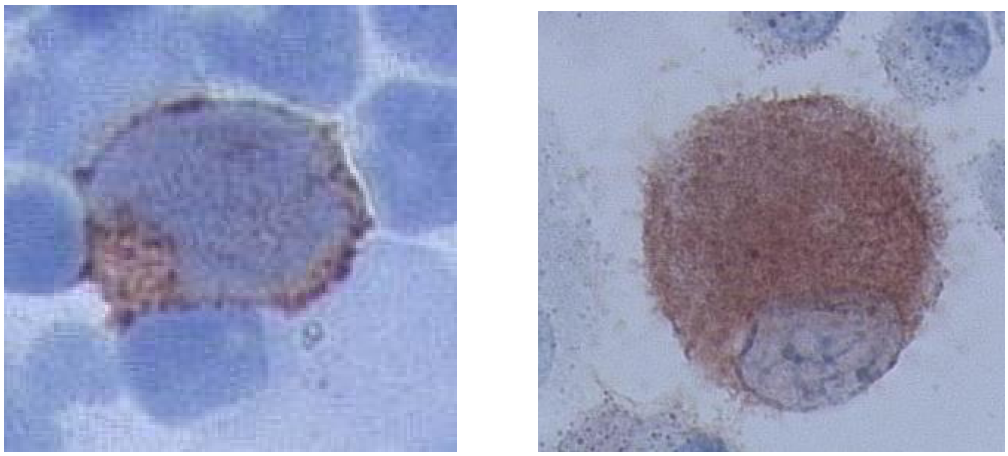


**Figure 15: Immunocytological staining for RHAMM/CD168:** a RHAMM/CD168 positive blast from the peripheral blood (PB) of AML patient No.4 (see Table 3, page 13; left) and an AML-DC (right) generated from this PB sample are positive for RHAMM/CD168 (original magnification x400) [Li et al. 2004].

### 3.10 MLPC

CD8+ T cells presensitized with the PRAME derived peptide P3 were able to recognize AML blasts testing positive for this LAAs in quantitative RT-PCR assays. AML-DCs generated thereof were recognized at a higher lytic level by the P3 primed T lymphocytes (Figure 16).

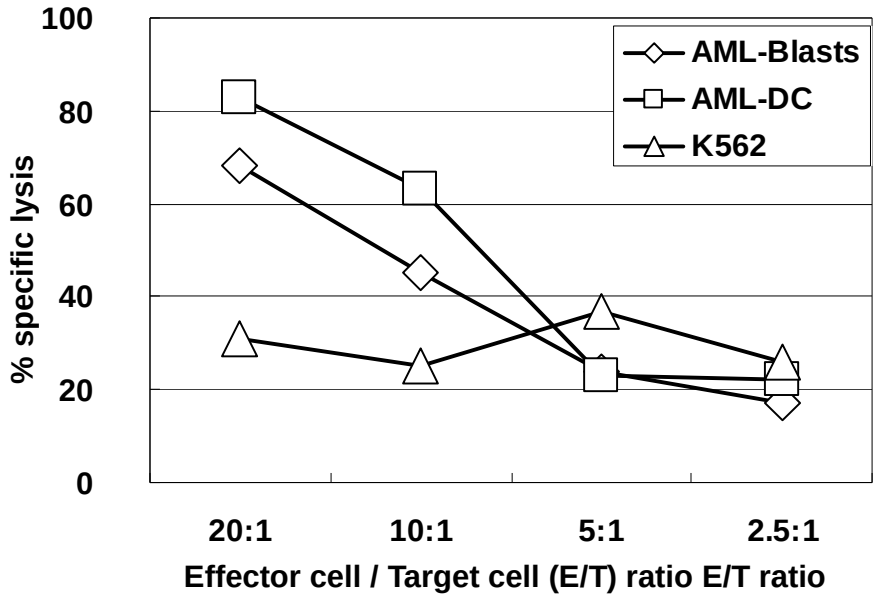



**Figure 16: Cr-51 release assay for the recognition of AML blasts and AML-DCs from patient No.11 (see Table.3, page 13) by T cells presensitized by the PRAME derived peptide P3 [Li et al. 2004].**

# 4 Discussion

As the most professional antigen presenting cells, DCs play a major role in the immune system (Banchereau et al. 2000, Grammer et al. 2000). Clinical trials using autologous DCs loaded with tumor cell lysate, antigen peptides or total RNA extracted from tumor cells are on-going for patients with melanoma, renal cell cancer or prostate carcinoma (Fong et al. 2000). New hope for immunotherapies with DCs emerged from phase I/II trials in which immunological and even clinical responses could be observed (Murphy et al. 1999, Thurner et al. 1999). AML-DCs might express constitutive LAAs such as PRAME (Ikeda et al. 1997), RHAMM/CD168 (Greiner et al. 2002) or WT-1 (Oka et al. 2000, Thurner et al. 1999, Maurer et al. 1997) which have been demonstrated to elicit specific T cell responses subsequently resulting in the lysis of leukemic blasts. Therefore autologous AML-DCs might be used as a cancer vaccine for leukemia patients either for maintenance therapy or in the case of relapse for the *ex vivo* generation of specific donor lymphocyte infusions.

