Surface marker expression profiles of dendritic cells (DC) generated from blasts in patients with acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) are valuable tools to characterize and quantify DC in experimental settings

vorgelegt von

Julia Dreyßig

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Direktor: Prof. Dr. med. Wolfgang Hiddemann

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Julia Dreyßig

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Berichterstatter:	Prof. Dr. rer. nat. Helga Schmetzer
Mitberichterstatter:	Priv. Doz. Dr. Dr. Fuat Oduncu
	Priv. Doz. Dr. Claudia Haferlach
Mitbetreuung durch den promovierten Mitarbeiter:	
Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser, FACR, FRCR

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Abbreviations

%	percent
>	bigger than
<	less than
Σ	sum
α	alpha
β	beta
µ	micro
Ab	antibody
ALL	acute lymphatic leukemia
AML	acute myeloid leukemia
AML-M0	AML FAB-type M0
AML-M1	AML FAB-type M1
AML-M2	AML FAB-type M2
AML-M3	AML FAB-type M3
AML-M4	AML FAB-type M4
AML-M4eo	AML FAB-type M4 with eosinophilia
AML-M5	AML FAB-type M5
AML-M6	AML FAB-type M6
APC	Allophycocyanin
Bla	blast
Bla_{con}	converted blasts
BM	bone marrow
Ca	calcium
CD	differentiation antigen (cluster of differentiation)
CLL	chronic myeloid leukemia
CML	chronic lymphatic leukemia
CMML	chronic myelomonocytic leukemia
CSF	colony stimulating factor
CTL	cytotoxic T-cells
d	days

DC	dendritic cells
DCA	DC antigen
DC_{leu}	leukemia-derived dendritic cells
DC_{opt}	optimum of DC
dgn	diagnosis
del	deletion
der	derivat
DNA	deoyribonucleic acid
EDTA	ethylendiamintetraacetic acid
e.g.	for example
FAB	French American British
FACS	Fluorescent-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FISH	fluorescent in situ hybridization
FISH-IPA	FISH-immunophenotyping
GM-CSF	granulocyte/macrophage stimulating factor
GVHD	graft-versus-host-disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ins	insertion
inv	inversion
MCM	monocyte-derived medium
MDS	myelodysplastic syndrome
Mg	magnesium
MHC	major histocompatibility complex
ml	milliliter
MLR	mixed lymphocyte reaction
MNC	mononuclear cell
moAb	monoclonal antibody
MPO	Myeloperoxidase

MRD	minimal residual disease
n	number
ng	nanogramm
NK-cell	natural killer cell
no.	number
pAML	primary AML
p	short arm of a chromosome
PB	peripheral blood
PBS	phosphate buffered saline
PC5	Cy5-PE-conjugation
PE	phycoerythrin
PGE_2	prostaglandin E_2
q	long arm of a chromosome
RA	refractory anemia
RAEB	refractory anemia with excess blasts
RAEBt	refractory anemia with excess blasts in transformation
RAS	refractory anemia with ringed sideroblasts
RNA	ribonucleic acid
sAML	secondary AML
SCF	stem cell factor
SCT	stem cell transplantation
t	translocation
TAA	Tumor-associated antigen
t-MDS	therapy-related MDS
TNF	tumor necrosis factor
U	Unit
WHO	World Health Organisation

1 Abstract

Dendritic cells (DC) have a central role in connecting innate with specific adoptive immunity resulting in target specific activation T-cells. As professional antigen presenting cells (APC) DC specifically stimulate T-effector cells, especially tumor-cytotoxic T-cells. Therefore they are regarded as interesting candidates for anti-tumor or anti-leukemic vaccination strategies. The insufficient expression of costimulatory antigens, MHC molecules and tumor-associated antigens (TAA) on the surface of cancer cells and disturbed mechanisms of apoptosis are the main reason for an ineffective immune response in oncologic diseases. It was shown that acute myeloid leukemic cells can be differentiated to leukemia-derived DC (DC_{leu}), regaining the stimulatory capacity of professional DC while potentially presenting the whole leukemic antigen repertoire. Thus, vaccination strategies, using ex vivo or in vivo generated DC, might induce a highly specific anti-leukemic T-cell response circumventing the cumbersome identification of leukemia-associated antigens.

In this thesis DC antigen (DCA) expression profiles of mononuclear cells (MNC) and dendritic cells (DC) generated from these MNC should be analyzed. The generated MNC and DC should be compared with respect to their DC antigen (DCA) expression profiles and the DCAs value to detect and quantify (leukemia-derived) DC in different AML/MDS subtypes and under different culture conditions. Therefore MNC and DC were generated from 137 patients with acute myeloid leukemia (AML) and 49 patients with myelodysplastic syndromes (MDS) under 6 different serum free culture conditions. DCA studied were: CD1a/1b/1c, CD206, CD25, CD137L, CD83, CD86, CD80 and CD40.

DC-generating media were chosen according to their different mechanisms of inducing DC-differentiation: 1. ‚Basic method': TNFα/GM-CSF/IL-4, 2. MCM-Mimic, 3. Ca Ionophore, 4. Picibanil, 5. Poly I:C and 6. Cytokines. Quality and quantity of generated DC was estimated by Flow cytometry applying a specified, 'DC-based' gating-strategy. Expression and coexpression profiles of 10 different DCA as well as various costimulatory molecules, maturation markers and blast antigens were evaluated. Only those DCA qualified for the quantification of leukemia-derived DC that were not expressed on uncultured MNC fractions.

AML patients presented with an average of 58 % blasts, MDS patients with 13 % blasts in MNC fractions. DCA were expressed on average on less than 7% of uncultured MNC,

however some of the markers could be expressed on up to 77% of uncultured cells in single AML cases. Consequently these DCA did not qualify for detection of DC in those cases. Highest expression rates were found for CD86 and CD40 in naïve AML and for CD137L and CD40 in naïve MDS samples. Other DCA (e.g. CD1a, 1b, 1c) were only rarely found on naïve blasts. DCA expression on uncultured AML and MDS MNC varied with FAB types and cytogenetic risk.

After culture in different DC-differentiating media, on average 28% DC could be generated from AML MNC and 30% from MDS MNC, depending on methods used, with an average DC viability of more than 60% and an average DC maturity of 49% (AML) and 56% (MDS). On average 36% of leukemic blasts could be converted to DC. Proportions of DC_{leu} in the total DC fraction varied from 40-58% and were on average 49% (AML) and 43% (MDS) after culture. Average results of all culture methods tested were comparable, however every method failed to create DC in some individual cases.

The most important results of this thesis are:

1. It could be shown that DCA are expressed on naïve blasts in AML and MDS in individual patients. That means that the individual patients' DCA-profiles have to be evaluated before DC-culture to find suitable DCA to detect and quantify (leukemia-derived) DC after culture.

2. Different methods of DC-generation qualify with varying individual efficiency to generate leukemic, mature, migratory and viable DC in individual cases.

3. To select the best DC-generating method the best DC-marker (no expression on naïve blasts, high expression on DC) has to be chosen to quantify DC in individual samples.

4. The use of only one method is not sufficient to create DC in every single AML and MDS sample. However, a successful, quantitative DC/DC_{leu} -generation is possible in every case of AML and MDS by the combination of 3 different DC-generating media, but not every blast is convertible to DC_{leu}.

5. There is a need for new, specific DC-markers that are not expressed on naïve blasts.

Zusammenfassung

Dendritische Zellen (DC) sind professionelle antigenpräsentierende Zellen (APC) und spielen eine zentrale Rolle in der Verknüpfung von unspezifischer und spezifischer Immunabwehr, die zu einer zielgerichteten Aktivierung von T-Zellen führt. T-Effektor-Zellen, v.a. tumor-zytotoxische T-Zellen, werden durch DC spezifisch stimuliert. Aus diesem Grund stellen DC-basierte Immuntherapieansätze interessante Optionen für neue Vakzinierungsstrategien bei der Behandlung von malignen Erkrankungen, wie z.B. Leukämien, dar.

Die ineffektive Immunantwort bei onkologischen Erkrankungen ist u.a. bedingt durch die ungenügende Expression von kostimulatorischen Molekülen, MHC-Molekülen und tumorassoziierten Antigenen auf der Oberfläche von Krebszellen, aber auch durch gestörte Apoptosemechanismen. Vakzinierungsstrategien mit DC könnten diese Mechanismen umgehen: es konnte bereits gezeigt werden, dass Blasten von Patienten mit akuter myeloischer Leukämie (AML) zu leukämischen DC (DC_{leu}) differenziert werden können. Dabei können sie weiterhin Antigene des spezifischen Blastenphänotyps exprimieren, aber zudem noch das immunstimulatorische Potential von originären DC erlangen. Vakzinierungsstrategien mit ex vivo oder in vivo generierten DC könnten dadurch die Stimulation einer hochspezifischen antileukämischen T-Zell-Antwort ohne Kenntnis von leukämischen Antigenen auf Blasten ermöglichen.

Im Rahmen dieser Arbeit wurden DC-Antigen (DCA) Expressionsprofile von mononukleären Zellen (MNC) sowie von aus diesen MNC generierten dendritischen Zellen (DC) analysiert. Die generierten MNC und DC wurden bezüglich ihrer DCA Expressionsprofile und der Eignung der verschiedenen exprimierten DCA zur Detektion und Quantifizierung von leukämischen DC verglichen. Zu diesem Zweck wurden MNC und DC von 137 Patienten mit AML und 49 Patienten mit myelodysplastischem Syndrom (MDS) unter Verwendung von 6 verschiedenen serumfreien Kulturmedien generiert. Es wurden 10 verschiedene DCA untersucht: CD1a/1b/1c, CD206, CD25, CD137L, CD83, CD86, CD80 und CD40.

Zur DC-Generierung wurden 6 verschiedene serumfreie Kulturmedien verwendet, die aufgrund ihrer unterschiedlichen Mechanismen einer Differenzierung von DC aus MNC ausgewählt wurden: 1. Basis-Methode: TNFα/GM-CSF/IL-4, 2. MCM-Mimic, 3. Ca Ionophore, 4. Picibanil, 5. Poly I:C und 6. Zytokine. Die Qualität und Quantität der generierten DC wurde mittels Durchflusszytometrie mit einer speziellen, ‚DC-basierten' Gating-Strategie bestimmt. Die Expression und Koexpression von 10 verschiedenen DCA

sowie kostimulatorischen Molekülen und Blastenantigenen wurde evaluiert. In die Analyse zur Quantifizierung von leukämischen DC wurden nur diejenigen DCA eingeschlossen, die nicht auf unkultivierten MNC exprimiert wurden.

Im Durchschnitt fanden sich in den unkultivierten MNC-Fraktionen der AML-Patienten 58% Blasten (MDS-Patienten: 13% Blasten). Die untersuchten DCA wurden durchschnittlich auf weniger als 7% der unkultivierten Zellen exprimiert, es fand sich jedoch eine große individuelle Variabilität: manche DCA wurden in einzelnen AML-Fällen auf bis zu 77% der Zellen exprimiert. D.h. dass diesen Fällen die untersuchten DCA nicht zur anschließenden Quantifizierung von DC geeignet waren. CD86 und CD40 zeigten die höchsten Expressionsraten auf unkultivierten AML- Proben (MDS-Proben: CD137L und CD40). Andere DCA wie CD1a, CD1b und CD1c wurden nur in geringem Maß auf naïven Blasten exprimiert. Die Expression von DCA auf unkultivierten AML- und MDS-Proben variierte in Abhängigkeit von verschiedenen FAB-Typen und zytogenetischen Risikogruppen.

Nach Kultur mit 6 verschiedenen Medien zur DC-Generierung konnten durchschnittlich 28% DC in AML-Proben und 30% DC in MDS-Proben generiert werden. Die durchschnittliche Rate an lebenden DC betrug sowohl in AML als auch MDS-Fällen 67%. In den AML-Fällen wurden durchschnittlich 49% reife DC gefunden, in MDS-Fällen 56%. Im Durchschnitt konnten 36% der leukämischen Blasten zu DC konvertiert werden. Die durchschnittlichen Anteile von DC_{leu} in den absoluten DC-Fraktionen waren 49% in AML- und 43% in MDS-Fällen. Die durchschnittlichen Ergebnisse bzgl. der Generierbarkeit von DC waren vergleichbar, allerdings versagte jede Methode in einzelnen Fällen bei der DC-Generierung.

Die wichtigsten Erkenntnisse dieser Arbeit waren:

1. DCA werden auf naiven Blasten von AML- und MDS-Patienten patientenabhängig variabel exprimiert. D.h. dass eine Evaluation der individuellen DCA-Expressionsprofile von AML- und MDS-Patienten vor Kultur erfolgen muss, um nach Kultur geeignete DCA zur Detektion von leukämischen DC für jeden einzelnen Patienten zu finden.

2. Die Verwendung von verschiedenen serumfreien Medien zur DC-Generierung kann bei verschiedenen Patienten unterschiedliche Ausbeuten von leukämischen, reifen, lebenden und migratorischen DC zur Folge haben.

3. Um die beste DC-Generierungsmethode zu finden, ist eine Evaluation des individuell besten DC-Markers (d.h. ohne Expression auf naiven Blasten, aber hohe Expressionsraten nach DC-Kultur) zur Quantifizierung von Zellen bei jedem einzelnen Patienten notwendig.

4. Die Anwendung nur einer Methode ist nicht ausreichend, um in jedem einzelnen Fall von AML und MDS DC erfolgreich zu generieren. Allerdings ist eine erfolgreiche, quantitative DC/DC_{leu}-Generierung in jedem Fall von AML oder MDS mit mindestens einer von 3 vorab getesteten Methoden möglich.

5. Es besteht ein großer Bedarf an neuen, spezifischen DC-Markern, die nicht auf naïven Blasten exprimiert werden.

2 Introduction

2.1 Definition and classification of leukemia

The term ‘leukemia’ refers to a group of neoplastic disorders characterized by malignant transformation of hematopoietic or lymphatic cells. These transformed cells are characterized by an increased rate of self-renewal and an aberrant differentiation. Accumulation of leukemic cells in the bone marrow ultimately suppresses normal hematopoiesis, resulting in anemia, thrombocytopenia and/or granulocytopenia, fatigue, hemorrhage and immune deficiency (Dietel et al., 2008).

Leukemia is not a consistent group of disorders and clinically and pathologically subdivided into several groups. Classification criteria are the course of disease (acute vs. chronic), the blasts‘ differentiation (lymphocytic vs. myelogenous/myeloid/nonlymphocytic) and the grade of differentiation of cells (mature vs. immature) (Dietel et al., 2008).

Cases of chronic leukemia normally present with rather differentiated malignant cells, whereas most cases of acute leukemia are characterized by immature cells. Chronic forms of leukemia are mostly geriatric disorders. Acute leukemia can be found in patients of all ages including children and adolescents. According to the affected cell type, leukemia is subdivided into four main groups: acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL)**,** acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (Wilmanns et al., 1994).

Besides, there are clonal disorders of the hematopoietic systems which cannot be classified in acute leukemia subgroups, but overlap in some characteristics, e.g. myeloproliferative and myelodysplastic syndromes (MDS) (Hiddemann and Haferlach, 2003). Morphologic and cytogenetic similarities can be found between AML and MDS. MDS are regarded as a heterogeneous family of clonal ‚preleukemia‘ disorders of hematopoietic stem cells resulting in ineffective hematopoiesis and susceptibility to AML (List, 2002;List et al., 2004). In 13% of AML cases, a precedent MDS can be found (Fialkow et al., 1987).

2.2 Acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS)

2.2.1 Epidemiology

The annual incidence of AML in Germany is about 9.100 persons, which is 2.1% of all newly diagnosed malignant diseases (Robert-Koch-Institut and Gesellschaft der epidemilogischen Krebsregister in Deutschland e.V., 2008). About 15% to 20% of acute leukemia in children and 80% of acute leukemia in adults are classified as ‚AML'. The median age at diagnosis is 67 years for male and 70 years for female patients with leukemia (Robert-Koch-Institut and Gesellschaft der epidemilogischen Krebsregister in Deutschland e.V., 2008).

The age-specific incidence of AML increases from 3.5/100.000 under the age of 45, then ascends to 15/100.000 in persons older than 70 years and to 35/100,000 over the age of 90. Only 15-20% of AML-patients are children. AML is slightly more common in male than in female persons. Besides, geographical differences can be shown: the highest incidences of AML are found in North America and Europe, the lowest incidences in Asia and Latin America (Fuchs, 2002).

Primarily, MDS is a disorder of the elderly; in children and adolescents, MDS are accounting for less than 5% of hematopoietic malignancies, mostly as a consequence of chromosomal fragility or in line with a secondary neoplasia (Aul et al., 1998;Niemeyer and Baumann, 2008;Solenthaler and Tobler, 2004). More than 80% of MDS patients are older than 60 years (Aul et al., 1998). Patients with therapy-related MDS (t-MDS) often present with an earlier onset of the disease (Aul et al., 1998).

The incidence of MDS ranges from 3.5-12.6/100,000 per year in the USA, with an increasing relative risk with age, which is 15-50/100,000 per year in persons older than 70 years (Aul et al., 2002). It is expected that the incidence of MDS will rise over the next decades (Dansey, 2000). Men have a slightly higher MDS risk than women (Wilmanns et al., 1994).

2.2.2 Etiology and pathogenesis

AML is a neoplastic disorder characterized by clonal proliferation of myeloid precursors associated with an impaired cell differentiation. The conversion of a normal stem cell to a leukemic blast requires a multistep process, however, etiology of AML is not yet completely clarified (Giles et al., 2002). In general, primary and secondary AML are distinguished. The

term ‘secondary leukemia’ refers to the development of AML following the history of a previous disease, such as MDS or chronic myeloproliferative disorders. Secondary leukemia also includes therapy-related AML (t-AML) defined as AML subtypes caused by mutation-inducing therapies like radiation therapy, chemotherapy or a combination of both (Godley and Larson, 2008;Larson, 2007). According to two hypotheses the neoplasia’s origin is either a mutated primitive pluripotent stem cell or a mutated more differentiated progenitor cell (Fuchs, 2002). In both cases, genomic mutations and modifications (chromosomal aberrations, activated oncogenes, inactivated tumor-suppressor genes) lead to uncontrolled cell division (el-Deiry, 1997).

In most cases, the cause of leukemia is unknown. However, some predisposing factors and inciting agents are well established. E.g., ionizing radiation is the most conclusively identified leukemogenic factor (Committee on the Biological Effects of Ionizing Radiations, 1990). Noxa like chemotherapeutics (especially alkylating agents or topoisomerase II inhibitors), or the exposure to chemicals like benzene or pestizides, which are known or are suspected to impair hematopoietic progenitor cells, are other potential risk factors for AML. Besides, smoking is an established risk factor for AML (Godley and Larson, 2008;Ishimaru et al., 1979;Natelson, 2007;Pyatt et al., 2007). A genetic disposition for AML is being discussed: clinical observations have identified higher susceptibility to AML in monozygotic twins (Linet, 1985). Some heritable disorders and genetic syndromes like Down’s syndrome and Bloom’s syndrome are associated with an increased risk for leukemia (Bischof et al., 2001;Linabery et al., 2008). Besides, a viral etiology of leukemia is discussed: e.g., infections with the human T-cell leukemia virus type I (HTLV-I) are etiologically linked to the genesis of adult T-cell leukemia (ATL) (Harhaj et al., 2005).

Etiology of MDS is quite similar to AML (Hirai, 2002). Analogous to AML, the conversion of a normal stem cell into a malignant blast requires a multistep process (Hirai, 2003). This process results in clonal malignant cells that can suppress normal hematopoiesis (Heaney and Golde, 1999). Again, primary and secondary MDS are distinguished. Secondary forms of MDS are observed especially after chemotherapy or radiation therapy (Rossi et al., 2000). E.g., patients with Hodgkin lymphoma have a 10% risk for a therapy-related MDS (t-MDS) (Sanz et al., 1997). Mutations can be found more frequently in patients with t-AML and t-MDS than in patients with de novo AML or MDS (Mauritzson et al., 2002). Similar to AML, an exposition to environmental carcinogens, e.g. smoking, or chemicals like benzene go along with an increased risk of MDS (Bjork et al., 2000;Travis et al., 1994). Besides, some

hereditary diseases and syndromes are associated with a higher risk of MDS, like Fanconi's anemia, Shwachman-Diamond Syndrome, Bloom's syndrome and Down's Syndrome. However, the mechanisms that lead to MDS in these cases are mostly unknown (Aul et al., 1998;Hiddemann and Haferlach, 2003).

2.2.3 Classification

Most classification systems of AML are based on the morphological criteria of FAB classification. The French-American-British (FAB) Cooperative Group provided clear and useful criteria for the pathologic classification of AML in 1976, dividing AML into six subgroups (FAB classification) (table1) (Bennett et al., 1976;Handin et al., 1995). Since 1976, FAB classification of AML has been adapted and discussed, and alternative classification systems have been proposed that incorporate immunophenotyping, cytogenetic, and myelodysplastic changes (Bene et al., 1995;Bennett et al., 1985;De Vita (editor), 1997).

The World Health Organisation (WHO) proposed another classification, subdividing AML into 4 big subgroups (table 2), including morphologic, immunologic, cytogenetic and clinical features (Arber, 2001;Harris et al., 2000). Some differences exist between the WHO and FAB classifications: the blast threshold required for diagnosing AML was reduced from 30 to 20% in WHO classification. Besides, new AML categories have been added for cytogenetic abnormalities, the presence of multilineage dysplasia as well as a history of chemotherapy and subtypes for acute basophilic leukemia, acute panmyelosis with myelofibrosis, and myeloid sarcoma (Wakui et al., 2008).

Table 1: FAB classification of AML.

	abbr	**name**	**characterization**	**cytochemistry**	**% of AML cases**
MO	AUL	undifferentiated leukemia	expression of CD13, CD33 and other myeloid markers	POX <3%	5
M1		myeloblastic leukemia without maturation	<10% promyelocytes or monocytes	>3%POX or SBB+	20
M2		myeloblastic leukemia with maturation	>10%promyelocytes, monocytes; <20% monocytic cells	POX+, SBB+, PAS-	30
M3 M3 v	APL	promyelocytic leukemia	>20% abnormal hypergranular promyelocytes; Auer rods common microgranular variant	POX+, SBB+, PAS-	10
M4 M4 eo	AMML	myelomonocytic leukemia Myelomoncytic leukemia with eosinophilia	>20% promonocytes and monocytes; >20% granulocytic component increase in abnormal marrow eosinophils (>5%)	POX+, NASDA+ POX+, NASDA+, eosinophils are PAS+	20 5
M5 M5 a M5 b	AMOL	monocytic leukemia monoblastic, undifferentiated monocytic, differentiated	 >80% of monocytic cells are monoblasts <80% of monocytic cells are monoblasts	POX+, NASDA+	 5 5
M6	EL	Erythroleukemia (DiGuglielmo's disease)	megaloblastoid, erythroid and myeloid blasts	PAS+, ringed sideroblasts with iron stain	5
M7		megakaryoblastic leukemia	large polymorphic blasts, cytoplasmic blebs	POX-, SBB-, NASDA+	1

abbr abbreviation. POX myeloperoxidase. SBB Sudan black B stain. NASDA naphtol-ASD chloracetate esterase. PAS periodic acid-Schiff stain.

Table 2: WHO classification of AML (Fauci et al., 2008).

I. AML with recurrent genetic abnormalities
- AML with t(8;21)(q22;q22);*RUNX1/RUNX1T1*
- AML with abnormal bone eosinophils [inv(16)(p13q22) or t(16;16)(p13;q22);*CBFB/MYH11*]
- Acute promyelocytic leukemia [AML with t(15;17)(q22;q12))(*PML/RARA*) and variants]
- AML with 11q23 (*MLL*) abnormalities
II. AML with multilineage dysplasia
- following a MDS or myeloproliferative disorder
- without antecedent MDS
III. AML and MDS, therapy-related
- alkylating agent-related
- Topoisomerase type II inhibitor-related
- other types
IV. AML not otherwise categorized
- AML minimally differentiated
- AML without maturation
- AML with maturation
- acute myelomonocytic leukemia
- acute monoblastic and monocytic leukemia
- acute erythroid leukemia
- acute megakaryoblastic leukemia
- acute basophilic leukemia
- acute panmyelosis with myelofibrosis
- myeloid sarcoma

t translocation. p short arm of a chromosome. q long arm of a chromosome.

The classification system for MDS developed by the FAB Cooperative Group is still the central system to classify subtypes according to morphological characterization(Bennett et al., 1982) (table 3) (Lee et al., 1999;Souto et al., 1997). Besides, the WHO provides another useful schema for the classification of MDS (table 4) (Germing et al., 2001).

Table 3: FAB classification of MDS (Handin et al., 1995).

subtype	**abbr**	**PB**	**BM**
Refractory anemia	RA	<1% blasts	<5% blasts
Refractory anemia with ringed sideroblasts	RAS	<1% blasts	<5% blasts
Refractory anemia with excess of blasts	RAEB	5% blasts	5-20% blasts
Refractory anemia with excess of blasts in transformation	RAEBt	>5% blasts	20-30% blasts or Auer rods
Chronic monomyelocytic leukemia	CMML	monocytes (>1000/L)	

abbr abbreviation. PB peripheral blood. BM bone marrow.

Table 4: WHO classification of MDS.

subtype	blasts in BM
1. Refractory cytopenia with unilineage dysplasia (Refractory anemia, Refractory neutropenia, and Refractory thrombocytopenia)	<5%
2. Refractory anemia with ringed sideroblasts (RARS)	<5%
3. Refractory anemia with ringed sideroblasts - thrombocytosis (RARS-t) (provisional entity)	<5%
4. Refractory cytopenia with multilineage dysplasia (RCMD), includes the subset Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS)	<5%
5. Refractory anemia with excess blasts (RAEB) I and II	RAEB I: 5-9% RAEB II: 10 -20%
6. 5q- syndrome	<5%
7. Myelodysplasia unclassifiable	<5%
8. Refractory cytopenia of childhood (dysplasia in childhood)	<5%

BM bone marrow. q long arm of a chromosome.

2.2.4 Diagnosis

AML patients often present with typical symptoms that begin either abruptly or gradually including weight loss, fatigue, bleeding and recurrent infections. Nearly 50% of patients have been symptomatic before diagnosis.

The diagnosis of AML is based on the demonstration of immature myeloid cells in Pappenheim stained peripheral blood (PB) and bone marrow (BM) smears (Aul et al., 1983). Standard initial diagnostic evaluation of AML patients includes patient's history, physical examination, complete blood count with manual differential cell count, chemistry tests and smears of PB and BM stained with May-Grünwald-Giemsa stain and finally applying cytochemical tests like MPO reaction, unspecific esterase (NSE) reaction and PAS (glycogene) stain. Typical findings in PB samples are normocytic, normochromic anemia, thrombocytopenia and leukocytosis or more rarely leucopenia. The median leukocyte count is about 15,000/µL. No blasts in PB samples are detectable in about 5% of AML patients.

Typical BM findings include a hypercellular aspirate with more than 30% blasts (according to FAB criteria, 20% according to WHO classification). Abnormalities in the erythroid, granulocytic and megakaryocytic lines can be found especially in M6 and M7 cases. The blasts' morphology varies in different subsets. Cytoplasm often contains nonspecific granules. Abnormal rod-shaped granules (Auer rods) are not always found, but their presence proofs a myeloid origin of cells (Fauci et al., 2008;Handin et al., 1995). Flow cytometric analyses facilitate classification to FAB subtypes and can verify myeloid lineage by demonstrating positive expression of myeloid markers like CD13 and CD33, if otherwise a differentiation between myeloid and lymphatic leukemia cannot be performed (Handin et al., 1995). Cytogenetic evaluation of AML cases can detect numeric or structural aberrations (with either classic cytogenetic analyses during metaphase or FISH-fluorescent in situ hybridization analysis during interphase) (Berger, 1992) and punctual mutations (PCR – polymerase chain reaction) (Kusec et al., 2006).

Anemia can be found in the majority of MDS patients, sometimes as part of a bi- or pancytopenia. Isolated thrombocytopenia or neutropenia is more uncommon in MDS cases. Even leukocytosis and thrombocytosis can be diagnosed in some MDS patients. Further common findings are dysmorphic erythrocytes (e.g. akanthocytes), hypogranulated thrombocytes and hypogranulated neutrophils with abnormally shaped or ringed nuclei in the PB smear. Circulating myeloblasts correlate with marrow blast yields in most cases. The patients' BM is either normal or hypercellular, 20% of MDS cases present with hypocellular marrow (Fauci et al., 2008). Other typical findings are dysmyelopoiesis (signs of dysplasia in 50% of cells in 2 or more cell lines) and a blast threshold between 5 and 30% (according to FAB criteria or 20% in WHO classification, respectively) (Bennett et al., 1982).

2.2.5 Prognosis

On average, 74% of AML patients under age 50 achieve complete remission (CR) after induction therapy, resulting in an average two year survival of 46%. With a CR rate of 52% and a two year survival of only 23%, prognosis of elderly patients is even worse (Jabbour et al., 2006;Woiciechowsky et al., 2001). One of the most important prognostic factors for AML patients is the achievement of CR after induction therapy. Moreover, age over 60 years,

certain chromosomal abnormalities, t- AML and sAML are clearly associated with lower remission rates (Haferlach et al., 2003;Handin et al., 1995;Karakas et al., 1998). The detection of aberrant karyotypes at diagnosis are important diagnostic factors. Frequently found chromosomal abnormalities can be classified into three different cytogenetic risk groups (table 5); defined FAB subtypes don't contribute to refine prognostic estimations (Haferlach et al., 2003).

The prognosis of most MDS patients is desolate, median survival times range from 0.4 to 5.7 years depending on individual risk factors (Greenberg et al., 1997). Especially therapy-related forms of MDS have an extremely poor prognosis (median survival 3 to 8 months) (Michels et al., 1985). The FAB classification has prognostic significance. E.g. Mufti et al. could show that median survival varies between different FAB subtypes (from 5 months in RAEBt to 76 months in RAS cases) (Mufti et al., 1985). Cytogenetic studies have provided most relevant prognostic information (Nevill et al., 1998). Chromosomal aberrations can be found in 30 to 50% of newly diagnosed MDS cases (Tassin et al., 1998), most of them are similar to those in AML and can be classified into the 3 previously mentioned cytogenetic risk groups (Greenberg et al., 1997) (table 6). However, defined deletions on the long arm of chromosome 5 predict a favorable prognosis, summarized as cases with '5q-minus syndrome'. Transformation to sAML, high-grade cytopenia as well as high yields of marrow blasts are associated with a worse prognosis (Greenberg et al., 2002).

Table 5: Frequent chromosomal abnormalities in AML patients and their prognostic significance.

cytogenetic risk group	karyotype	associated FAB subtypes
favorable	t(8;21) t(15;17) inv(16)/t(16;16)	M2 M3/M3v M4eo
intermediate	normal karyotype other abnormalities	
poor	t(11q23) 17q aberrations inv(3)/t(3;3) complex abnormalities -5/5q- -7/7q-	M4, M5a M0, M4

Table 6: Prognostically relevant cytogenetic categories of MDS.

cytogenetic risk type	karyotype	associated FAB subtypes
favorable	normal karyotype/-Y del(5q) del(20q)	RA RAS CMML
intermediate	other abnormalities	RA RAS RAEB RAEBt CMML
poor	complex abnormalities chromosome 7 abnormalities	RAEB RAEBt CMML

Table 5 and 6: t translocation. inv inversion. q long arm of a chromosome. del deletion.

2.2.6 Therapeutic options

Treatment options for AML and MDS with cytotoxic regimens and stem cell transplantation (SCT) are still unsatisfying due to early relapse or persistence of disease. In addition, therapeutic options are often limited due to the advanced age of the patients.

The main goal of therapy in AML patients is the eradication of the leukemic cell population while preserving normal hematopoiesis. Although CR (<5% detectable blasts in PB or BM and restoration of normal marrow function) can be achieved in about 80% of AML patients by intensive chemotherapy-based treatments including SCT, long-term survival stays rather short (5-year overall survival 20-25%) due to the persistence of minimal residual disease (MRD) in about 80% of AML patients (Houtenbos et al., 2006;Li et al., 2003;Venditti et al., 2000). Established therapeutic regimen in AML are conventional chemotherapy and SCT (Bishop, 1997). The first therapeutic step is induction therapy, which is normally carried out with Daunorubicin, Cytarabine and/or Etoposide. Postinduction treatments include further conventional chemotherapy, SCT strategies and experimental approaches. However, MRD does still exist in most patients who achieve CR. The aim of consolidation (repetitive course of the induction regimen) and intensification (higher doses of drugs than applied during induction) following induction therapy is to eradicate MRD. Both cause severe myelosuppression (Schmetzer et al., 2000). Maintenance chemotherapy includes several cycles of therapy over months or years to maintain remission and is less myelosuppressive (Fauci et al., 2008;Handin et al., 1995;Winton and Langston, 2004). Both autologous and allogeneic SCT are possible. It is still discussed whether polychemotherapy or SCT should be favored for consolidation (Levi et al., 2004). However, it has been shown that SCT is correlated with a higher rate of complications but better disease-free survival (Zittoun et al., 1995). Despite side effects, SCT is the only curative therapeutic option for AML patients. The chimeric state after allogeneic SCT provides an ideal platform for adoptive immunotherapy using donor-derived cells, especially donor lymphocyte infusions (DLI) to restore complete chimerism and offers many advantages, e.g. minimizing therapy-related toxicitiy and mortality (Gorin et al., 2000;Kolb et al., 2004). More aspects of immunotherapy in AML and MDS patients are given in 2.3.2.

Therapy of MDS patients ranges from low-intensity treatment for symptom management/ hematologic improvement including supportive care, biological response modifiers (BRM) and low-intensity chemotherapy to high-intensity treatment including chemotherapy and SCT. Especially older patients do not benefit from high-intensity-treatment in most cases. Supportive care, including red blood cell transfusions, platelet transfusions and antibiotic prophylaxis, may allow maintenance of a normal lifestyle and prevention of necessary morbidity and mortality (Greenberg et al., 2002). High-dose chemotherapy carried out with Cytarabine, Daunorubicin or other drugs can be applied in younger patients, however in most cases patients relapse after a short time (Bennett et al., 2002). Both allogeneic and autologous SCT are possible in MDS patients, however, long-term disease control is only achieved in 30 to 50% of patients (Giralt, 2004).

There is a need for new therapeutic strategies, e.g. immunotherapies with a minimum of toxic side effects in order to maintain stable remissions in AML and at least a stable disease in MDS-patients.

2.3 Immunotherapy

2.3.1 Short survey of the immune system and tumorbiology

The immune system is a complex system protecting the organism with layered defenses of increasing specificity. The innate immune system provides an immediate, but non-specific response, whereas the adaptive immune system requires the recognition of specific “non-self” antigens during antigen presentation. This improved response is then retained after elimination of the pathogen as an immunological memory, and allows the adaptive immune system to mount enhanced attacks each time this pathogen is encountered (Mayer, 2006). The innate immune system is based on cell-mediated and humoral components such as complement system, cytokines, chemokines, growth factors etc. Cellular components are macrophages, natural killer (NK) cells, mast cells, eosinophils, neutrophils, basophils and DC. These cells identify and eliminate pathogens and are also important mediators in the activation of the adaptive immune system (Janeway CA, 2005;Mayer, 2006). B-cells are involved in the humoral part of adaptive immune response (production of antigen-specific antibodies), whereas T-cells (killer T-cells, helper T-cells) are part of cell-mediated adaptive immune response (Janeway CA, 2005).

One important role of the immune system is to detect and eliminate tumor cells. Tumor-associated antigens (TAA) can be detected by antibodies or T-cells, which may lead to either lysis of tumor cells (complement system) or phagocytosis (macrophages, NK-cells, DC). However, tumors can evade mechanisms of the immune system in different ways. It has been shown that function and yields of cells of the innate and adaptive immune system can be reduced in various malignancies (Mohty et al., 2001;Ratta et al., 2002). The main reason for the phenomenon that tumor cells are aible to undergo mechanisms of immune defense is the complexity of carcinogenesis comprising "immune escape mechanisms" (Brossart et al., 2000;Gemsa et al., 1997;Whiteway et al., 2003). Tumor cells often have a reduced number of MHC class I molecules on their surface, thus avoiding detection by killer T-cells (Seliger et al., 2006). Some tumor cells also release products that inhibit the immune response, e.g. by secreting TGF-β which suppresses the activity of macrophages and lymphocytes (Frumento et al., 2006). In addition, immunological tolerance may develop against tumor antigens (Seliger, 2005).

Another problem in tumor immunology is the fact that only few TAA are specific for one certain tumor; most TAA are group specific (i.e. expressed on tumors of the same group) or unspecific (i.e. expressed on various types of cells/tissues). Besides, deranged mechanisms of apoptosis play a role in carcinogenesis (Greenberg, 1998).

2.3.2 Current options of immunotherapy in AML and MDS

The limited possibilities of common antitumor therapy described in 2.3.1 reveal that there is a need for new strategies to improve survival in AML and MDS patients. Strategies to influence the immune system for therapeutical options are subsumed by the term "immunotherapy" (Römpp, 2000). Some targeted immunotherapies are based on monoclonal antibodies which requires the identification of appropriate TAA (Greiner et al., 2006b). Conjugated antibodies, which are immunoglobulins conjugated to a cytotoxic agent (Steele, 2000), are already used in AML and MDS therapy, e.g. gemtuzumab, a monoclonal CD33-antibody (Sakamaki, 2008). Recent studies also use bispecific antibodies that not only bind to TAA, but additionally to other structures like surface markers on DC or T-cells to enhance cytotoxic reativity against tumor-cells (Balaian and Ball, 2001;Kaneko et al., 1993). Other immunotherapeutic approaches in AML and MDS comprise cytokines to stimulate the immune system against leukemic blasts: Maraninchi et al. demonstrated that clinical

remission in AML patients can be induced by IL-2, a cytokine for T-cell stimulation (Maraninchi et al., 1998). Active immunotherapy based on cytotoxic T-cells (CTL) and/or DC are promising new therapeutic options in AML and MDS patients. In case of relapse or MRD, DLI is a therapeutic option to induce anti-leukemia effects (graft-versus-leukemia (GVL) effect) (Kolb et al., 1995;Kolb et al., 2003). These immunological effects demonstrate the potential of donor lymphocytes to reject leukemic blasts (Greiner et al., 2006a). DC specifically stimulate T-effector cells, especially tumor-cytotoxic T-cells and are regarded as interesting candidates for anti-tumor or anti-leukemic vaccination strategies: DC could help to enhance specific T-cell responses against leukemic blasts in or ex vivo. A more detailed survey of DC-based immunotherapy is given in chapter 2.4.3.

2.4 Dendritic cells (DC) and DC-antigens (DCA)

2.4.1 Definition and function of DC

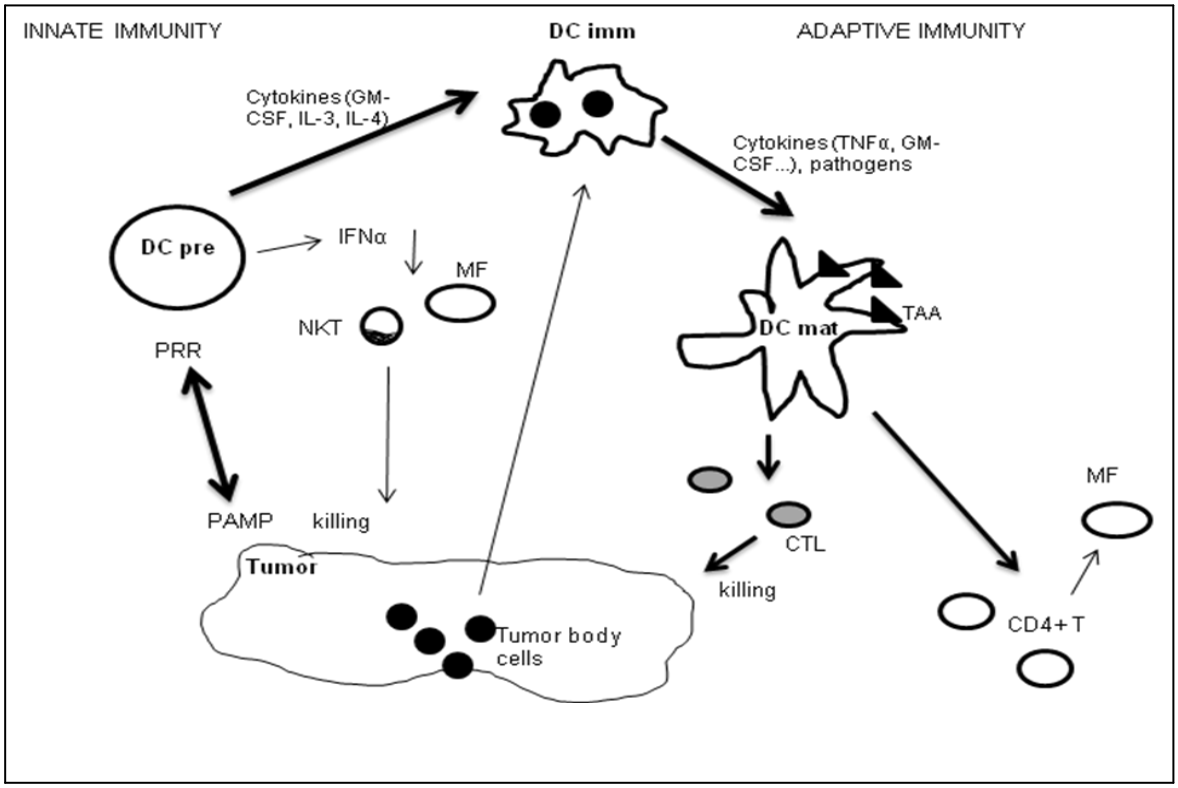
DC are antigen-presenting cells (APC) with a unique potential to induce primary immune responses. Besides, they play a central role in the stimulation and regulation of T-cell responses and in the induction of immune tolerance (Banchereau et al., 2000).