In this study we compared the expression of HLA class I and II molecules and the costimulatory molecules CD40, CD80, CD86 on leukemic blasts and DCs from AML patients with PBMC and DCs from healthy volunteers. Our findings in this and in an earlier study (Vollmer et al. 2003) demonstrate a deficient expression of costimulatory molecules in leukemic blasts which might hamper sufficient stimulation of T cell responses. The expression of CD40 is considered to be crucial for T cell activation and expansion (Mazouz et al. 2002), so especially the absence of CD40 on blasts might be responsible for the insufficient recognition of blasts by the immune system of the AML patient. Moreover, the expression of CD80 on blasts was almost absent. Enhancing the expression of CD80 on leukemia blasts increases their costimulatory activity for autologous T cells (Notter et al. 2001). For both HLA and all costimulatory molecules on AML-DCs a strong upregulation was detected after 8 days of DCs culture, therefore providing the prerequisites for eliciting T cell responses.

The comparison of AML-DCs with HV-DCs demonstrated a similar expression of

HLA-ABC, -DR and CD80, but lower expression of CD40 and CD86 on AML-DCs. This indicates that AML-DCs are more potent than AML blasts to elicit T cell responses. However, HV-DCs might be even more efficient through the higher expression of costimulatory molecules. On the other hand, HV-DCs lack the expression of LAAs which would make procedures such as LAAs peptide pulsing or LAAs-RNA transfection necessary for immunotherapy. The upregulation of HLA and costimulatory molecules on AML-DCs makes them valuable professional antigen presenting cells for the stimulation of specific CTL responses to LAAs presented on the surface of leukemic blasts. Therefore, we initiated at our institution a phase I/II clinical trial using autologous DCs applied intradermally for the treatment of elderly patients with AML in the palliative setting. The first patient showed a 100% increase of CTL recognizing leukemic blasts after three vaccinations with autologous DCs, but did not show a clinical response (Reinhardt et al. 2002)

Autologous AML-DCs generated from AML blasts might be used as a vaccine potentially eliciting LAAs-specific T cell responses. To design such experiments and clinical trials, the origin of DCs from leukemic blasts and the maintenance of LAAs expression has to be demonstrated. Choudhury and co-workers showed earlier (Frohling et al. 2002) that DCs originated from AML blasts by FISH analysis of cytogenetic aberrations. However, FISH analysis does not constitute an appropriate method to demonstrate the origin of AML-DCs in clinical practice. Firstly, only about 50% of AML patients (not only of those included in the present study) show chromosomal aberrations (Neumann et al. 1998). Moreover, at least $10^7$ DCs must be generated for FISH analysis which requires up to $10^9$ PBMC from AML patients only for this pre-diagnostic procedure, a number which opposes clinical feasibility. Immunophenotyping of AML blasts and AML-DCs is not appropriate to prove the origin of AML-DCs either, e.g. the stem cell marker CD34 is expressed only in 10-20% of AML patients and vanishes as a result of maturation during the eight days of DCs culture. To ensure a rapid method for the evaluation of LAAs expression in AML-DCs with only $10^5$ cells we established RT-PCR for testing LAAs in AML (Li et al. 2003). In previous studies (Greiner et al.

2002, Li et al. 2003, Mailander et al. 2004), we detected the exquisite expression of the LAAs (PRAME, RHAMM/CD168 and WT-1) on AML blasts in contrast to PBMC from healthy volunteers. In our present RT-PCR assays, at least one of these three LAAs/TAAs was maintained in each of the 15 AML-DCs preparations. In the majority of the cases, a stronger PCR signal was observed after eight days of DCs culture. This result might be explained by a stronger expression of the LAAs/TAAs by the AML-DCs when compared with the AML blasts, or rather by a higher percentage of the blast derived DCs in the whole AML-DCs preparation when compared with the blast percentage in the original AML sample.