DC represent a heterogeneous population of leukocytes that is defined by phenotypic, morphologic and functional criteria (Banchereau and Steinman, 1998). The DC system includes at least three different subsets, comprising two within the myeloid lineage (including Langerhans cells, interstitial DC and monocyte-derived DC) and one within the lymphoid lineage (Banchereau and Steinman, 1998;Young, 1999). Within each of these subsets, cells differentiate from precursors to immature DC resident in peripheral tissues, and to mature DC which are especially found in secondary lymphoid organs. Different DC subsets play different roles in immune response and immune tolerance: resident lymphoid DC induce self tolerance, whereas myeloid DC, especially migratory DC, are activated by antigens/pathogens and initiate immune response (Banchereau and Steinman, 1998) (figure 1). Immature DC are characterized by a high potential of antigen uptake and processing, but low T-cell stimulatory capacity. In contrast, mature DC have a high stimulatory function, but poor antigen uptake potential and processing ability. Several cytokines such as TNFα, IL-1 and PGE_2 and bacterial products like lipopolysaccharides stimulate DC maturation, whereas IL-10 inhibits it (Bell et al., 1999;Rescigno et al., 1999). Only differentiated and mature DC finally are able to trigger

potent cytotoxic T-cell reponses (Sallusto and Lanzavecchia, 2002). DC express MHC class I molecules which are essential for stimulation of $CD8^+$ CTL, but also MHC class II molecules to interact with $CD4^+$ T-helper cells (Gatti and Pierre, 2003). This fact is essential for eliciting immune responses, because DC have to be activated by $CD4^+$ cells, e.g. via CD40/CD40L or other interactions, before they are able to stimulate CD8+ cells using a CD80/CD86-CD28 signal transduction ("cross-priming") (Cho and Bhardwaj, 2003) (Dhodapkar and Dhodapkar, 2005).

Figure 1: DC as an important link between innate and adaptive immunity in antitumor response. (Banchereau et al., 2000;Bell et al., 1999).

Precusor DC (DC pre) recognize tumor-associated molecular patterns (PAMP) by their pattern-recognizing receptors (PRR) and release interferon (IFNα) to activate macrophages (MF) and natural killer T-cells (NKT). NKT that kill tumors lead to the release of tumor cell bodies which are captured by immature DC (DC imm), leading to maturation of DC (DC mat) and displaying of tumor-associated antigens (TAA). TAA are recognized by tumor-specific cytotoxic lymphocytes (CTL) and CD4+ T-cells which activate macrophages.



DC are characterized by a distinct morphology: in situ, both immature and mature DC have a stellate shape; isolated DC display fine dendrits in many directions from the cell body. The shape and motility of DC perfectly suit their function of capturing antigens and selecting antigen-specific lymphocytes (Bell et al., 1999).

DC express a variety of molecules on their surface: migration receptors like CCR7 and E-cadherin, adhesion and costimulatory molecules (e.g. CD50, CD54, CD86, CD80), receptors for antigen-uptake and antigen-presenting molecules (MHC I, MHC II, CD1) (Bell et al., 1999). A more extended survey of CD (cluster of differentiation) antigens that are expressed on DC is given in chapter 2.4.2. At the moment, only a combination of antigens can define a population of DC, but no specific antigen is known which permits unequivocal assignment of a given cell to the DC family (Bell et al., 1999).

2.4.2 Function of different DC-antigens (DCA)

As already described in 2.4.1, DC express a variety of molecules on their surface to suit their different functions, e.g. antigen capture and presentation or migration. Different subsets of DC can only be distinguished from each other and from other cell types by a combination of surface markers, but not by only one specific antigen (Bell et al., 1999) (table 7). For this thesis, expression profiles of 10 different CD molecules which are typically expressed on DC were analyzed on naïve AML and MDS samples and cultured MNC fractions.

CD1 molecules are regarded as a hallmark of DC. They are involved in antigen presentation and essential in the regulation of T-cell-responses (Banchereau et al., 2000). CD1 antigens are nonpolymorphic cell surface proteins and have been shown to present peptides as well as microbial, nonpeptide antigens to T-cells. They share functional and physical characteristics with MHC molecules and are import connectors between innate and adaptive immunity (Bell et al., 1999). In humans, five CD1 proteins are expressed on DC: Cd1a, CD1b, CD1c, CD1d and CD1e (Peiser et al., 2003). CD1a, CD1b and CD1c isoforms are recognized by conventional T-cells, whereas CD1d activates a restricted set of T-cells as well as NK-cells (Banchereau et al., 2000;Gelin et al., 2008). CD1c is variably expressed on Langerhans cells, whereas high yields of $CD1b^{+}$ cells are especially found among dermal and migrating Langerhans cells (Bell et al., 1999). GM-CSF, TNFα, IL-6 and IL-1β induce the expression of CD1a (Athanasas-Platsis et al., 1995). CD1a molecules are not only found on DC, but also on

cortical thymocytes, and disappear at later stages of T-cell maturation (Bell et al., 1999). In some malignancies, $CD1^+$ cells also play an important role in the initiation of immune response (La et al., 2004).

CD83 is one of the most important antigens to identify mature DC and not detectable on other APC like immature DC, resting B-lymphocytes or monocytes (Hirano et al., 2006). DC which express CD83 are mainly found within T-lymphocyte areas of lymphoid organs and express high levels of MHC II molecules (Bell et al., 1999). It is known that CD83 is essential for antigen presentation and for the stimulation of antigen-specific T-cells, especially $CD8^+$ T-cells: in vitro studies could show that CD83L is induced both on $CD4^+$ and $CD8^+$ T-lymphocytes by CD28-mediated costimulation. Engagement with CD83 supports the expansion of newly primed naïve T-cells, enhances in vitro generation of CTL and enables long-term-survival of antigen-specific T-cell cultures (Hirano et al., 2006). Various cellular signals, including cytokines (e.g. TNFα), and other agents like bacterial lipopolysaccharide (LPS) and monocyte-conditioned medium (MCM) have been shown to lead to high levels of CD83 expression on cultured DC (Mosca et al., 2000).

One way of DC to capture antigens is mediated via the mannose receptor CD206 (Sallusto et al., 1995). Antigen-uptake by DC via CD206 results in an amplified (about 100fold) antigen-presentation and thus a more efficient activation of T-cells, as compared to antigens that are internalized via fluid phase (Engering et al., 1997). Expression of CD206 has been regarded a differentiation hallmark of immature dendritic cells, whereas other APC like monocytes and mature dendritic cells normally do not express CD206 (Wollenberg et al., 2002).

CD25 is expressed on DC-precursors, immature and mature DC (Cella et al., 1997;von Bergwelt-Baildon et al., 2006). Besides, CD25 is found on T-cells (Cools et al., 2007) and on specialized, antigen-presenting B-cells (Brisslert et al., 2006).

CD137L is a member of the TNF superfamily and is involved in the determination of cell proliferation, differentiation and apoptosis (Locksley et al., 2001;Salih et al., 2004). Interaction of CD137L with CD137, which is expressed on activated NK-cells, T-cells and DC, induces T-cell activation (Cheuk et al., 2004;Mittler et al., 2004;Sica and Chen, 1999). CD137L is expressed on several types of APC and can be induced on T-cells. Aberrant expression has been reported on leukemic cells, carcinoma and lymphoma (Hentschel et al., 2006).

Table 7: DC surface phenotype (Fong and Engleman, 2000).

	DC precur-sors	**Langer-hans cells**	**activated DC**	**monocyte-derived DC**	**monocytes**	**activated B-cells**
MHC						
Class I	+	+	+	+	+	++
Class II	+	++	+++	++	+	++
myeloid markers						
CD14	-	-	-	-	+	+/-
CD33	+	+	+	+	+/-	-
costimulatory molecules						
CD80	-	+	++	+	-	++
CD86	+	+	+++	+	+	++
CD40	+/-	+	+	+	+	++
antigen receptors						
CD206 (mannose receptor)	-	+	+	+	+	-
CD36	+	+		+	+	-
adhesion molecules						
CD58						
CD54	+	+	+	+	+	+
	+	+	++	+	+	++
DC markers						
CD83	-	+/-	+	+/-	-	+
CD1a	-	+	+	+/-	+/-	-
CD1b	-	-	+	+	+/-	-
CD1c	-	+/-	+	+	+/-	-

MHC major histocompatibility complex.CD cluster of differentiation.

Maturation of DC is associated with upregulation of the surface markers CD80, CD86 and CD40 (Banchereau et al., 2000). They are important costimulatory molecules which constitute T-cell activation (Bell et al., 1999;Nicod et al., 2005;Rogers et al., 2005). Interaction between CD28 on T-cells and CD86 and CD80 influences T-cell regulation: CD28-CD80 interaction skews towards type 1 responses, whereas CD28-CD86 interaction orientates towards type 2 responses (Bell et al., 1999). CD86 seems to be the most critical factor for amplification of T-cell responses (Banchereau et al., 2000).

CD40 ligand (CD40L) is expressed on activated T-cells, basophils, B-cells, and DC. CD40-CD40L-interaction is not only a one-way interaction for activating T-cells, but also the most effective stimulus for DC maturation (i.e., upregulation of CD80 and CD86). Besides, engagement with CD40L increases viability of DC (Bell et al., 1999). After CD40-CD40L interaction, DC release large amounts of cytokines like IL1, TNFα, chemokines and IL-12, which is important for the enhancement of T-cell-responses (Bell et al., 1999;Cella et al., 1996;Sasaki et al., 2005).

Expression profiles of DCA might be different on artificially generated DC. E.g. it has been shown that antigens typically expressed on DC (e.g. CD1a, CD83) are expressed to a lower degree on artificially generated DC (Pietschmann et al., 2000). Moreover, leukemic cells can show aberrant expression of DCA. Therefore a refined strategy has to be applied to quantify DC and especially leukemia-derived DC in individual cases by selection and combination of suitable DC with leukemic antigen markers (Schmetzer et al., 2007).

2.4.3 Current options of DC-based immunotherapy in cancer patients

DC are the most potent antigen-presenting cells (APC) of the immune system and important players in immune response against neoplastic cells (den Brok et al., 2005). This fact has moved DC-based immunotherapy to the center stage in active immunotherapy (Bocchia et al., 2000;Mashino et al., 2002). Since the first clinical trial of DC vaccination has been published in 1996 (Hsu et al., 1996), more than 100 studies reported about more than 1000 DC-based vaccination trials in different types of solid tumors (e.g. malignant melanoma, prostate cancer, colorectal carcinoma) (Lodge et al., 2000;Mackensen et al., 2000b) and hematologic

malignancies (e.g. multiple myeloma) (Nestle et al., 1998;Ridgway, 2003;Satthaporn and Eremin, 2001).

DC vaccines can either be prepared by pulsing DCs with tumor lysates or TAA (Mackensen et al., 2000b), by transfection with tumor DNA (Bocchia et al., 2000), or by creating tumor cell/DC fusions (Koido et al., 2008). Pilot DC vaccination studies showed specific anticancer responses and, in about 50% of trials, clinical responses (Fong and Engleman, 2000;Nestle et al., 2001;Ridgway, 2003).

In contrast to solid tumors, a big advantage in DC-based immunotherapy of AML is that leukemia derived DC (DC_{leu}) can be generated in vitro by converting leukemic cells directly to mature APC giving rise to leukemic cells coexpressing DC-typical antigens (Schmetzer et al., 2007). It has already been demonstrated that DC can be generated successfully in vitro from $CD14^+$ monocytes or from $CD34^+$ progenitor cells or leukemic cells, e.g. in the presence of cytokines (Lee et al., 2002;Westers et al., 2003;Woiciechowsky et al., 2001), Calcium-ionophores (Houtenbos et al., 2003), nucleic acid fragments (Rouas et al., 2004) or bacterial lysates (Sato et al., 2003) with different mechanisms of inducing DC differentiation. The addition of Flt-ligand increases the harvest of DC (Kufner et al., 2005b;Woiciechowsky et al., 2001). The use of fetal calf serum (FCS)-free media for DC-generation should be preferred to avoid immune reactions against FCS-peptide-associated, xenogeneic antigens or anaphylactic reactions (Mackensen et al., 2000a).

Clinical trials on DC-based vaccination in patients with hematologic disorders are still in early stages, however, some recently published in vivo studies on DC vaccination in leukemia showed promising results (Reichardt and Brossart, 2005). First results of Phase I/II clinical trials vaccination with autologous DC in AML patients showed that vaccinations with DC are feasible and safe, although not regularly clinically effective. It could be demonstrated that concentrations of leukemic RNA can decrease and T-cell responses can be boosted after vaccination with autologous DC in many cases. However, regulatory or inhibitory effects may be limiting in some cases (Houtenbos et al., 2006;Roddie et al., 2006). In MDS, in vitro DC-generation studies could show that DC can be generated as effectively as in AML patients (Kufner et al., 2005a).

DC-based vaccination provides a promising approach to cancer immunotherapy, however, there is still much debate about DC preparation, antigen loading, dosing, injection sites and monitoring of immune and clinical responses (Nestle et al., 2001;Ridgway, 2003).

2.5 Aim of this thesis

Due to persistence of MRD in about 80% of AML patients, long-time survival of AML patients is limited (5-year overall survival 20-25%) (Houtenbos et al., 2006;Li et al., 2003;Venditti et al., 2000). As the median age of MDS patients is rather high, allogenic SCT is available only for a minority of these patients, and treatment options are mainly supportive (Hofmann and Koeffler, 2005;List, 2002). Thus, the achievement of long-term remission stays the central therapeutic challenge in most AML and MDS patients. New immunotherapeutic strategies, especially anti-tumor vaccination, are promising tools to improve long-term remission. DC play a central role in anti-tumor response and are regarded as interesting candidates for anti-tumor or anti-leukemic vaccination (Sallusto and Lanzavecchia, 2002). However, results of several in vitro studies showed that the differentiation from leukemic blasts to APC is not successful in 30-70% of cases (Kufner et al., 2005a;Roddie et al., 2006;Roddie et al., 2002;Westers et al., 2003).

The aims of this thesis were:

1. the evaluation of DCA expression profiles on uncultured, naive AML and MDS MNC;
2. the generation, quantification and characterization of DC from AML and MDS MNC with special regard to the variety of DCA expression after culture;
3. to develop a strategy to find suitable DCA (in combination with blast markers) to detect and quantify leukemia-derived DC after culture;
4. the comparison of efficacies (or failure rated) of different culture systems to generate DC in different subtypes of AML applying DCA/blast marker expression profiles;
5. the evaluation of a DC generation and quantification strategy applying different DC-generating media in combination with DCA/blast marker expression profiles in every given patient.

All in all, this thesis should contribute to the further optimization of DC-generating studies with regard to future in vivo studies to improve treatment options for AML and MDS patients.

3 Material and Methods

3.1 Characteristics of AML and MDS patients

For this study, samples from 137 AML patients and 49 MDS patients in active stages of disease and 43 healthy donors were collected after obtaining informed consent. Most of these patients had been diagnosed and treated in the course of clinical studies. In addition, DC were generated from three AML cell lines: Kazumi and HL60, both AML-M2, and MUTZ-3 (AML-M4) (Table 8a-c).

Table 8: Characteristics of patients with AML (a) and MDS (b) and AML cell lines (c).

(a) AML patients (n=137)

pat. no.	sex	dgn	stage	age at dgn	clonal markers	cytoge-netic risk	ic blasts*	blast phenotype (CD)
2	m	pM1	fd	69	-	i	94	117,33,13,64
6	f	pM1	rel	67	-	-	79	13,33,117,34,56
7	m	pM2	pers	59	normal	i	62	13,33,65,117
8	f	pM2	fd	43	t(2;3)(pq)	i	94	13,33,34,117,2,65,15
9	m	pM0	rel	60	normal	-	17	33,Dr
10	f	pM4eo	fd	37	inv(16pq),del(7q)	f	43	13,33,117,34,Dr
11	f	sM5a	fd	57	+8,+13,+20,t(9;11)(pq23),inv(17pq)	p	74	7.1,13,33,86,Dr,15,56,64
12	m	sM4	pers	76	+8,+9,+14	p	91	7,13,33,Dr,14,64,34
17	w	pM1	fd	73	normal	i	89	33,13,Dr
18	m	pM5b	rel	80	normal	-	40	33,13,64,34
20	m	pM4eo	fd	57	inv(16pq)	f	81	117,33,34,13,Dr
22	m	pM2	fd	59	t(8;21)	f	21	33,34,13
23	f	pM5	fd	53	t(11;22)(qq)	p	37	-
25	m	pM0	fd	61	normal	i	39	33,13,34,7,117
29	m	pM4	fd	56	normal	i	31	14,15,33,64,Dr
33	m	sM2	pers	54	t(3;18)(qq),ins(4;9)(q?),t(4;19)(p?),-5,t(7;11)(qq),+9,del(9q),t(9;12)(pq),t(5;10)(?p),-11,t(5;14)(?p),del(17p),-18,-19,-20	p	91	7,13,33,34,56,117
36	f	pM4	fd	41	t(11;16)(pp)	i	45	13,33
37	m	sM2	fd	67	-	-	80	33,117
38	f	pM1	fd	75	-	-	59	7,13,34,117,Dr
41	m	pM5	rel	43	normal	-	48	4,15,33,56,64,65,Dr
43	f	pM3	fd	29	t(15;17),del(7qq)	f	58	13,33,64,Dr
44	m	pM5	fd	37	normal	i	46	15,33,56,64,Dr
46	f	pM4eo	fd	33	normal	i	75	4,15,33,56,64,Dr
48	m	pM4eo	fd	40	normal	i	88	13,15,33,34,65,Dr
49	f	sM2	fd	55	normal	i	49	2,33,34
50	f	pM0	fd	72	-	-	10	4,10,13,14,33,117,Dr
51	f	sM6	pers	31	-7	p	48	34
53	m	pM1	fd	24	normal	i	87	4,13,33,117,Dr
54	m	sM6	pers	60	ins(5;17),del(5q),t(6;17)	p	11	33,34
56	m	pM2	fd	36	normal	i	38	13,33,117,Dr
60	f	pM3	fd	56	t(15;17)	f	87	13,33,64,Dr-
61	f	pM0	fd	68	normal	i	4	13,33,34,117
64	m	pM4eo	fd	69	inv(16pq)	f	25	13,14,33

65	f	sM5	fd	74	r(1pq)	i	79	15,33,64,65
66	m	sM2	fd	74	normal	i	85	13,14,33,64,116,Dr
89	m	sM2	fd	67	-	-	30	13,33,34,117,Dr
92	m	pM0	fd	73	-	-	5	34,38,117
93	f	pM4	fd	52	normal	i	52	13,33,64,117,Dr
104	f	pM2	fd	20	t(3;5)(qq)	i	33	15,33,123
107		pM5	fd	48	normal	i	73	13,14,15,33,64
121	f	pM1	fd	69	normal	i	40	13,117
126	m	pM2	fd	37	t(8;21)(qq)	f	86	13,33
130	m	pM0	fd	53	t(9;18)(pq)	i	76	2,7,13,15,33,65,117
131		pM4	fd	56	+4,+9etc	p	30	33,34,38
135	f	pM4	fd	26	normal	i	52	13,33,34,38,MPO
136	m	pM1	fd	29	normal	i	62	13,33,117
137	f	pM1	pers	68	normal	i	69	13,33,MPO
138	m	sM4	fd	61	-7	p	36	13,14,15,33,34,65
140	f	pM2	fd	82	normal	i	42	13,33,MPO
143		pM2	fd	75	normal	i	50	13,33
146		pM5	fd	68	-	-	84	33,34
148	m	pM1	rel	55	normal	i	95	33,123
153		pM0	fd	61	normal	i	80	13,33
161	m	pM1	fd	50	normal	i	90	4,13,33,117
166	m	pM1	fd	60	del(3pp)	i	66	4,13,33,34,65
168		pM0	fd	70	-	-	8	7,13,33
172	m	sM2	fd	75	-	-	75	13,15,33,117
175	f	sM1	fd	69	normal	i	90	4,13,14,15,33,64,65
176	m	pM2	fd	58	normal	i	79	13,33,117,135
181	m	sAML	rel	63	t(3;6)(qq),-7,del(16q),t(5;17)qq,t(6;10)(qp)	-	92	34,33,13
182	f	pM2	fd	51	normal	i	18	7,33,34
184	m	pM4	fd	64	normal	i	71	-
188	f	pM1	fd	40	normal	i	96	56,123
189	f	pM1	fd	31	normal	i	22	13,33,117,65,15,7,2,c3
191	m	sM2	fd	72	+8	i	12	34,33,117
195	m	pM0	rel	53	normal	i	21	34
196	f	pM5	fd	67	-	-	91	64,33,13,4,15
197	f	pM5	fd	59	t(9;11)(q21;q23)	p	86	64,15,65,33,13
198	m	sM1	fd	68	+8	i	67	33,65,56,15,64
200	f	pM2	fd	32	normal	i	23	117,13,33,65
201	m	pM5	fd	47	normal	i	93	-
202	m	pM0	fd	62	+8	i	95	13,33,117,64,7
205	m	pM2	fd	38	t(8;21)	f	85	13,33,34
207	m	pM4	fd	63	+11,inv11(pq13)	i	61	33,65,14,4,56,135
210	f	pM2	fd	75	normal	i	6	-
211	m	M0	pers	52	inv3,-7	p	84	33,34,13,117,4,7
214	f	pM1	fd	70	normal	i	64	33,13,15,40
229		pM2	fd	54	add(12p)	i	70	33,13,34,7
232		M2	fd	-	normal	-	94	-
236		sM0	rel	70	-	-	22	33,13,117,7
238	f	pM1	fd	72	-	-	53	13,33,117,34
239	f	sM4	rel	54	t(9;11)(q21;q23)	-	49	13,33,117,135
243		sAML	-	64	-	-	-	-
244		pM2	rel	44	t(8;21)(q24;q22)	f	26	33,13,117
245		pM0	fd	54	-	-	22	34,13,7,117,65
248		AML	-	71	-	-	81	33,13
249		AML	-	41	-	-	-	13,34,33,117
253		M3	fd	26	t(15;17)	f	85	33,Dr-
254		M1	fd	50	normal	i	34	33,13,117
256		AML	-	67	-	-	75	13,34,33,117
258		M1	fd	81	-	-	70	56,33,15,64,4
259		M4	rel	57	-X	i	95	15,33,64,4
260		M2	rel	30	-	-	-	15,34,33,65,13
261		M1	-		-	-	-	-
262		M2	fd	61	-	-	85	33,13,65,15,117,34

264		sM3	fd	61	-	-	-	33,65,64,117,34,56,4
265		sM5	fd	61	-	-	64	33,13,64
269		sM2	-	66	-	-	-	33,13,117,34,4
270		sM4	fd	63	-	-	80	65,15,56,64
272		sAML	fd	65	-	-	49	33,65,64,15
273		M5	fd	34	-	-	-	65,33,64,15,56,4
275		M1	fd	37	-	-	90	33,15,65,64,4
280		AML	rel	62	-	-	68	13,33,65,15,34,117,4
285		M2	fd	40	-	-	-	-
291		M1	-	82	-	-	-	-
293		M1	-	71	-	-	-	-
292		M1	-	67	-	-	-	-
295		M1	fd	46	-	i	70	-
298		pM2	rel	52	-	-	90	-
300		M2	fd	84	normal	i	85	13,33,65,15
301		M0	rel	51	del(2p),+11,+10,+10,+13,+13,+14,+21,+21,+22,+22,t(7;10)q,t(2;10)p,t(13;16),del(16q)	-	82	-
305		M2	pers	62	Y,t(8;21),t(2;6)(qq),t(3;11)(pq23),t(7;9;15)	f	50	7.1
307		M1	fd	36	-	-	95	13,33,64,117
309		M2	rel	-	-	-	-	13,33,34,117
311		M2	fd	48	+8,t(8;21)	f	-	33,15,13,65,34,19
312		sM4	fd	72	normal	i	-	33,54,65,15,34
313		M2	rel	35	+21,+21,+21	i	-	7,33,34
315		M4	fd	66	normal	i	14	13,33,7,15
317		M2	fd	29	-Y,ins(21;8)qq	f	-	56,13,33,15,117,34
318		M0	fd	64	+13,-7	p	-	13,33,65,15,4,34,117
320		M4	fd	44	normal	i	-	33,13,65,14,64,15,4
321		M2	rel	56	normal	-	34	13,33,117,65
322		sM2	fd	59	-3,-7,+10,-16,-18,del(5q),t(6;17)(pq);t(3;12)pq,i(8q),t(3;10)ßq,i(11q),t(3;6)(?p)	p	80	13,33,34,65
323		sM0	fd	59	Inv(9pq)	i	30	33,13,34
324		M4	fd	64	-	-	95	33,65,13,15,4,56,117
325		pM2	fd	63	normal	-	95	33,13,64,117
328		sM2	rel	59	del(5qq),+8	-	65	34,33,13,117
330		M4	fd	72	-	-	90	56,33,65,13,15,14
331		pM4	fd	41	-	-	71	34,33,13,117,19,5,2
332		pM2	fd	65	del(5qq)	i	-	-
336		pM3	fd	70		f	60	33,14,Dr
344		M5	fd	-	+21	i	81	15,13,33
347		sM5	fd	-	t(9;11)(pq23)	p	94	-
348		M1	fd	-	normal	i	-	13,33,14,117,34
351		M5	fd	-	-	-	95	33,65,15,64,7,117
352		sM2	rel	-	normal	-	34	-
353		sM2	fd	-	normal	i	85	117,33,13,15

(b) MDS patients (n=49)

5	m	RAEB	pers	66	-	-	12	34, 117
15	m	RAEB	pers	49	normal	f	10	34, 117
16	w	MDS	pers	70	del(5q)	f	2	34, 117
19	f	RAEB	fd	73	t(3;5)(pq)	i	11	117,33
26	m	RAEBt	fd	63	normal	i	17	15
27	m	RAEBt	fd	51	normal	i	25	33,7,117,34
30	f	RAEB	pers	78	-	-	4	33,34
40	f	RAEB	fd	77	-	-	2	34, 117
52	m	RAS	fd	60	-	-	4	34, 117
57	f	CMML	fd	67	-	-	23	13,33,34,117,Dr
63		RAEBt	fd	51	normal	f	30	13,34,117
67		RAEBt	fd	71	-	-	25	33,13,64,15
69	m	RAEB	fd	51	t(2;10)(qp),-9,der(16q)	p	15	33,34
70	m	RAEB	fd	53	dic(5;17)(qp),+8,t(19;20)(qp),-20,+22,t(11;14)(?p)	p	15	13,14,33
75	f	CMML	fd	67	-21	i	15	13,117
77	m	RAEBt	fd	67	normal	f	25	13,33
78	f	RA	fd	39	del(5q)	f	-	34, 117
83	m	RA	pers	68	normal	f	15	34, 117
84	f	RAS	pers	43	+19	i	4	117
88	m	MDS	fd	70	-	-	3	34, 117
96	m	RAEB	fd	60	normal	f	15	34, 117
97	m	RA	fd	76	normal	f	2	34, 117
99	m	RAEB	fd	76	normal	f	11	34, 117
102	m	RAEB	fd	67	XXYY	i	10	34, 117
110	f	RAEB	fd	77	normal	f	9	34, 117
123	f	RAEB	fd	62	-	-	18	34, 117
127	f	RAEB	fd	63	der(5;17)(qq),-7,del(9q),t(9;12)(qq)	p	3	34, 117
129	f	RA	fd	49	normal	f	3	34, 117
133	f	RAEBt	fd	68	del(5q)	f	21	33,34
134	f	RA	pers	78	del(5q)	f	8	33,34
141	f	RAEBt	fd	63	i(17q)	i	24	33
145	m	RA	fd	76	normal	f	3	34, 117
158	f	RAEBt	fd	74	-	-	21	34, 117
160	m	RAEBt	fd	65	normal	f	20	13,33,64,65
169		RAEBt	fd	74	-	-	21	34
179	m	RAEBt	fd	82	+1,+2,+4,+8,+9,+14,-16,+19,t(8;11)(pq),dic(q;11)(qp);t(9;19)(?q)	p	15	34, 117
190	m	RAEB	fd	68	-y	f	12	34
208	f	RAEB	fd	83	normal	f	21	34, 117
213		RAEBt/ NHL	fd	40	normal	f	22	34, 117
215	f	RAEB	fd	45	normal	f	2	117,34,33
223	m	RAEB	fd	75	-	-	15	34, 117
225	m	RAEBt	fd	80	-	-	7	34, 117
227	w	RAEBt	fd	51	-	-	6	117,33,13,7
234	m	RAEB	fd	54	normal	i	18	117,34
257		MDS	-	67	-	-	-	34, 117
277		RA	pers	66	-	-	5	34, 117
288		MDS	fd	52	-	-	-	33,13,15,64
302		RAEB	fd	40	inv(1pq),inv(3qq)	p	14	34, 117
319		RAEBt	pers	53	-	-	29	13,65,15,3364,117

(c) AML cell lines

name	FAB type	clonal markers	blast phenotype
HL 60	pM2	-X,-8,-16,-17,+18,+22,ins(1;8)(p?;q), t(5;17)(q;q),del(9)p(13)t(9;14) (q;q),t(9;14)(q ;q),t(16;17)(q;q), sideline with:-2,-5,-15,del11(q;q)	13, 15, 34, 65
Kasumi	pM2	-9,-13,-16,t(8;21)(q;q),t(9;?)(p22;?) t(?9)(p22;?)t(?9;15)(p;q)	4, 13, 33, 34
Mutz-3	pM4	t(1;3)(q;q),inv(3)(q;q),t(2;7)(q;q), inv(7)(p;q),t(12;22)(p;q)	4, 13, 33, 34

pat.no. patient number. m male. f female. dgn diagnosis. pM0 primary AML FAB type M0. sAML secondary AML no FAB type available. stage stage of disease. fd first diagnosis. rel relapse. pers persisting disease. – no data available. f favorable cytogenetic risk. i intermediate cytogenetic risk. p poor cytogenetic risk. CD cluster of differentiation. * % of blasts found in the analyzed material (bone marrow or peripheral blood) by FACS analysis, alternatively % of CD34+cells in MDS cases.

Diagnostic reports like morphology, cytochemistry, karyotype, immunophenotype and classification to cytogenetic risk groups were provided by the leukemia diagnostics laboratories of the Med III, University Hospital Großhadern, Munich, and the university hospitals of Berlin, Oldenburg and Ulm. Most of cell preparations (MNC, T-cells) and combined cell testings were performed by other members of our group. Many of the specified FACS-analyses and the complete statistical evaluation were performed by me. Results of functional assays (MLC and cytotoxicity assays) were provided for analyses for this thesis by other members of our group.

99 AML patients studied presented at first diagnosis, 8 in persisting disease and 19 at relapse. In the cohort of MDS patients, 40 presented at first diagnosis and 9 in persisting disease. The median age of the AML-Patients was 56 years (range 20 - 84 years), the female:male ratio was 0.8. The median age of the MDS-patients was 64 years (range 39 – 83 years), the female:male ratio was 0.9.

Diagnosis of AML and MDS cases was based on FAB classification (Bennett and Komrokji, 2005;Hayhoe, 1988) as described in 2.2.1. 33 AML presented with minimally differentiated leukemia (M0: n=16, M1: n=27), 41 with acute myeloblastic leukemia with granulocytic maturation (M2) and 5 with promyelocytic leukemia (M3). 39 AML patients had been diagnosed with monocytoid leukemia (M4: n= 18, M4eo: n= 5, M5: n=16) and 2 with acute erythroid leukemia (M6). Primary leukemia (pAML) was found in 69 cases, secondary leukemia (sAML) in 31 cases (primary or secondary AML not defined in 37 cases). 7 MDS patients had been diagnosed with refractory anemia (RA), 2 with refractory anemia with

ringed sideroblasts (RAS) and 19 with a refractory anemia with excess of blasts (RAEB). 15 MDS patients presented with refractory anemia with excess of blasts in transformation (RAEBt) and 2 with chronic myelomonocytoid leukemia (CMML). No FAB type was available for 7 AML and 4 MDS patients.

Cytogenetic analyses were performed according to standard protocols and criteria defined by the International System for Human Cytogenetic Nomenclature (Mitelman, 1995) (see 3.4). 14 of 137 AML patients were categorized as favorable cytogenetic risk, 59 patients as intermediate risk and 13 patients as poor cytogenetic risk. 19 MDS patients were categorized as favorable cytogenetic risk, 8 patients as intermediate and 5 patients as poor cytogenetic risk. No cytogenetic risk group was available for 51 AML and 17 MDS patients.

3.2 Experimental processing of AML, MDS and healthy samples

Bone marrow (BM) was obtained after informed consent by iliac crest puncture. Blood samples were taken by puncture of peripheral veins (PB). Anticoagulation of samples was carried out with heparin or ethylendiamintetraacetic acid (EDTA) (see 3.3). For morphologic and cytochemical diagnostic evaluation, PB or BMs smears were prepared and samples classified according to FAB classification criteria (see 3.4). For remaining analyses, mononuclear cells (MNC) were separated from whole blood samples by density gradient centrifugation (Böyum, 1984) using Ficoll-Hypaque, then washed and suspended in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (both Biochrom, Berlin, Germany).

Separated MNC fractions that were not processed at the same day were stored at +4 °C overnight. For some analyses we used MNC that had been defrosted after cryoconservation with nitrogen at -196 °C (Fliedner et al., 1977).

3.3 Cytogenetic analysis of AML and MDS samples

Cytogenetic analyses were performed in the diagnostic laboratories according to standard protocols (Schmetzer et al., 1997;Schoch et al., 1997) and criteria defined by the International System for Human Cytogenetic Nomenclature (Mitelman, 1995). Patients were categorized in cytogenetic risk groups as described in chapter 2.2.3 (Greenberg et al., 1997;Haferlach et al., 2003). Favorable risk' AML-patients had presented with a t(8;21), t(15;17), inv(16), t(16;16);

'poor risk' AML-patients with -5/5q-, -7/7q-, t(11q23), inv(3), t(3;3), 17p abnormalities or a complex aberrant karyotype (≥3 abnormalities); 'intermediate risk' AML-patients had presented with a normal karyotype or with any of the remaining aberrations. 'Favorable risk' MDS-patients had presented with a normal karyotype, a del(5q) only, a del(20q) only or a –Y only; 'poor risk' MDS-patients had presented with -7/7q aberrations or a complex aberrant karyotype; 'intermediate risk' patients were MDS-patients with any remaining aberration.

3.4 Generation of DC from MNC fractions with 6 different serum-free media

MNC were separated from AML and MDS samples as described in 3.2. Cell counts were quantified and MNC were pipetted in 12-well multi well tissue culture plates in 1ml Xvivo 15 (Bio Whittaker Europe, Verviers, Belgium) FCS-free medium. DC were generated with six DC differentiating media which were chosen according to their different mechanisms to induce blast differentiation towards DC (table 9).

3.4.1 Basic Method (standard medium)

MNC fractions were incubated in 'standard' medium containing GM-CSF, IL-4, TNFα and FLT3-ligand. FLT3-ligand has been shown to act synergistically with other cytokines and increases yields of DC after culture (Woiciechowsky et al., 2001).

$2.5x10^6$ MNC/ml were incubated in medium containing 800 U/ml GM-CSF (Sandoz), 500 U/ml IL-4 (Cell concepts, Umkirch, Germany), 40 ng/ml FLT3-ligand (PromoCell) for 10-12 days, adding 200 U/ml TNFα (Cell concepts) for the last two days (Kufner et al., 2005a;Woiciechowsky et al., 2001).

3.4.2 MCM Mimic

This cytokine-based DC-differentiation method is a defined cocktail of recombinant cytokines and PGE_2 mimicking the components of monocyte-conditioned medium ('MCM mimic') including TNFα, IL-1β, IL-6. PGE_2 is known to improve yields of DC, especially mature DC, and function of DC, e.g. migration capacity (Lee et al., 2002).

$2.5x10^6$ MNC/ml were incubated in medium containing 800 U/ml GM-CSF, 500 U/ml IL-4 and 40 ng/ml FL; the same cytokines were added after 4-5 days again. Half medium exchange was performed on day 7 or 8. At this time 150ng/ml IL-6 (Cell concepts), 5 ng/ml IL-1β (Cell concepts), 1µg/ml PGE_2 (Pfizer, Vienna, Austria) and 5ng/ml TNFα were added to the medium. Cells were harvested on day 10-12 (Lee et al., 2002;Woiciechowsky et al., 2001).

3.4.3 Picibanil (OK-432)

OK-432 is a biological response modifier (BRM) derived from the Su strain of Streptococcus pyogenes. This bacterial lysate stimulates immature DC towards maturation and can improve production of Th-1-type cytokines, e.g. IL-12, especially in combination with PGE_2 (Sato et al., 2003).

$1\text{-}1.25x10^6$ MNC/ml were incubated in medium containing 500 U/ml GM-CSF and 250 U/ml IL-4 and in addition OK-432 (Chugai Pharmaceuticals, Kamakura City, Japan). After 7-8 days in culture, 10µl/ml OK-432 and 1µg/ml PGE_2 were added. Cells were harvested after 9 to 11 days in culture (Sato et al., 2003).

3.4.4 Cytokines

MNC fractions were incubated in a medium containing a conventional cocktail of cytokines for DC differentiation: GM-CSF, TNFα, IL-3, SCF, FLT3-ligand and IL-4.

$2.5x10^6$ MNC/ml were incubated with a cytokine cocktail containing 250 U/ml GM-CSF, 50 U/ml TNFα, 20 ng/ml IL-3, 50 ng/ml SCF (Cell Concepts) and 50 ng/ml FLT3-ligand. Half medium exchange was performed every 3 or 4 days. At day 7, 250 U/ml IL-4 were added. Cells were harvested after 13 to 15 days in culture (Westers et al., 2003).

3.4.5 Poly (I:C)

Poly(I:C) is a synthetically fabricated double-stranded RNA acting through TLR3 expressed on DC. Poly(I:C) functions as a ‘danger signal’ and induces DC-differentiation with high levels of IL-12 (Rouas et al., 2004).

$2x10^6$ MNC/ml were added to culture with 800 U/ml GM-CSF and 1000 U/ml IL-4. After 6 to 7 days, 30 µg/ml poly(I:C) (Sigma-Medizin-Technik) were added, cells were harvested 48 hours later (Rouas et al., 2004).

3.4.6 Calcium Ionophore (A23187)

The Calcium Ionophore A23187 is a DC-differentiating substance bypassing the cytokine-driven DC-differentiation.

$7x10^5$ MNC/ml were incubated in medium containing 375ng/ml A23187 (Sigma-Medizin-Technik) and 250 U/ml IL-4 for 3-4 days (Houtenbos et al., 2003).

All of the substances used for DC-generation are approved for human treatment.