Ikeda et al. reported on PRAME as a melanoma associated tumor antigen recognized CTL in a HLA-A24- restricted manner (Ikeda et al. 1997). Expression of the gene PRAME was described in melanoma, sarcoma, lung squamous cell carcinoma, renal cell carcinoma (Ikeda et al. 1997, Kessler et al. 2001, Neumann et al. 1998), and in 35% of AML patients (Matsushita et al. 2003), whereas no expression was found in PBMC and bone marrow cells from healthy volunteers (Greiner et al. 2003, Baren et al. 1998). In this study PRAME was expressed at a rather low level in AML blasts while compared with the housekeeping gene TBP. However, a remarkable upregulation of the gene was detected in 7/12 AML-DCs, suggesting that these cells might be a useful tool to elicit T cell responses to PRAME which were otherwise difficult to obtain (Jan Kessler/ Cees Melief, Leiden University Medical Center, NL, 2001 and personal communication). This finding was confirmed by our data demonstrating a recognition of PRAME expressing AML blasts and AML-DCs by P3-primed CD8+ lymphocytes.

RHAMM/CD168, also designated CD168, is a receptor for hyaluronan, a glycoaminoglycan that plays a fundamental role in cell growth, differentiation, and motility (Wang et al. 1996). CD168 is a cell-surface receptor and belongs to the group of extracellular matrix molecules (Sherman et al. 1994, Yang et al. 1993). The molecule is highly expressed in different tumor cell lines (Abetamann et al. 1996, Teder et al. 1995) in multiple myeloma (Crainie et al. 1999) and AML/ CML (Greiner et al. 2002), but not in PBMC of health volunteers (Pilarski et al. 1994). RHAMM/CD168 was upregulated in 6/12 AML-DCs. We could confirm the

RHAMM/CD168 protein expression both on the surface and in the cytoplasm of these AML-DCs by immunocytology.

Recently, we defined CD8+ T cell epitopes of RHAMM/CD168 which were recognized by CD8+ T cells from AML patients (Greiner/Li et al. 2005). A clinical vaccination trial with the R3 peptide for patients with AML has been initialed at our institution. The Wilms' tumor antigen WT-1, a differentiation gene first described in pediatric kidney tumors (Call et al. 1990), is expressed in the majority (about 80%) of AML patients, but also in CD34 positive cells of the early hematopoiesis and its expression is correlated with a poor clinical prognosis (Bergmann et al. 1997, Maurer et al.1997, Ohminami et al. 2000). Because of the expression in CD34 positive cells, which has been a matter of debate for several years (Call et al. 1990, Hosen et al. 2002, Maurer et al. 1997, Ohminami et al. 2000, Oka et al. 2000), vaccination against WT-1 might stimulate autoimmune responses.

Our quantitative mRNA expression data suggest that RHAMM/CD168 might have a similar potential as WT-1 to elicit T cell responses, at least as far as AML-DCs are used for vaccination of patients. The down-regulation of WT-1 in AML-DCs when compared with AML blasts might be related to the maturation process of DCs as WT-1 is also downregulated during the maturation of CD34+ stem cells (Maurer et al. 1997).

Proteinase 3 is a myeloid tissue-restricted 26 kDa serine protease that is abundantly expressed in azurophilic granules in normal myeloid cells, is overexpressed by two- to five-fold in some leukemia cells, and may be important for maintenance of the leukemic phenotype (Bories et al. 1989, Dengler et al. 1995). Evidence that proteinase 3 is immunogenic was recently obtained in patients with CML and AML (Molldrem et al. 2002, Molldrem et al. 2003, Molldrem et al. 2000, Scheibenbogen et al. 2002). The quantitative RT-PCR for proteinase 3 revealed an expression of the gene in 8/12 of the AML blasts when compared with the housekeeping gene TBP. This characterizes proteinase 3 as a gene with an inhomogenous mRNA expression level in our cohort of AML patients. Only in 2/12 an expression ratio bigger than 1 (compared with TBP). When AML-DCs where

generated from the AML blasts, only 1/12 samples upregulated the mRNA expression of proteinase 3. In most of the cases (7/12), proteinase 3 mRNA was dramatically downregulated. This indicates that generating AML-DCs might not constitute a method to induce effectively T cell answers against proteinase 3. It remains to be elucidated whether the loss of proteinase 3 expression is associated with the maturation process as it is the case for WT-1.