Table 9: Survey of 6 different DC-generating methods for AML and MDS

DC-generating method	**DC-differentiation stimulating substances**	**mode of action**	**culture time**	**references**
'Basic Method'	GM-CSF, IL-4, TNFα, FLT3-ligand	differentiation towards DC in combination with stem cell driven DC differentiation	7-14d	Woiciechowsky 2001
'MCM-Mimic'	GM-CSF, IL-4, TNFα, IL-1β, IL-6, PGE2, FLT3-ligand	cytokine-based DC-differentiation, PGE_2 increases CCR7-expression and improves migration	10-14d	Lee 2001
'Cytokines'	GM-CSF, TNFα, IL-4, IL-3, SCF, FLT3-ligand	cytokine-based DC-differentiation via cytokine receptor expression on leukemic cells	10-14d	Westers 2003
'Picibanil'	GM-CSF, TNFα-lysat from Streptococcus pyogenes, PGE2	bacterial lysate and PGE_2 stimulate DC differentiation	7-8d	Sato 2003
'Poly(I:C)'	GM-CSF, IL-4, Poly(I:C)	double stranded RNA as 'danger signal' induces DC differentiation with high IL12-release	8d	Rouas 2004
'Ca-Ionophore'	IL-4, A23187	bypass of cytokine-driven DC differentiation	2-3d	Houtenbos 2003

GM-CSF granulocyte-macrophage colony-stimulating factor. IL interleukin. TNF tumor necrosis factor. PGE prostaglandine. SCF stem cell factor.

3.5 Flow cytometry

With regard to the patient's initial diagnose and blast phenotype, a basic FACS analysis was performed in each case of AML and MDS, which allowed to quantify fractions of B-, T-, NK-cells and monocytes (Rothe and Schmitz, 1996). In MDS cases, amounts of $CD34^+$ cells were regularly analyzed to evaluate amounts of undifferentiated cells/blasts (Buchner et al., 2003;Campana and Behm, 2000).

Flow cytometric analyses with a panel of mouse monoclonal antibodies (moAbs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem Cy5-PE-conjugation (PC5) or Allophycocyanin (APC) were performed to evaluate and quantify amounts and phenotypes of leukemic cells, B-, T- and NK-cells and DC in the PB/BM samples analyzed. To avoid differences in the detection of antigens, we used the same clone of antibodies with the same conjugated fluorochrome in each analysis. Antigens which are normally expressed in rather low degrees on the cell surface were tested with the strong fluorescent fluorochrome PE or PC5. On the other hand, we tested antigens with rather high expression rates on cells with the less potent fluorochrome FITC.

Table 10: Flow cytometric analyses with a panel of mouse monoclonal antibodies (moABs) directly conjugated with the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem Cy5-PE-conjugation (PC5) or Allophycocyanin (APC) were performed to evaluate amounts and phenotypes of various cell subsets, using moAbs purchased from different distributors.

CD antigen	**distributor**	**fluorochrome**
CD1a	BD, BC, Cal	PE, PC5, APC
CD1b	BD	FITC
CD1c	Mil	PE, PC5, APC
CD206	BC	PE
CD25	BC	FITC, PE
CD137L	Phar	FITC, PE
CD86	Cal, Ser	FITC, PE, PC5, APC
CD80	BD, BC	FITC, PE, PC5

CD40	Cal, Ser, BC	FITC, PE, PC5, APC
CD14	BC	FITC, PC5
CD83	BC, BD, Cal	FITC, PE, PC5, APC
CD33	BC	FITC, PE, PC5, APC
CD34	BC, Cal	FITC, PC5, APC
CD45	BC	APC
CD56	BC	PE, APC
CD117	BC	PC5, APC
CD13	Cal	APC
CD4	BC	APC
CD11c	BC	APC
CD45Ra	BC	APC
CD71	BC	PE, APC
CD3	BC	FITC, PE, PC5
CD15	BC	PE, PC5
CD19	BC	PE
CD28	BC	FITC
CD137	BD	PE
CD152	BC	PE
CD154	BD	FITC
CCR7	R&D	PE
CD209	R&D	FITC
HLA-Dr	BC	FITC, PC5
CD7.1	BC	PE

BC Beckman Coulter. Ser Serotec. BD Becton Dickinson. Phar Pharmingen. Mil Milteny Biotec. Cal Caltag.

Antibodies were purchased from Becton Dickinson (Heidelberg, Germany), Immunotec/ Beckmann Coulter, Milteny Biotec (Bergisch Gladbach, Germany), Caltag, Serotec (Duesseldorf, Germany), R&D and Pharmingen (table 10).

MNC or cultured cells were suspended in PBS with 20% FCS (Biochrome) and incubated with moAbs according to the respective manufacturer's instructions. Appropriate isotype controls were applied (<3% positive cells were tolerated). At least 5000 events were evaluated on a FACS Calibur Flow Cytometer (Becton Dickinson, BD) using Cell Quest data acquisition and analysis software (BD). For analysis and quantification of lymphocytes, monocytes and leukemic cells before culture, total MNC fractions were gated. CD antigens used for quantification of different cell subsets in naïve MNC fractions were CD3 for T-cells, CD14 for monocytes and CD19 or CD20 for B-lymphocytes. NK-cells were defined as $CD3^-$ /$CD56^+$ cells. An AML sample was considered as `positive´ for a leukemic surface marker, if the percentage of positive events in a gate surrounding blasts, lymphocytes and monocytes was more than 20% (Campana and Behm, 2000). Proportions of positive events in defined gates compared with the isotype controls were calculated using CellQuest Software (BD). Leukemic populations showed typical patterns in the Dot-Plot-picture 'Forward-Scatter' (y-axis) versus 'Sideward-Scatter' (x-axis) analogous to their cellular characteristics like cell size and granularity. For counting of cells a special gating strategy was applied (see next chapter).

3.5.1 Quantification and characterization of DC

3.5.1.1 Special gating strategy

DC were generated as described, harvested, counted and quantified by FACS analysis. For analysis and quantification of DC and especially leukemia-derived DC, a refined gating strategy was applied (Schmetzer et al., 2007). This strategy takes into consideration different scatter profiles of blasts and DC: DC are characterized by a different fluorescence behavior than unconverted blasts. As a consequence, the quantification of DC and remaining leukemic blasts is not possible within one analyzing gate (Figure 2a). Therefore, a blast gate (equal to the MNC gate on day 0) surrounding blasts, residual lymphocytes and monocytes was defined individually in every cases of AML and MDS to characterize remaining cells after culture. Using this special gating strategy, a sensitive detection and quantification of non-converted blasts, of leukemia-derived DC and of DC without proof of leukemic derivation is possible.

Figure 2: Convertibility of blasts to leukemia-derived DC (DC_{leu}) was quantified by FACS analysis with a special gating strategy.

(a) Blast- and DC-marker profiles before and after DC-culture

Given is schematic presentation of a blast population before (left side) and after conversion to DC (right side). Convertibility of blasts to leukemia-derived DC (DC_{leu}) can be demostrated: conversion of naïve blasts expressing individual blast antigens (e.g., CD34, CD117) to DC can be detected after culture by the gain of DC antigens (e.g. CD80, CD86, CD1a) (right side) that had not been expressed on naïve blasts. Using coexpression analyses of blast and DC antigens, non-converted blasts (expression of blast markers, no expression of DC markers), DC_{leu} (double positive cells expressing blast markers and DC antigens) as well as non-leukemia-derived DC (single positive cells without the expression of blast markers) can be differentiated.

Blast cell population before culture

Blast/DC populations after culture in DC differentiating media

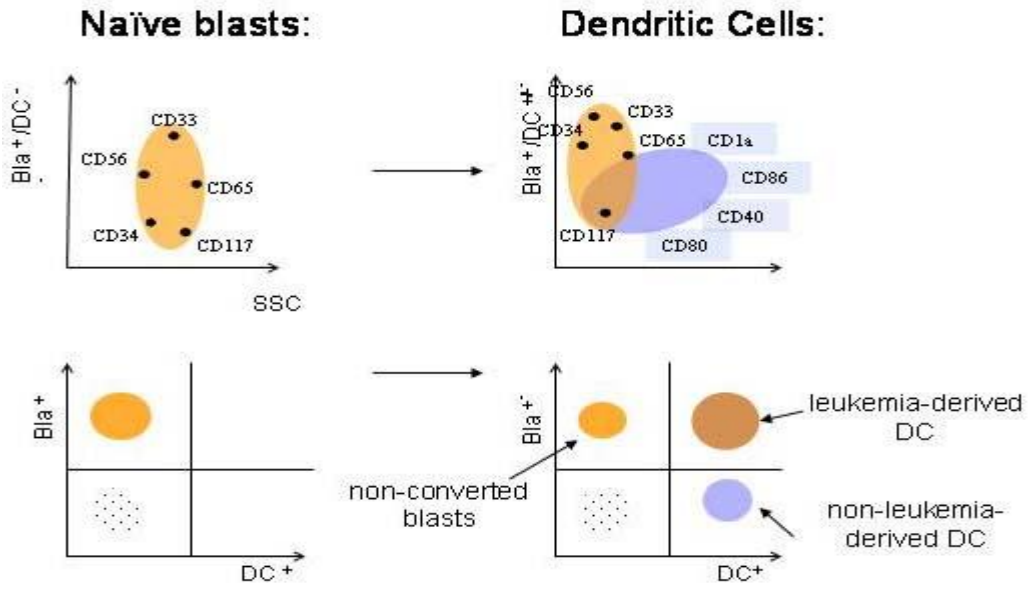
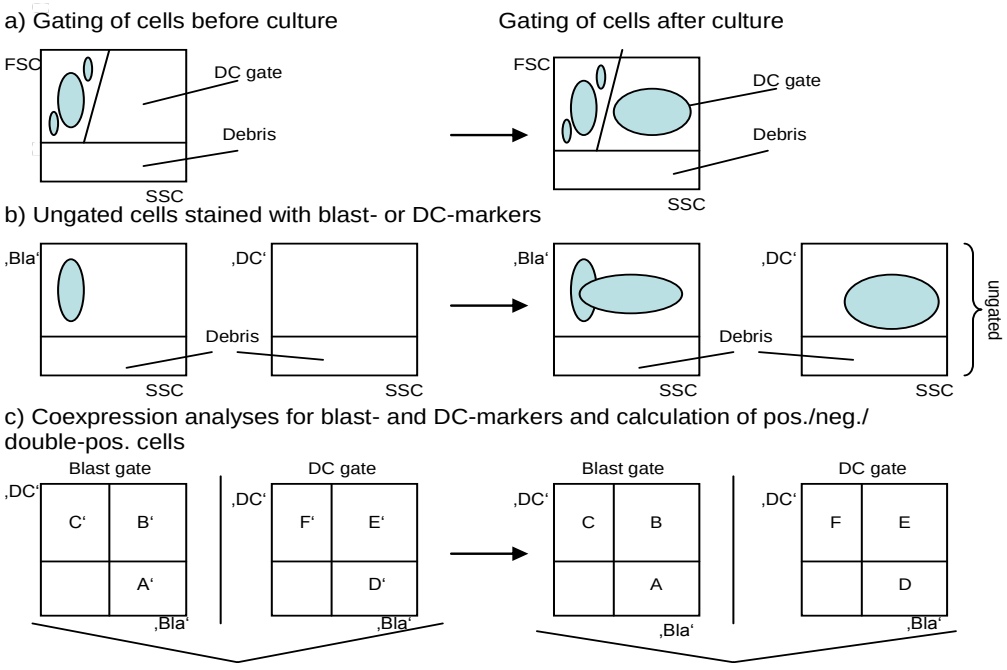


Figure 2 (b) Gating strategy to quantify DC, DC_{leu} and not to DC_{leu} converted blasts

Given is a schematic presentation of the applied special gating strategy to estimate amounts of DC, DC_{leu} and non-converted blasts using an individual blast gate and DC gate: proportions of blasts before culture (left side) and of DC, DC_{leu} and not to DC_{leu} converted blasts can be estimated by coexpression analyses for blast markers and DC markers.



Σ blasts = Σ (A'+D')

Σ DC = Σ (C'+B'+F'+E')*

* per definition < 7% in ungated setting

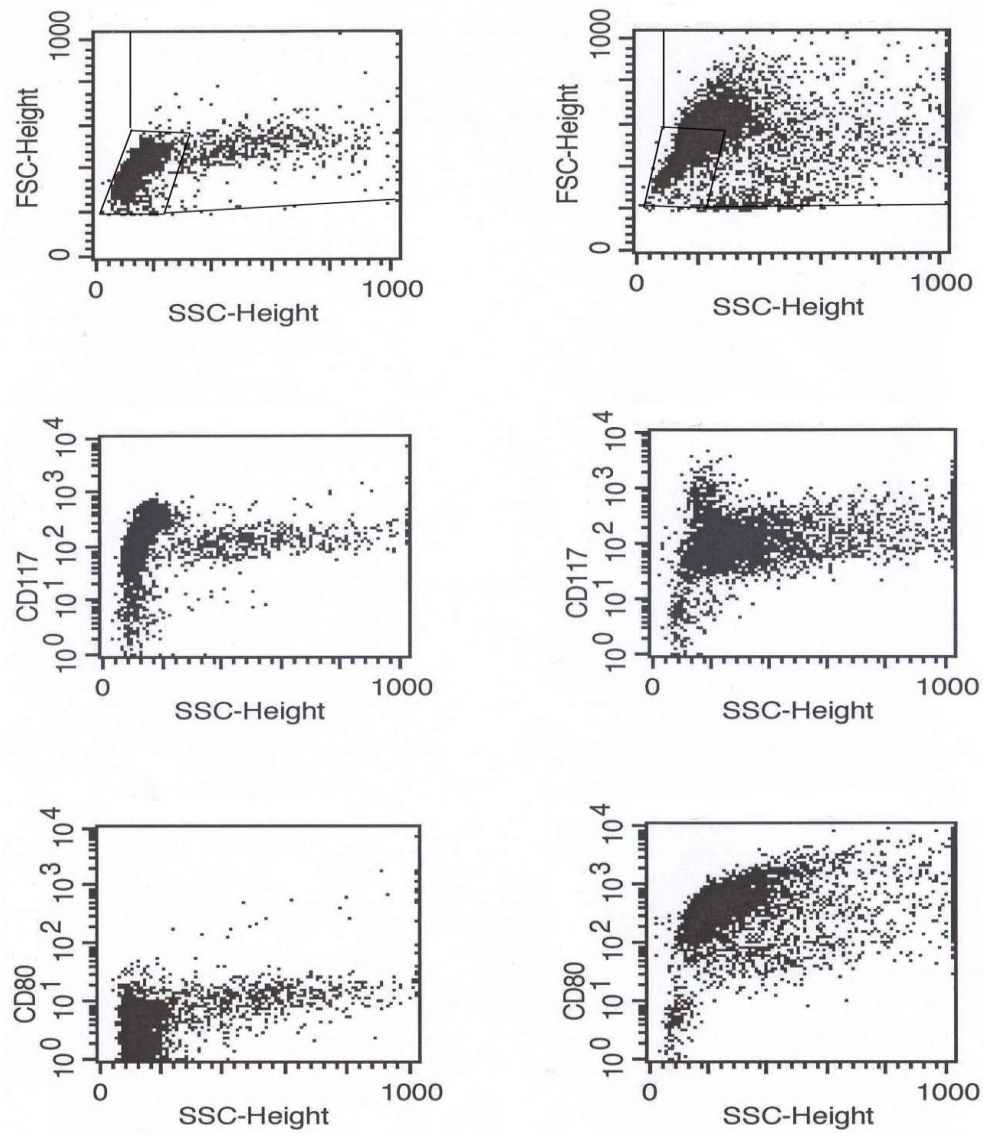
Σ blasts (not converted to DC) = Σ (A+D)

Σ DC = Σ (C+B+F+E)

Σ DC_{leu} = Σ (B+E)

Figure 2 (c) Convertibility of blasts to leukemia-derived DC (DC_{leu}) in a case of AML-M2

Convertibility of blasts to leukemia-derived DC (DC_{leu}): given is one example of immunophenotyping in a case of AML-M2, showing typical naïve blast cell populations (left side) and blast/DC-populations after culture in DC-differentiating media. The upper column shows FSC/SSC projections demonstrating the gain of higher SSC/FSC values of DC (right side) compared to blasts. Figures in the middle column demonstrate the (incomplete) conversion of CD117+ blasts (left side) to DC_{leu} (right side). Figures in the third column demonstrate the gain of CD80-positivity of converted blasts (right side).



FSC forward-scatter. SSC sideward-scatter. Bla^+ cells with positive expression rates for blast antigens. DC^- cells with negative expression rates for DC-antigens. DC_{leu} leukemia-derived DC.

Figure 2a schematically presents antigen characteristics of the involved cells: naïve blasts are characterized by the expression of patient-specific antigens (e.g. CD34, CD56, CD33, CD34) (figure 2a upper left). These cells can be presented in a dot plot analysis by their expression of blast markers (e.g., CD117), but no expression of DC markers (e.g., CD80) (figure 2a lower left). After culture with DC-differentiating methods, varying proportions of non-converted blasts can be detected (expression of blast markers, no expression of DC markers), characterized by a low sideward scatter (SSC) (figure 2b upper and lower right). Moreover, DC without proof of leukemic origin can be detected (expression of DC markers, but no coexpression of blast markers), characterized by a high SSC. Leukemia-derived DC (DC_{leu}) are characterized by a coexpression of blast markers and DC markers (figure 2a upper and lower right).

Figure 2b schematically presents applied 2-step gating strategy to estimate amounts of DC, DC_{leu} and non-converted blasts using an individual blast gate and DC gate: proportions of blasts before culture (left side) and of DC, DC_{leu} and not to DC_{leu} converted blasts can be estimated by coexpression analysis for blast markers and DC markers: the first gate surrounds the blast population characterized by a low FSC/SSC, the second gate surrounds all cells outside this gate. Debris and dead cells were excluded from analysis. For both gates, isotype controls were applied to separate positive from negative cell fractions in both gates. Positive events were quantified separately in each gate and summarized at the end (figure 2b column c). By applying coexpression analyses with a patient-specific blast marker and a DC-marker that had not been expressed on naïve MNC, a sensitive quantification of non-converted blasts, DC_{leu} and of DC without proof of leukemic origin is possible (figure 2b column c): to determine cell counts, the results of the two different gates were summed up. Amounts of DC_{leu} can be estimated by summing up double-positive cells in both gates that express a blast marker and a DC marker after culture (figure 2b column c right side). Amounts of DC after culture can be estimated by summing up single positive cells for a DC marker (DC+) in both gates (figure 2b column c, right side).

Figure 2c gives an example of immunophenotyping in a case of AML M2. Our special gating strategy was applied using a specific combination of the patients' individual blast marker with a DC marker that was not expressed on uncultured MNC. Before culture, a blast population with a low forward scatter (FSC) and SSC could be detected (figure 2c column A, left side),

that was characterized by the expression of CD117 (figure 2c column B, left side), but without expression of the DC marker CD80 (figure 2c column C, left side). After DC culture, a shift of the initial blast population to a population characterized by a higher FSC and SSC profile could be demonstrated (figure 2c column A, right side). By immunophenotype analysis, a cell population with a high SSC could be detected, which had gained positivity for the DC marker CD80 (figure 2c column C, right side). Staining of cells with the individual blast marker CD117 showed CD117-expression on a big proportion of the cell fraction with the high SSC (resembling DC with leukemic antigen presentation). However, a cell population expressing CD117 with a low SSC could be detected, representing a blast population without conversion to DC (figure 2c column B, right side). For the exact quantification of blasts, of DCleu , and of blast convertibility to DCleu, we applied our 2-step gating and quantification strategy: the first gate surrounds the blast population characterized by a low FSC/SSC (figure 2c column A, left side). The second gate surrounds all cells outside this gate (figure 2c column A, right side). For both gates, isotype controls were applied to separate positive from negatve cell fractions in both gates. Positive events were quantified separately in each gate and summarized at the end, as described for figure 2b. This contributes to a refined quantification of positive cells, subdivided in blasts, DC without proof of leukemic origin, and DC_{leu}.

To characterize the blasts' convertibility to DC_{leu} as well as proportions of DC_{leu} cells, only markers expressed on leukemic cells but not on DC (e.g. CD34, CD117, CD56, CD7, CD65) qualified for evaluation (Figure 2b left side, Figure 2c upper left). As described above, amounts of converted blasts were determined by counting of cells that expressed a specific blast marker and gained a DC marker after culture. In some cases, no specific blast marker was available. In those cases (with less than 5% $CD14^+$ cells in the naïve MNC fraction as a precondition), CD33 or CD13 were used to determine proportions of converted blasts. After culture, cells that expressed at least one typical DC surface marker (e.g. CD40, CD80, CD1a, CD86) which was not detectable on naïve blasts on day 0 (per definition $< 7\%$) were considered as DC (Schmetzer et al., 2007). In AML cases, DC_{leu} were defined as the proportion of DC coexpressing a specific leukemic surface marker (Figure 2b and 2c right side). After culture, a specific combination of the patient's individual blast markers with DC-antigens that were not expressed on uncultured MNC allowed the detection and quantification of DC_{leu} and of blasts that could not be converted to DC_{leu} (Figure 2b, Figure 2c lower right). In MDS cases with >10% $CD34^+$ or $CD117^+$ cells, those markers were used to evaluate proportions of converted blasts. Beside the described quantification of DC, amounts of

'optimum of DC' (DC_{opt}) were evaluated: for each individual AML and MDS sample, a DC_{opt} marker was evaluated, defined as the DC marker with highest expression rates on cultured MNC fractions (and without expression on naïve MNC). DC_{opt} describes the amount of cells after culture expressing the patients' individual DC_{opt} marker.

Moreover, counts of DC were estimated by microscopical counting of MNC before and after culture.

3.5.1.2 Analysis of viability, maturity and migratory capacity of DC

To evaluate amounts of viable DC after culture, 7-amino actinomycin D (7AAD) staining was used, which allows concurrent cell surface staining and a clear distinction between the cell subsets: populations of dead (7AAD-bright), apoptotic (7AAD-dim) and viable (7AAD-negative) cells can be quantified (Philpott et al., 1996). Viable DC were defined as $DCA^+/7AAD^-$ cells. Mature DC were characterized by coexpression of a selected DCA and CD83 (Bender et al., 1996). The migratory capacity was determined by coexpression of a DC-marker and CCR7 (Sanchez-Sanchez et al., 2006).

3.5.1.3 Criteria for a successful generation of DC and leukemia-derived DC (DC_{leu})

We postulated at least 10% generated DC and in addition 5% of DC_{leu} (DC that coexpressed blast markers) in the total cell fraction as 'successful' DC generation.

3.6 Mixed lymphocyte culture (MLC)

Positively selected $CD3^+$ T-cells (Milteney Biotech, Bergisch-Gladbach, Germany, 1×10^6 cells/well) from AML, MDS or healthy MNC fractions were cocultured and primed with irradiated (20Gy) AML blast-containing MNC (5×10^4) and in parallel with irradiated DC_{leu}-containing AML MNC (5×10^4) in 1 ml RPMI-1640 medium (Biochrom) containing 15% human serum (PAA) and IL-2 50 U/ml (Proleukin R5, Chiron). For the T-cell priming with DC/DC_{leu}, the whole cell suspension after conversion of blasts to DC was used, resembling a

mixture of DC_{leu}, nonconverted blasts, DC without proof of leukemic origin, and residual lymphocytes and monocytes. Total DC counts were adjusted to $5x10^4$ DC and T-cell counts adjusted to $1x10^6$/well.

Cells were harvested after 10 days of coculture and 2-fold restimulation with $5x10^4$ irradiated DC or $5x10^4$ irradiated blast-containing MNC and supplementation of IL-2 (Kufner et al., 2005a). Half medium exchange was carried out every 3 to 4 days. 6 days after the last restimulation, DC-primed T-cells (T^{*DC}) or blast-containing MNC-primed T-cells (T^{*MNC}) were harvested. By comparing the expression profiles of DC-ligands like CD28, CD137 or CD154 on T-cells before and after contact with DC, and by evaluating the proliferation activity of T-cells, the capability of leukemia-derived DC to activate T-cells was analyzed. T-cell proliferation was calculated by quantification of CD71- and CD28-coexpressing T-cells before and after DC-contact (Nguyen et al., 2003). Results of MLC and fluorolysis assays were provided for my analysis by other members of our group.

3.7 Cytotoxicity (fluorolysis) assay

The lytic activity of effector T-cells was measured by a Fluorolysis assay through counting viable target-cells, labelled with specific fluorochrome-antibodies, before and after effector-cell (E) contact. DC- or blast-containing MNC-primed donor-T-cells obtained from AML and MDS patients were cocultured in 1.5 ml Eppendorf tubes with blast-containing MNC as target-cells (T). The E:T ratio was adjusted to 1:1 and cells were incubated overnight at 37°C and 5% CO_2. Before culture, blast-containing MNC-target cells were stained for 15 minutes with two FITC- and/or PE conjugated `blast` specific antibodies and cocultured for 3 hours with effector cells (T-cells or DC as target cells were stained with T-/DC-specific antibodies). As a control, target- and effector-cells were cultured separately and afterwards mingled with T-cells. To evaluate amounts of viable ($7AAD^-$) target cells and to quantify the cell-loss after 24 h incubation, cells were harvested, washed in PBS and resuspended in a FACS flow solution containing 7AAD (BD, Biosciences Pharmingen) and a defined number of Fluorosphere beads (Becton Dickinson, Heidelberg, Germany). Viable cells were gated in a SSC/7AAD-gate. Afterwards, viable cells coexpressing specific blast markers were quantified, taking into account defined counts of calibration beads as described. Cells were analyzed in a FACS Calibur Flow Cytometer using CELL Quest software (Becton Dickinson,

Heidelberg, Germany). The percentage of lysis was defined as the difference between proportions of viable blasts before and after effector-cell contact (Kienzle et al., 2002;Kufner et al., 2005a).

3.8 Statistical methods

Mean and standard deviation, median and range, two-tailed t-test as well as variance analysis were performed with a personal computer using Microsoft ® Excel 2003 and 2007, WinStat for MS Excel and SPSS® Statistics 17 software. Differences were considered as significant, if the p-value was < 0.05.

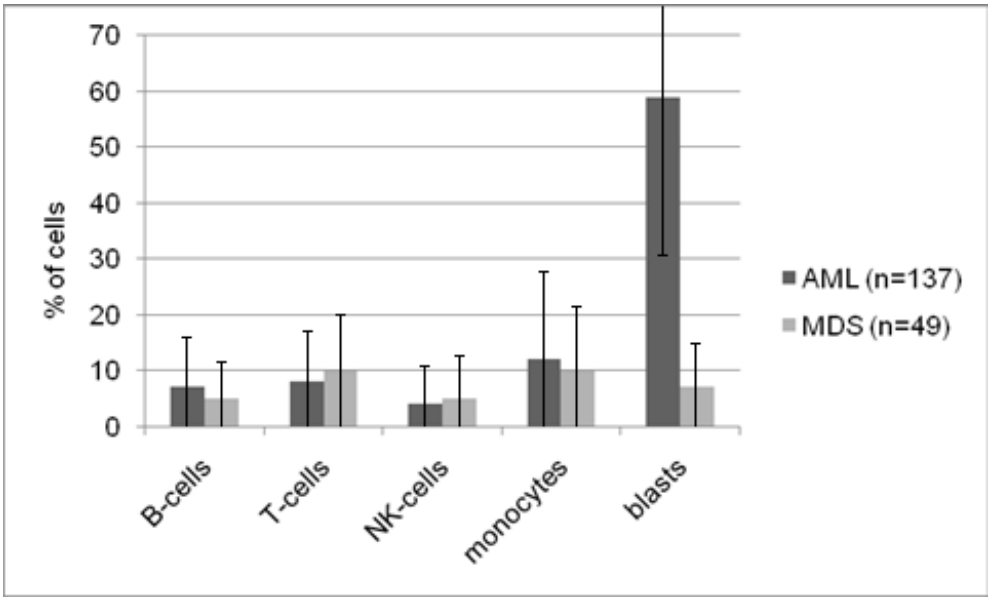
4 Results

4.1 Characterization of MNC fractions obtained from AML, MDS and healthy probands before culture

4.1.1 Cell subsets in MNC fractions before culture

To evaluate cell counts of monocytes, B-cells, NK-cells and blasts in AML, MDS and healthy MNC fractions, 137 AML, 49 MDS and 43 healthy samples were analyzed via flow cytometry before DC culture. On average, AML samples presented with 6 (±9)% B-cells, 8 (±9)% T-cells, 4 (±6)% NK-cells, 12% monocytes and 58(±16)% blasts (figure 3); in MDS samples, 5 (±6)% B-cells, 11 (±10)% T-cells, 5 (±7)% NK-cells, 10 (±11)% monocytes and 7 (±7)% blasts could be detected (figure 3). In MNC fractions obtained from healthy probands (n=43), on average 7% B-cells could be detected (NK-cells not available) (data not shown). Average counts of T-cells (37%) and monocytes (31%) were significantly higher compared to AML and MDS samples ($p < 0.05$).

Figure 3: Different cell subsets were evaluated in 137 uncultured AML and 49 uncultured MDS MNC fractions: amounts of B-cells ($CD19^+$, $CD20^+$), T-cells ($CD3^+$), NK-cells ($CD56^+$), monocytes ($CD14^+$) and blasts (specific blast markers), given are average results (mean ± standard deviation).

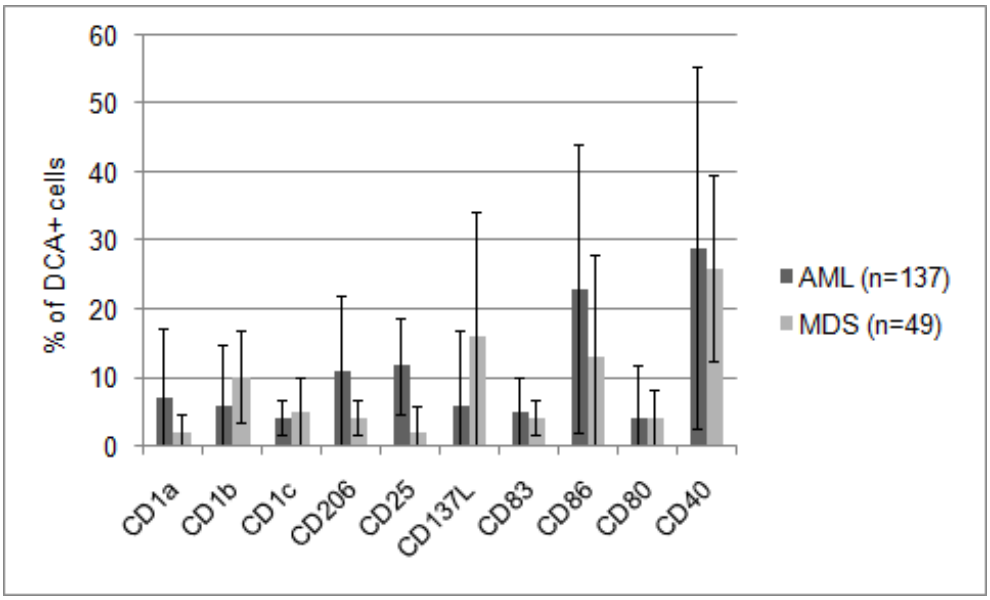


4.1.2 DCA are regularly expressed on uncultured AML and MDS MNC fractions with variations in subtypes

Expression of DCA on individual AML and MDS samples before culture was analyzed (figure 4). On average, 4% (CD1c) to 29% (CD40) DCA^+ cells in AML, 2% (CD1a and CD25) to 26% (CD40) DCA^+ cells in MDS samples (figure 4) and 3% (CD83) to 43% (CD40) DCA^+ cells in healthy samples (data not shown) could be detected in uncultured MNC fractions.

Different DCA are expressed in varying degrees on uncultured MNC fractions. Highest average expression rates could be demonstrated for CD40 on uncultured AML and MDS samples (29±27% and 26±14%). High average expression rates were also found for CD86 (23±21% in AML, 13±15% in MDS samples) and CD137L in MDS samples (16±18%). All groups presented with low expression rates especially for CD1a (2-7%), CD1c (4-5%), CD83 (3-5%) and CD80 (4%). However, no significant differences between expression rates of different DCA were found (AML: p = 0.95; MDS: p = 0.81)

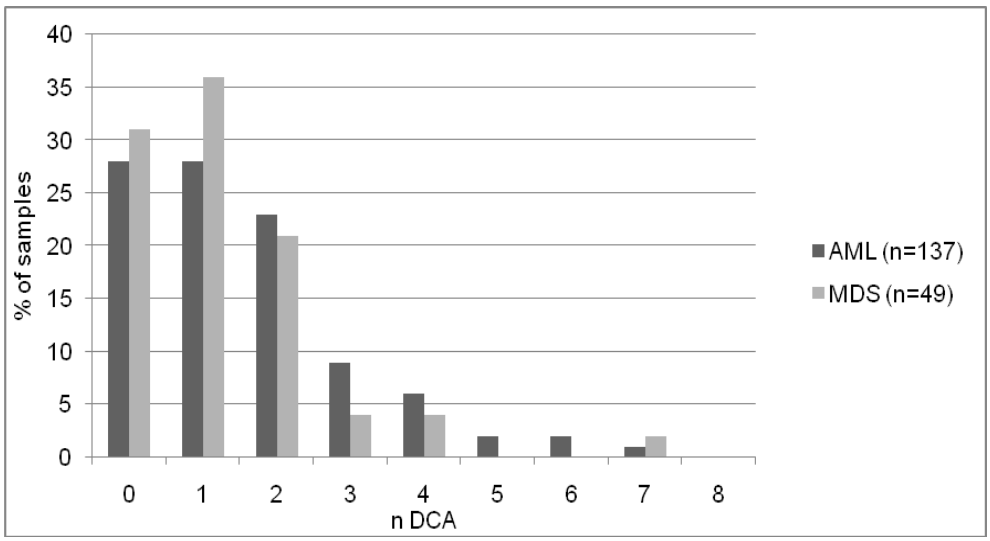
Figure 4: Proportions of 10 different DCA were analyzed in 137 uncultured AML and 49 MDS MNC fractions. Given is the average (± standard deviation) of DCA^+ cells in the total cell fraction.



DCA+ DC-antigen-positive cells. No bar shown: no data available.

As already described in the 'Materials and Methods' section, DCA only qualify to be used for detecting leukemia-derived DC after culture if they are not expressed on uncultured MNC fractions (per definition < 7% DCA^+ cells). Thus, not only average DCA expression rates were evaluated before culture, but also the number of negative and positive DCA for every individual sample. Data presented in figure 5 show that 39 of 137 analyzed AML samples (28%) and 14 of 49 tested MDS samples (31%) presented with less than 7% DCA^+ cells defined as 'negative' for every DCA. Vice versa, 70% of all tested samples expressed at least 1 DCA, some of them 3 or more DCA. About 50% of AML and MDS samples expressed 1 or 2 DCA. One AML and one MDS sample expressed 7 of the 8 tested DCA. In 37 of the 43 healthy samples, only 3 DCA were available. In this group, 35 of 37 samples were positive for 1 DCA before culture. 2 samples showed no expression of any DCA (data not shown). In 6 cases of healthy persons, 9 DCA were available before culture. In this group, every case showed positive expression rates for at least 2 DCA.

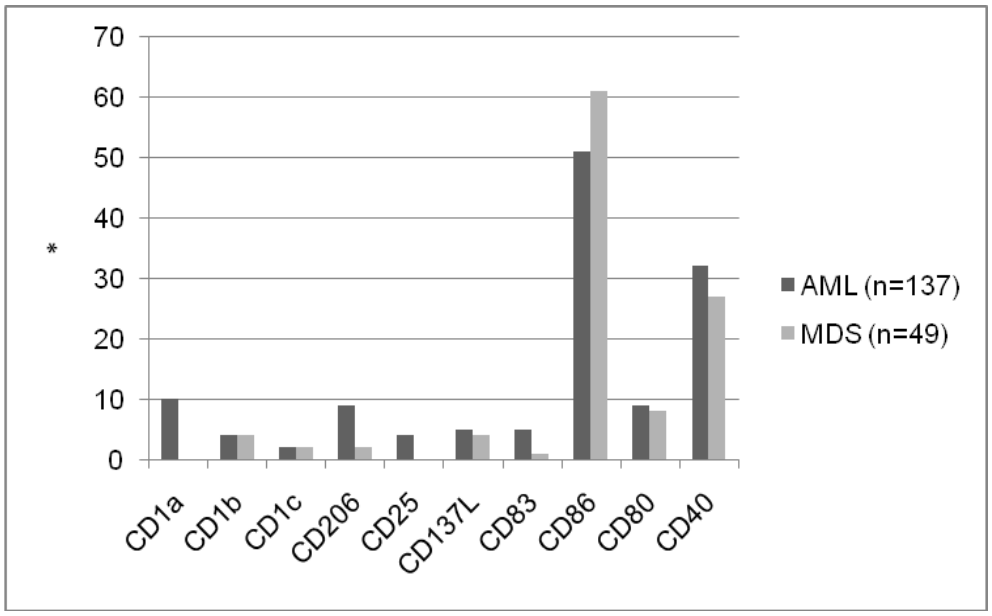
Figure 5: In each individual case of 137 AML and 49 MDS samples, the number of DCA that were already expressed on naïve MNC fractions (per definition >7% of cells) was evaluated. It could be demonstrated that uncultured AML and MDS samples can express up to 7 DCA.



n DCA number of DCA that are expressed on one individual AML or MDS sample.

A detailed analysis was performed for each tested DCA to evaluate the frequency of positive expression rates of this marker on uncultured samples. More than half of both AML and MDS samples presented with positive expression rates for CD86, and about one third of the samples presented with positive CD40 expression rates. On the other hand, only up to 10% of naïve AML and MDS samples showed positive expression of any of the other tested DCA. No case of MDS was found with positive expression of CD1a or CD25 before culture.

Figure 6: 137 naïve AML and 49 MDS MNC fractions were analyzed with regard to either positive or negative expression rates for 10 different given DCA: positive expression of a DCA was defined as >7% DCA^+ cells in an uncultured MNC fraction.

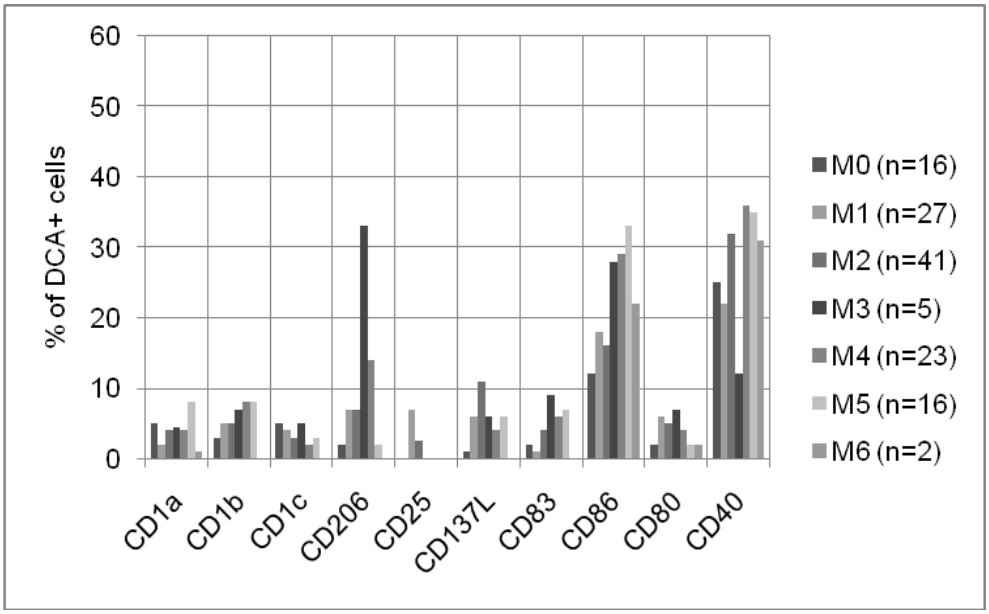


* % of uncultured AML or MDS samples with DCA expression rates >7% for the given marker.