In this study, quantitative RT-PCR showed an upregulation of the LAAs PRAME and RHAMM in AML-DCs compared with AML blasts in the majority of the patients. This might be due to the maturation process of the DCs or due to the higher percentage of AML-DCs in the AML-DCs preparation when compared to the percentage of blasts in the peripheral blood of the patients. The stronger expression of LAAs by AML-DCs when compared to AML blasts make AML-DCs better antigen presenting cells (APCs) for a stronger stimulation of a specific T cell response. In addition, vaccination with AML-DCs can circumvent the problem of low expression of a certain LAAs, as AML-DCs might still express another LAAs efficiently (Table 4).

In summary, we have proven the origin of DCs from AML blasts and the maintained or even upregulated expression of LAAs in AML-DCs using a technique which requires only a small number of DCs. The results were confirmed for RHAMM/CD168 on the protein level by immunocytology. As HV-DCs did not show any expression of the LAAs tested we conclude that their expression is not related to differentiation into DCs *per se*. Taken together with the expression of HLA and costimulatory molecules (Li et al. 2003, Vollmer et al. 2003), the expression of multiple LAAs in AML-DCs is a prerequisite for the stimulation of a LAAs-specific T cell reaction as we demonstrated for the PRAME derived peptide P3 in cytotoxicity assays. LAAs expressing autologous AML-DCs might be a promising vaccine for AML patients for maintenance therapy in complete remission or as a specific immunotherapy for minimal residual disease (MRD).

For the selection of patients for clinical trials with AML-DCs, the production of antibodies against LAAs of interest for immunocytology as well as the definition of

T cell epitopes of more LAAs (e.g. RHAMM/CD168) for immuno-monitoring would be highly desirable. Such studies are ongoing in our laboratory.

## 4.1 Conclusions

In the light of the presence of HLA and costimulatory molecules as prerequisites for LAAs presentation and because of the strong LAAs expression detected here, AML-DCs might constitute a powerful tool in immunotherapy for AML patients. Real-time PCR allows a quick and quantitative assessment of LAAs expression even with a limited number of DCs.

- DCs can be generated in the majority (80%) of AML patients,
- AML-DCs provide with the expression of at least one LAAs and a strong expression of HLA and costimulatory molecules the prerequisites for eliciting T cell responses.
- qRT-PCR allows a quick and quantitative assessment of immunologically relevant LAAs expression with only $10^5$ DCs and might be helpful for the decision whether the AML-DCs vaccination strategy is favourable or not.
- RHAMM/CD168 protein expression was evaluated by immuno-cytochemistry, recognition of AML-DCs through PRAME epitope specific T cells was evaluated by chromium release assay. AML-DCs might constitute a powerful tool in immunotherapy for AML.

# 5 Summary

Acute myeloid leukemia (AML) is a clonal disease of hematopoesis with poor clinical outcome despite recent improvement of chemotherapy and stem cell transplantation regimens. Immunotherapy with dendritic cells (DCs) eliciting specific T cell responses to leukemia-associated antigens (LAAs) might be a therapeutical option. DCs should express human leukocyte antigen (HLA) class I/II molecules and the costimulatory molecules cluster of differentiation 40 (CD40), CD80 and CD86 to activate effectively T cells for the subsequent lysis of leukemic blasts. Here, the expression of these antigens was compared on DCs generated from the peripheral blood (PB) of 15 AML patients (AML-DCs) versus 15 healthy volunteers (HV-DCs) by FACS analysis. All DCs displayed the typical morphology and tested negative for B, T and NK cell markers. The origin of AML-DCs from AML blasts was proven by the maintained expression of mRNA of LAAs such as preferentially expressed antigen in melanoma (PRAME), the receptor for hyaluronic acid mediated motility (RHAMM/CD168) or Wilms tumor gene 1 (WT-1). In comparison with AML blasts, the expression of CD40, CD80, CD86 and HLA-DR was upregulated during DC culture to a median of 80% to 98% on AML-DCs. HLA-ABC was preserved on AML-DCs (median 95%). Expression of CD40, CD80 and CD83 remained lower on AML-DCs than on HV-DCs. AML-DCs provide with the expression of at least one LAAs and a strong expression of HLA and costimulatory molecules the prerequisites for eliciting specific T cell responses.