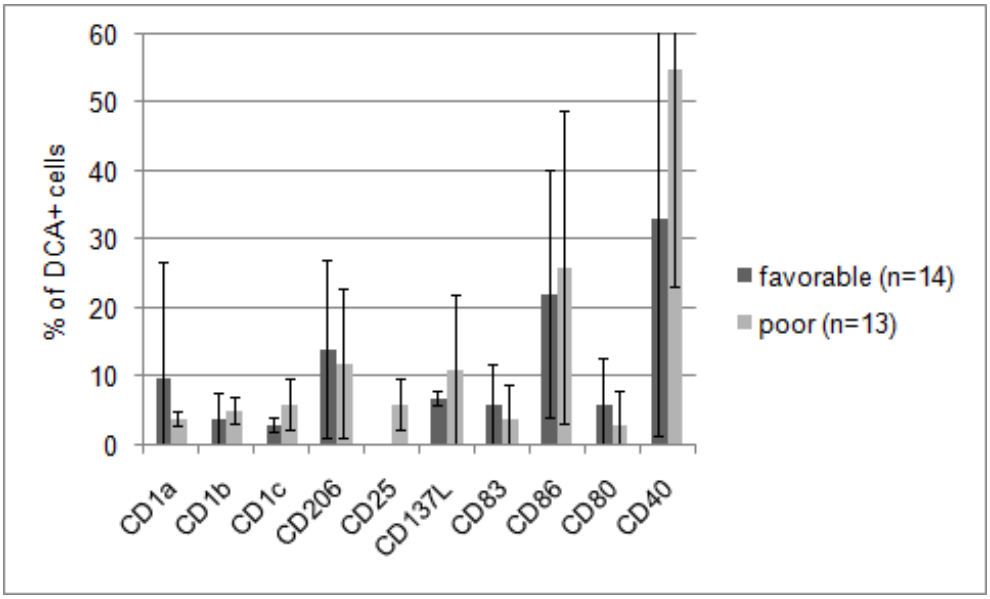
Subdividing the AML patients' cohort in FAB subtypes, expression rates for CD86 ranged from 12(±11)% in M0 to 33(±21)% in M5 cases, for CD137L between 1(±1)% in M0 and 11(±17)% in M2 samples (figure 7a). All FAB subtypes presented with rather high expression rates for CD86 and CD40 (between 12% in M3 and 35% in M5 cases). CD206 expression rates were much higher in M3 cases compared to other FAB subtypes, whereas expression rates of other DCA like CD1b, CD1c and CD80 were quite similar in the different FAB groups (< 10%).

Figure 7: Expression profiles of 10 different DCA (CD1a, CD1b, CD1c,CD206, CD25, CD137L, CD83, CD86, CD80, CD40) in uncultured AML MNC fractions were analyzed depending on FAB subtype (a) and cytogenetic risk group (b). DCA are expressed in varying degrees on uncultured AML MNC fractions (n=137) depending on FAB subtypes (a) and cytogenetic risk groups (b). Given are average results.

(a)



(b)



No bar shown: no data available.

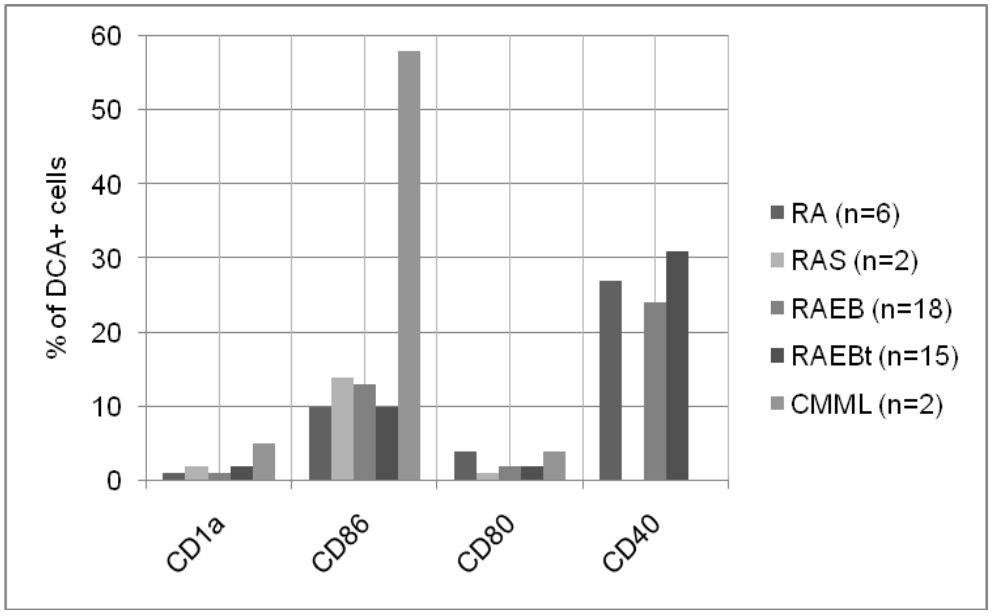
However, average expression rates of single DCA in individual cases were very variable. On average, when pooling all expression results, DCA expression on uncultured AML MNC was similar, and low in the poor cytogenetic risk group, compared to the favorable cytogenetic risk group (13% vs. 11% DCA^+ cells, data not shown). High average proportions of CD40 and CD86 were found especially in the poor risk group (55±32% and 26±15%), whereas expression rates for CD1a, CD1b, CD1c, CD83 and CD80 were rather low in both cytogenetic risk groups (< 10%) (figure 7b). Expression rates of the given DCA did not significantly differ between different cytogenetic risk groups or FAB subtypes (p-value always > 0,05).

Subdividing MDS cases in FAB subtypes, a detailed comparison between MDS FAB subtypes was only available for CD1a, CD86, CD80 and CD40 (figure 8). Expression rates of CD86 on naive MNC fractions did significantly differ between MDS FAB subtypes (p-value 0.02): high average CD86 expression rates in the two CMML cases (58% $CD86^+$ cells) and low expression rates in the six RA cases (10% $CD86^+$ cells) could be demonstrated (figure 8). The remaining FAB subtypes presented with similar average expression rates for CD1a (≤ 5% $CD1a^+$ cells), CD80 (1-4%) and CD40 (24-31% $CD40^+$ cells) (p-value always > 0.05). In MDS cytogenetic risk groups, only 3 DCA were available for a comparable analysis. Average expression rates of CD1a (2% in cases with favourable cytogenetic risk and 1% in cases with poor cytogenetic risk), CD86 (10%/12%) and CD80 (3%/2%) were comparable in both cytogenetic risk groups (data not shown). A more extended differential analysis of DCA expression profiles was not possible due to a low case number and/or low number of tested DCA per sample.

Low proportions of CD1a were found for the cell lines HL60 and Kasumi. HL60 showed an expression of CD86 (84%). The other 3 tested cell lines presented with low proportions of CD86 before culture (between 5% and 7%, data not shown).

In summary, DCA are already expressed in high degrees on naïve MNC fractions in many cases and are expressed in varying degrees in different subtypes of uncultured AML and MDS MNC. Consequently, it is essential to evaluate expression profiles of DCA in individual, uncultured AML or MDS MNC samples to find out those DCA that are not expressed on naïve MNC and can be used for the quantification of DC after culture of MNC.

Figure 8: Expression profiles of 10 different DCA in uncultured MDS MNC fractions was analyzed depending on FAB subtypes. Given are average results (mean).



No bar shown: no data available.

4.2 DCA expression rates and gain of DCA$^+$ cells after conversion of MNC to DC in AML and MDS samples – results of all methods pooled

To compare capabilities of different DC-differentiating media to generate DC and leukemia-derived DC (DC_{leu}), MNC from AML, MDS and healthy donors were cultured in 6 different media. Those media were chosen according to their different modes of action to determine differentiation towards DC, which have already been described in the 'Material and Methods' section. After culture, average DC counts and DC subsets were evaluated with FACS analysis. Only data from samples with DCA expressed on less than 7% DCA$^+$ cells in uncultured MNC fractions qualified for analysis.

Data of a parallel comparison of 6 methods were not available in every case of AML and MDS, in some cases only one or few methods were tested. In this section, results of all methods pooled are given, i.e., data of all patients were included, independent from the number of methods used for culture of DC. A detailed analysis of a parallel comparison of 5

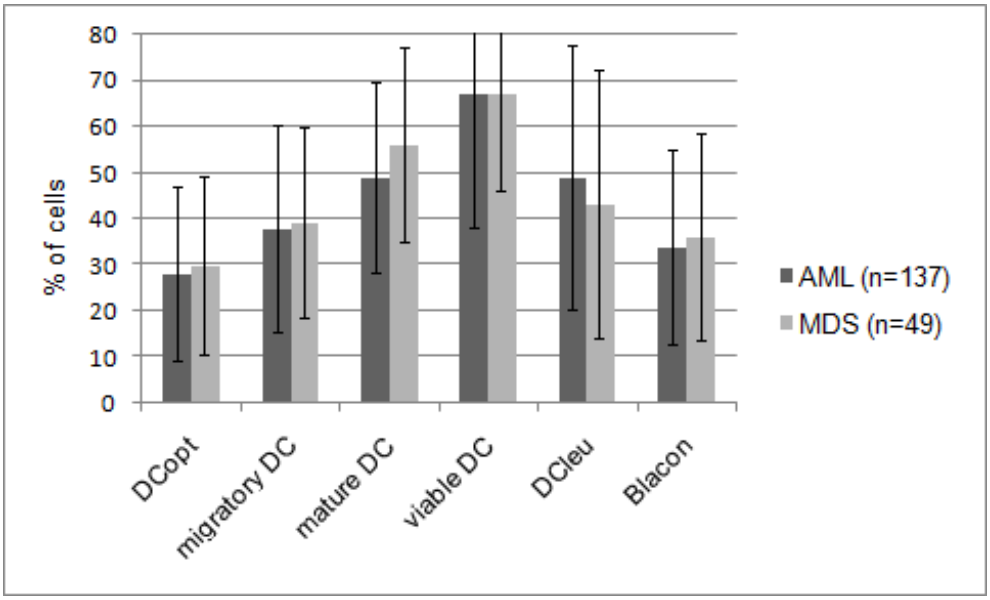
DC-generating media, where data of patients was only included if all 5 methods had been tested, is given in chapter 4.3.

4.2.1 Mature, viable, migratory and leukemia-derived DC (DC_{leu}) can be generated from AML and MDS MNC fractions

4.2.1.1 Comparable average amounts of DC subtypes can be generated from AML and MDS MNC fractions

One of the most important goals of this thesis was to find the 'optimum DC marker' (DC_{opt}) for each individual sample, i.e., the DCA that is not expressed on uncultured blast fractions but with highest expression on cultured DC. Using this 'DC_{opt}-marker' and pooling all results obtained with different DC-differentiating methods, on average 28(±19)% DC_{opt} could be generated from AML samples, (cell lines between 16 and 46%) and 30(±20)% DC_{opt} from MDS samples (figure 9). On average, 49(±23)% mature DC (coexpression of a DCA and CD83) could be generated from AML MNC (AML cell lines: between 24% and 98%) and 56(±24)% from MDS MNC. 38(26)% migratory DC (coexpression of a DCA and CCR7) from AML MNC (cell lines between 6% and 94%) and 39(±23)% from MDS MNC fractions could be generated. Proportions of viable DC ($7AAD^-/DC^+$ -cells) were similar in AML and MDS MNC fractions (both 67%). Quantifying amounts of leukemia-derived DC (DC_{leu}) by the combination of a DCA and an individual blast marker, it could be shown that on average 49(±27)% DC_{leu} could be generated from AML MNC (cell lines: Mutz 3: 85%, Kasumi 2: 71%) and 43(±28)% from MDS-MNC. The convertibility of blasts to DC_{leu} could be estimated by the gain of DCA on the blast population (Bla_{con}). On average, 36% of blasts both in AML and MDS were convertible to DC_{leu} (figure 9).

Figure 9: Quantification of DC from AML and MDS MNC after culture in different media. DC were generated from blast-containing AML and MDS MNC using 6 different DC-generating methods (Basic method, MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly I:C). Quantification of different subsets of DC was performed after culture using combinations of DC, costimulatory, maturation and migration antigens. Average results (mean± standard deviation) are given from all methods pooled. Proportions of flow cytometrically estimated DC_{opt}, migratory DC (CCR7+), mature DC (CD83+), viable (7AAD-), DC_{leu} and Bla_{con} were analyzed in AML (n=137) and MDS (n=49) samples. DC_{opt} optimum of DC, i.e. for each individual sample, the DCA with lowest expression on naïve cells and with highest expression rates after culture was evaluated; DC_{opt} means the percentage of cells that expressed the specific DC_{opt} marker after culture (percentage of cells in the total cell amount).



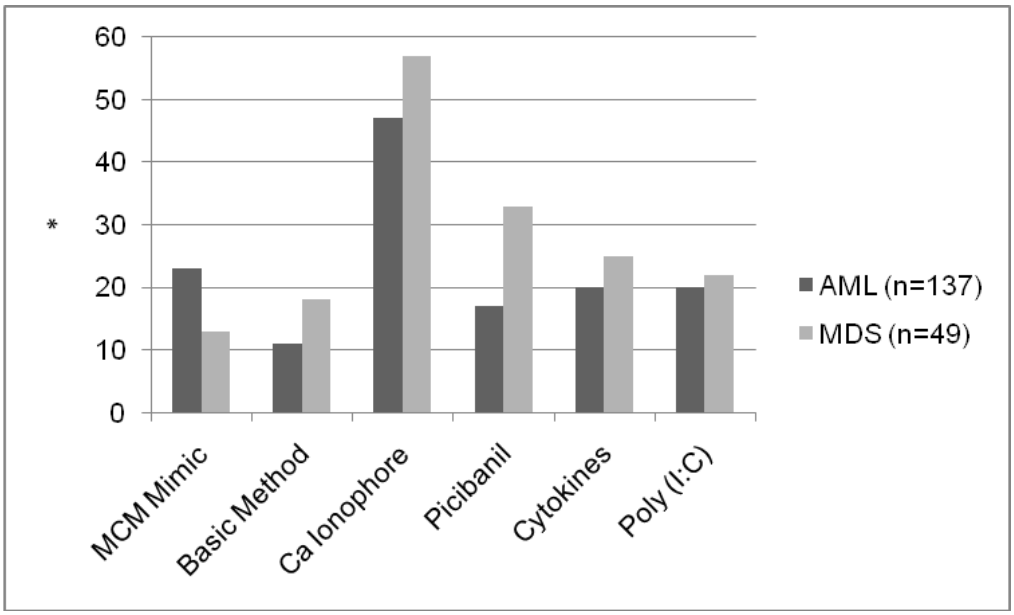
DC_{opt} optimum of DC. DC_{leu} leukemia-derived DC. Bla_{con} converted blasts.

4.2.1.2 Each DC-generating method regularly fails to generate DC, however DC can be generated in any given case after pretesting of a combination of 3 methods

To evaluate the success of DC generation from AML and MDS samples, not only average counts of DC after culture were analyzed. Additionally, the failure rate of each method in DC generation was evaluated: DC generation was defined as successful if more than 10% DC (using the DC_{opt} marker) could be found in cultured AML and MDS fractions. Comparing the different methods, some methods failed more frequently in generating DC than others (figure 10). In AML cases, the failure rate ranged between 11% (Basic method) to 47% (Ca Ionophore), in MDS cases it ranged from 13% (MCM Mimic) to 57% (Ca Ionophore). It

could be shown that by a combination of merely two methods, DC could not be generated successfully in many cases. However, if three or more DC-generating methods were used in parallel, a successful DC generation could be demonstrated in every patient with at least one method.

Figure 10: Failure rate of various DC-generating methods to generate DC successfully (per definition >10% DC using the DC_{opt} marker): for each of the 6 given media for generation of from AML and MDS MNC, the number (and then rate) of cases with less < 10% detectable DC after culture was evaluated.



* failure rate in % of AML or MDS cases.

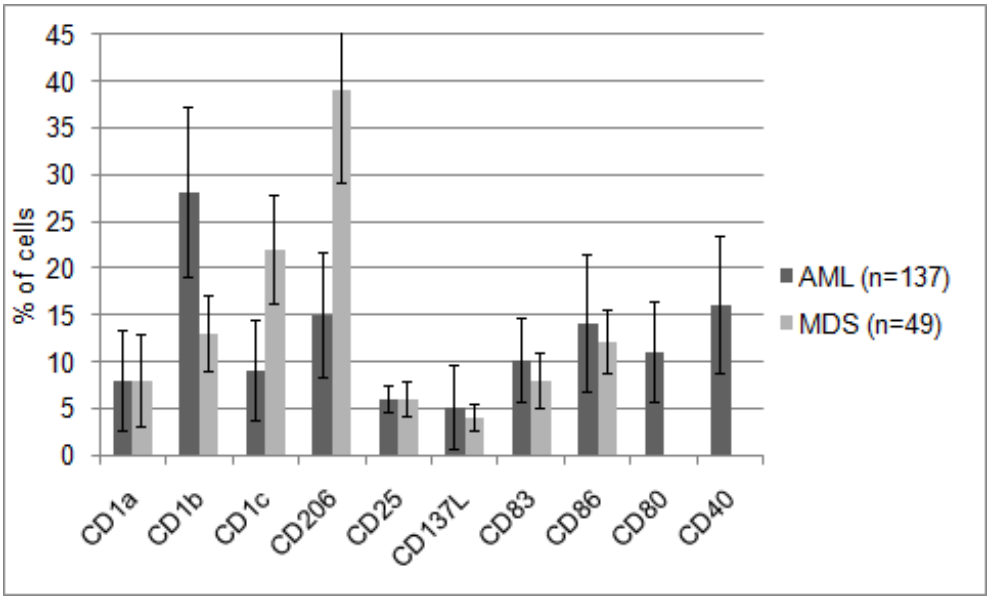
4.2.2 Expression of DCA on cultured AML and MDS MNC is highly variable in FAB subtypes or cytogenetic risk groups

Expression profiles of 10 DCA on cultured AML and MDS MNC fractions were evaluated. Pooling results of all methods, both AML and MDS samples presented with lowest expression rates for CD25 (6%) and CD137L (5%/4%) after culture (figure 11). Highest average expression rates of DCA could be detected for CD1b in AML (28±18%) and CD206 in MDS (39±20%). AML and MDS cases presented with similar expression rates for CD1a (8%), CD25 (6%), CD137L (5%/4%) and CD83 (10%/8%). No significant differences could be shown between DCA expression rates both in AML and MDS samples. Comparing AML and

MDS samples, different expression rates could be shown for CD206 (15±14% in AML samples, 39±20% in MDS samples), CD1b (28±18%/13±8%) and CD1c (8±11%/2212%) ($p < 0.05$).

In healthy samples, average expression rates of DCA were rather low and ranged from 3% (CD137L) to 11% (CD83) (data not shown).

Figure 11: DCA expression in AML and MDS MNC after culture in different media is variable: expression rates of different DCA were analyzed after culture of 137 AML and 49 MDS samples with 6 different methods (Basic method, MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly I:C). Average results (mean ± standard deviation) are given of all methods pooled. Only cases were included with less than 7% DCA^+ cells before culture.



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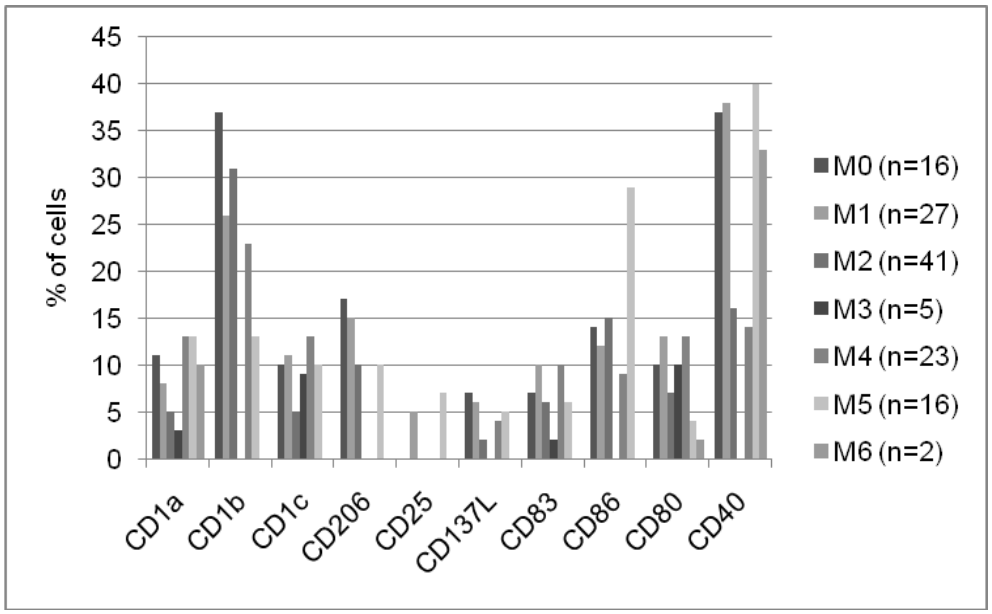
Subdividing AML cases according to FAB subtypes and pooling results obtained with all methods, undifferentiated AML cases (M0, M1) presented with high average expression rates of CD1b (37±16% and 26±18%) and CD206 (figure 12a). Average expression rates of CD1b (13%-37%), CD86 (9%-29%) and CD40 (40%-38%) were rather high in all FAB subtypes, whereas CD25, CD137L and CD83 were expressed in lower degrees on cultured cells, (≤ 10% in all FAB subtypes). All analyzed DCA, except CD137L, were expressed in higher degrees on AML cases with poor cytogenetic risk (no data available for CD40 in the poor

cytogenetic risk group and for CD25), especially CD1b (Ø 37±16%) and CD206 (Ø 24±14%), however, significant differences between AML cytogenetic risk groups could only be demonstrated for CD1a (p 0.02).