Moreover, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed for the following LAAs: PRAME, RHAMM/CD168, WT-1 and proteinase 3. RHAMM/CD168 protein expression was evaluated by immuno- cytochemistry, recognition of AML-DCs by PRAME epitope specific T cells was evaluated in a chromium release assay. qRT-PCR for AML-DCs versus AML blasts showed an alteration in mRNA expression of LAAs. An elevated PCR signal for PRAME was detected in 7/12 AML-DCs preparations. 6/12 AML-DCs preparations showed a significant upregulation of the PCR signal for

RHAMM/CD168. A stronger WT-1 and proteinase 3 signal was observed in PCR for only 2/12 respectively 1/12 AML-DCs. All preparations showed a strong expression of at least one of the LAAs examined. AML-DCs tested positive for RHAMM/CD168 protein. PRAME positive AML-DCs were recognized by specific T cells. AML-DCs might constitute a powerful tool in immunotherapy for AML patient. qRT-PCR allows a quick and quantitative assessment of immunologically relevant LAAs expression with only $10^5$ DCs and might be helpful for the decision whether in a particular patient the AML-DCs vaccination strategy is favourable or not.

## 5.1 Zusammenfassung

Die Akute Myeloische Leukämie (AML) stellt eine klonale Erkrankung des hämatopoetischen Systems dar, die trotz Verbesserung der Chemotherapie- und Stammzelltransplantations-Strategien immer noch durch eine schlechte Prognose charakterisiert ist. Eine Immuntherapie mit Dendritischen Zellen (DCs), die spezifische T-Zell-Antworten auf Leukämie-assoziierte Antigene (LAAs) evozieren können, stellt eine therapeutische Option für Patienten mit AML dar. DCs sollten Humane Leukozyten-Antigen (HLA)-Moleküle der Klassen I und II, sowie die kostimulatorischen Moleküle cluster of differentiation 40 (CD40), CD80 und CD86 exprimieren, um effektiv T-Zellen zur nachfolgenden Lyse leukämischer Blasten zu stimulieren. In dieser Arbeit wurde die Expression dieser Oberflächen-Antigene in der Durchflusszytometrie verglichen zwischen DCs, die aus dem peripheren Blut (PB) von 15 AML-Patienten (AML-DCs) versus 15 normal gesunden Probanden gezüchtet wurden (AML-HV). Alle DCs wiesen eine DC-typische Morphologie auf und waren negativ für B-, T- und Natürliche Killer (NK)-Zell-Marker. Der Ursprung der AML-DCs aus AML-Blasten wurde nachgewiesen durch die aufrechterhaltene Expression von mRNA für diese LAAs wie preferentially expressed antigen in melanoma (PRAME), receptor for hyaluronic acid mediated motility/CD168 (RHAMM/CD168) und Wilms-Tumor -Gen 1 (WT-1). Im Vergleich mit AML-Blasten wurde die Expression von CD40, CD80, CD86 und HLA-DR im Verlauf der DC-Kultur auf einen Median von 80% bis 98% auf AML-DCs aufreguliert. HLA-ABC blieb auf AML-DCs erhalten (Median 95%). Die Expression von CD40, CD80 und CD83 wurde von AML-DCs niedriger exprimiert als auf HV-DCs. AML-DCs erfüllen mit der Expression zumindest eines LAAs und einer starken Expression von HLA- und kostimulatorischen Molekülen die Voraussetzungen zur Stimulation einer spezifischen T-Zell-Antwort.

Darüber hinaus wurden quantitative Echtzeit-Reverse Transkriptase Polymerase-Keltten-Reaktions-Arbeiten (qRT-PCR) durchgeführt für die folgenden LAAs: PRAME, RHAMM/CD168, WT-1 und Proteinase 3. Die RHAMM/CD168 Protein-Expression wurde mittels Immunhistochemie evaluiert, die Erkennung

von AML-DCs durch PRAME-Epitop spezifische T-Zellen im Chrom-Freisetzungs-Assay. Die qRT-PCR für AML-DCs versus AML-Blasten zeigte eine Veränderung der mRNA-Expression von LAAs: Ein verstärktes PCR-Signal für PRAME wurde in sieben von 12 AML-DCs-Präparationen detektiert. Sechs von 12 AML-DCs-Präparationen zeigten eine signifikante Aufregulation von RHAMM/CD168. Ein stärkeres Signal in der PCR für WT-1 bzw. Proteinase 3 wurde nur in zwei respektive einer von 12 AML-DCs-Präparationen festgestellt. PRAME-positive AML-DCs wurden von spezifischen T-Zellen erkannt. AML-DCs könnten somit einen wichtigen Bestandteil einer Immuntherapie für AML-Patienten darstellen. Die qRT-PCR erlaubt einen schnelle und quantitative Analyse von immunologisch relevanter LAAs-Expression mit lediglich $10^5$ DCs und könnte hilfreich sein für die Entscheidung, ob in einem bestimmten Patienten eine AML-DCs-Vakzinierungs-Strategie sinnvoll ist oder nicht.

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### Original Articles

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