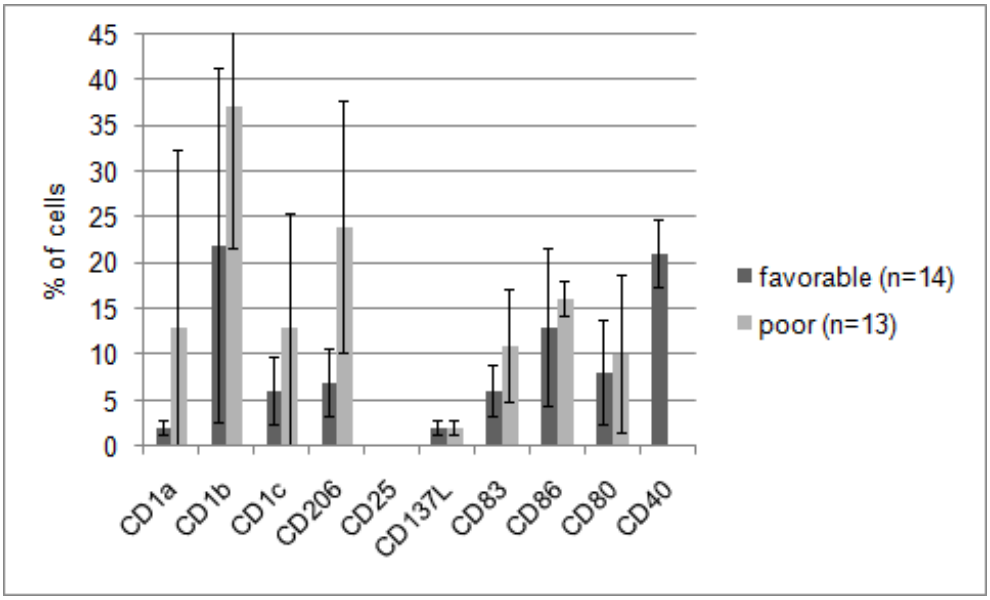
In different MDS FAB subtypes, only few data were available. CD1a showed the highest expression rates in the two cases with CMML (Ø17% $CD1a^+$ cells); CD206 was expressed the highest in the 17 cases with RAEBt (Ø37% $CD206^+$ cells) and the 6 cases with RA (Ø41% $CD206^+$ cells, data not shown). A more extended, differential analysis of DCA-expression profiles (e.g. with the remaining other antigens or subdiving cases in cytogenetic risk groups) was not possible.

Figure 12: DCA expression in AML FAB subtypes (a) and cytogenetic risk groups (b) after culture in different media is variable. Expression profiles of 10 different DCA were analyzed on AML samples (n=137) after culture with 6 different DC-differentiating methods (Basic method, MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly I:C). Average results (mean ± standard deviation) are given, results of all methods pooled. Only cases were included with less than 7% DCA^+ cells before culture.

(a)



(b)



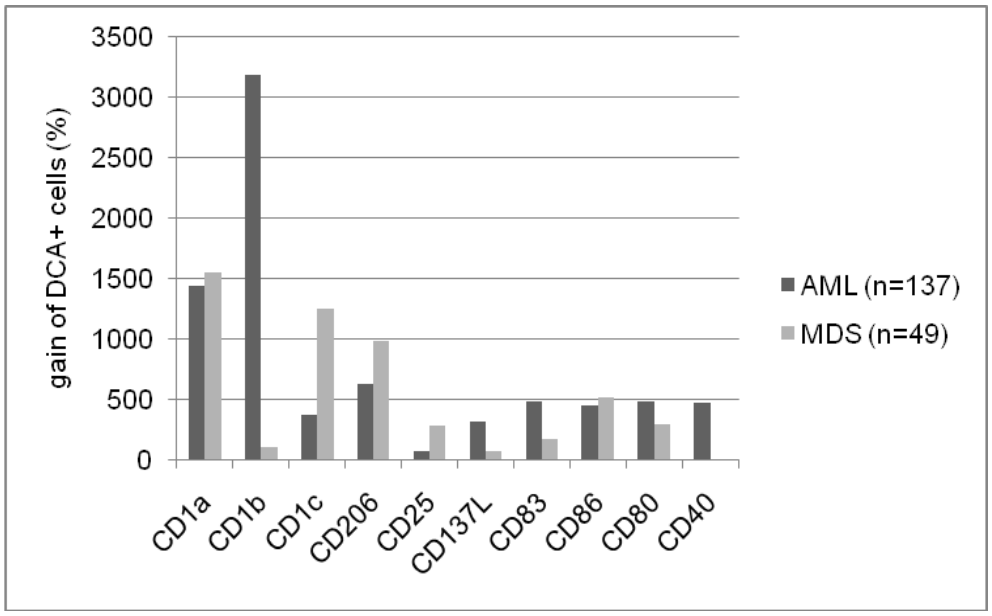
No bar shown: no data available.

4.2.3 Upregulation of DCA after DC culture is highly variable in AML and MDS samples

The gain of DCA^+ cells, demonstrated by cells upregulating distinct DCA after culture in cases with AML and MDS, was analyzed. Only cases with less than 7% DCA^+ cells in uncultured MNC fractions were included. Again, results of various methods given were pooled.

Neither AML nor MDS or healthy samples presented with a loss of DCA^+ cells compared to the expression profiles before culture, although big variations were seen between different DCA (figure 13). The DCA with highest upregulation were CD1b in AML samples (+3190% gain of $CD1b^+$ cells), CD1a in MDS cases (+1550%) and CD80 in healthy samples (+718%) (figure 13). The DCA with lowest upregulation were CD25 in AML cases (+72%) and CD137L (+70%) in MDS cases.

Figure 13: Gain of DCA^+ cells in AML and MDS MNC after culture is variable. The average gain (mean) of 10 selected on 137 AML and 49 MDS samples was analyzed after culture with 6 different methods (Basic method, MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly I:C). Results obtained with different methods were pooled. Only cases were included with less than 7% DCA^+ cells before culture.



DCA+ DCA positive. No bar shown: no data available.

Subdividing cases in FAB subtypes, an upregulation of all DCA in all AML FAB subtypes except for CD137L, CD1a and CD83 in M3 cases could be demonstrated (figure 14a). Highest rates of DCA upregulation could be demonstrated for CD1a in M1 cases (+3090%), CD1b in M2 and M5 cases and CD86 in M3 samples (+1470%).

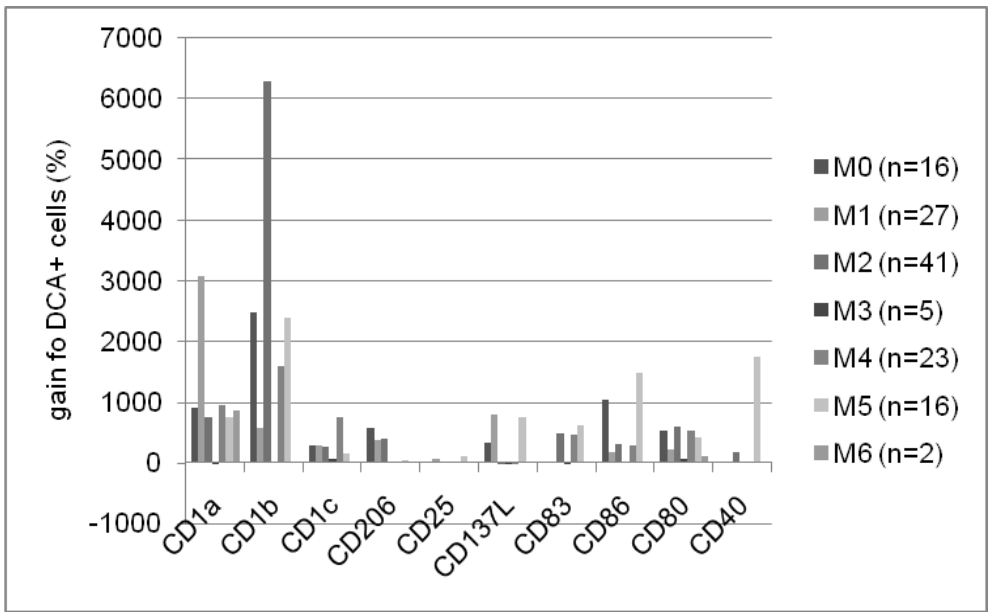
In AML cases, both favorable and poor cytogenetic risk groups showed an upregulation of DCA except CD137L in the favorable risk group (-64%) (figure 14b). Highest yields of gained DCA^+ cells could be demonstrated for CD1b in both AML cytogenetic risk groups (+5400% in cases with favorable cytogenetic risk).

An average upregulation of all available DCA in all MDS FAB subtypes except CD137L in RAEBt could be shown. A more extended, differential analysis of DCA upregulation (e.g. with the remaining other antigens or subdividing cases in cytogenetic risk groups) was not possible due to a low case number.

This means, that except CD1a, CD83 and CD137L in M3 cases and CD137L in RAEBt cases, any of the analyzed DCA principally qualify to be used as ‘markers to detect and quantify DC’ according to criteria to quantify DC.

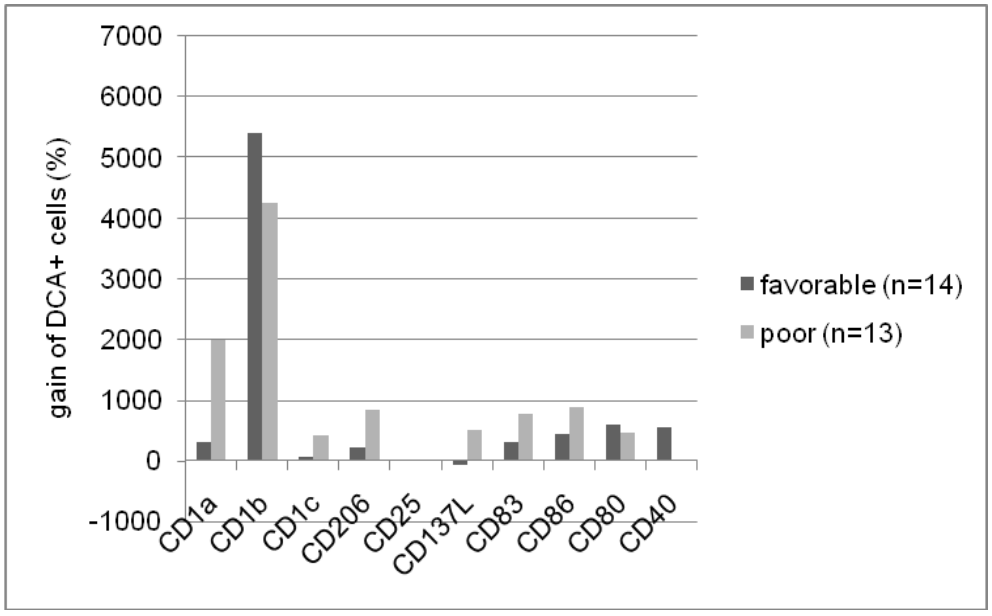
Figure 14: Gain of DCA$^+$ cells in different AML FAB subtypes (a) and cytogenetic risk groups (b). After culture with different DC-differentiating media, the average gain of 10 different DCA (CD1a, CD1b, CD1c, CD206, CD25, CD137L, CD83, CD86, CD80, CD40) was analyzed in 137 AML samples, depending on different FAB subtypes (a) and cytogenetic risk groups (a). All AML were included in which FAB subtype and/or cytogenetic risk group were available, and with less than 7% DCA$^+$ cells before culture. Average results (mean) obtained with all methods pooled are given.

(a)



DCA+ DCA positive. No bar shown: no data available.

(b)



DCA+ DCA positive. No bar shown: no data available.

As the average upregulation of DCA after culture isn't informative concerning the number of upregulated DCA in individual cases, the number of upregulated DCA in every single AML and MDS case as well as the failure rate to upregulate DCA of various methods was analyzed. In AML samples, the Basic method failed in 12 cases, MCM Mimic in 7 cases, and Poly (I:C) in 3 cases, whereas Cytokines, Ca Ionophore and Picibanil each failed in only 1 case to upregulate at least one DCA (data not shown). In MDS samples, the Basic method failed to upregulate at least one DCA in 3 cases, MCM Mimic in 1 case. All other methods were successful in upregulating at least one DCA in every case of MDS. Vice versa, individual AML or MDS cases could be detected where up to 7 DCA were upregulated after DC generation with one method. On average, 3 DCA were upregulated in AML and MDS cases. It can be concluded that with a pretesting of 3 (MCM Mimic, Ca Ionophore, Picibanil) of the 5 tested different media, it was possible to upregulate at least one DCA on one sample in every case of AML, MDS or healthy samples.

Summing up, it is not sufficient to use only one DC-medium to generate DC successfully in every AML and MDS case. Rather, it is necessary to test at least three DC-media in parallel for their potential to differentiate DC. A pretesting of MCM Mimic, Ca Ionophore and Picibanil seems to be the most effective way to create DC in every case of AML and MDS sufficiently.

4.3 Parallel comparison of 5 different DC methods shows comparable average DCA expression rates and upregulation after conversion of MNC to DC in AML, MDS or healthy samples

It has been shown that in 6%-60% of healthy, AML or MDS cases, less than 10% of DC could be generated. In order to find at least one DC-generating method for every individual patient and to detect method-specific characteristics, 37 AML-, 3 MDS- and 6 healthy MNC were cultured in parallel in 5 different DC-media. This means that only those cases qualified for this analysis where all 5 media (MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly (I:C)) had been tested in parallel.

4.3.1 Similar average amounts of mature, viable, migratory and leukemia-derived DC can be generated from AML, MDS and healthy MNC fractions under different parallel culture conditions

Comparing yields of DC subtypes, we found similar average results as gained by pooling results from all methods (p-value always > 0.05).In AML samples (n=43) especially average amounts of DC_{opt} (on average between 27(±18)% DC_{opt} after culture with MCM Mimic and 30(±19)% after culture with Poly (I:C)) were very similar after culture with 5 different methods (figure 15a). Yields of mature DC ranged from 43(±21)% (Picibanil) to 52(±23)% (MCM Mimic). Highest amounts of DC_{leu} were found after culture with Ca Ionophore (60±23%), whereas yields of DC_{leu} were lower after culture with MCM Mimic (on average 45±26%) or Cytokines (47±30%). Average amounts of viable DC always were ≥ 63% in AML samples (figure 16a). Yields of Bla_{con} ranged from 31(±23)% (Ca Ionophore) to 38(±22)% (MCM Mimic) in AML samples (figure 15a).

A parallel comparison of average DC amounts after culture was possible in only 3 cases of MDS: yields of DC_{opt} ranged from 19(±12)% (Ca Ionophore) to 27(±10)% (Poly I:C) (figure 15b). Highest numbers of migratory DC were found after culture with Ca Ionophore and Cytokines (50%). Yields of mature DC ranged from 45(±19)% (MCM Mimic) to 65(±28)% (Cytokines). Highest numbers of viable DC and DC_{leu} were found for MCM Mimic (70±29% and 60±6%). Yields of Bla_{con} in cultured MDS samples ranged from 24(±12)% (MCM Mimic) to 37(±29)% (Poly I:C).

Figure 15: Quantification of DC subsets in AML (a) and MDS (b) after culture, parallel comparison of 5 different methods: DC were generated from blast-containing AML and MDS MNC using 6 different DC-generating methods. Quantification of different subsets of DC was performed after culture using combinations of DC, costimulatory, maturation and migration antigens. Average results (mean± standard deviation) are given. Proportions of flow cytometrically estimated DC_{opt}, migratory DC (CCR7+), mature DC (CD83+), viable (7AAD-), DC_{leu} and Bla_{con} were analyzed in AML (n=137) and MDS (n=49) samples. DC_{opt} optimum of DC, i.e. for each individual sample, the DCA with lowest expression on naïve cells and with highest expression rates after culture was evaluated; DC_{opt} means the percentage of cells that expressed the specific DC_{opt} marker after culture (percentage of cells in the total cell amount).

(a)

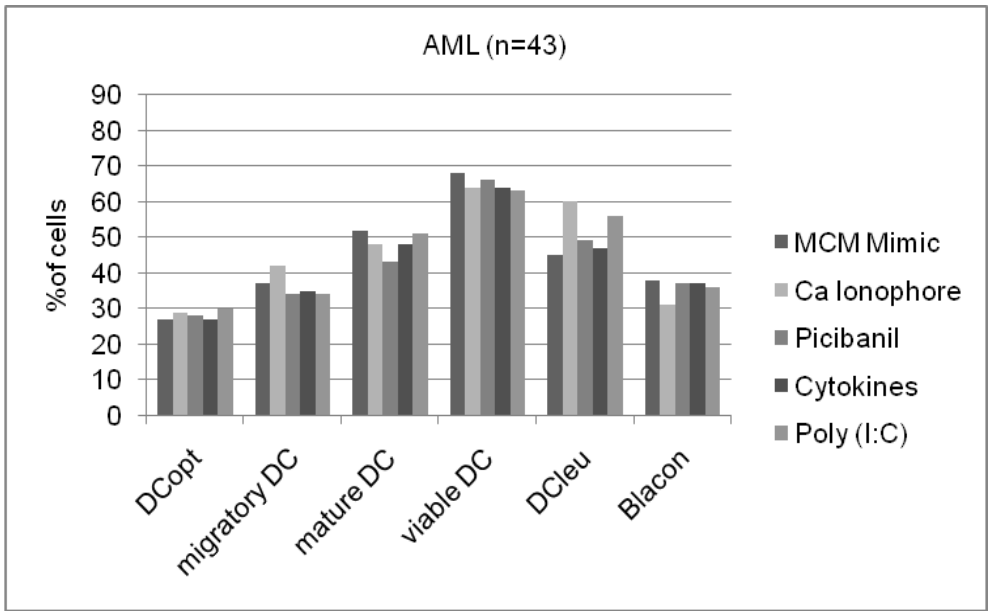
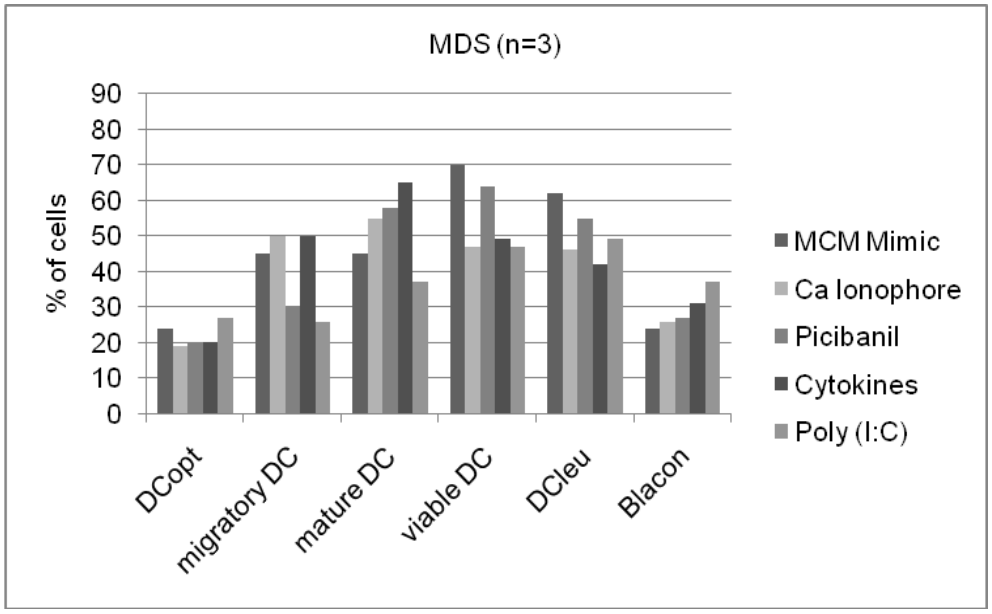


Figure 15 (b)



DC_{opt} optimum of DC. DC_{leu} leukemia-derived DC. Bla_{con} converted blasts.

4.3.2 Similar average expression of specific DCA can be found on DC from AML MNC under different, parallel cultured conditions

To compare the average expression of DCA^+ cells for the given 5 methods that were tested in parallel, average expression rates of all available DCA for every single method were pooled.

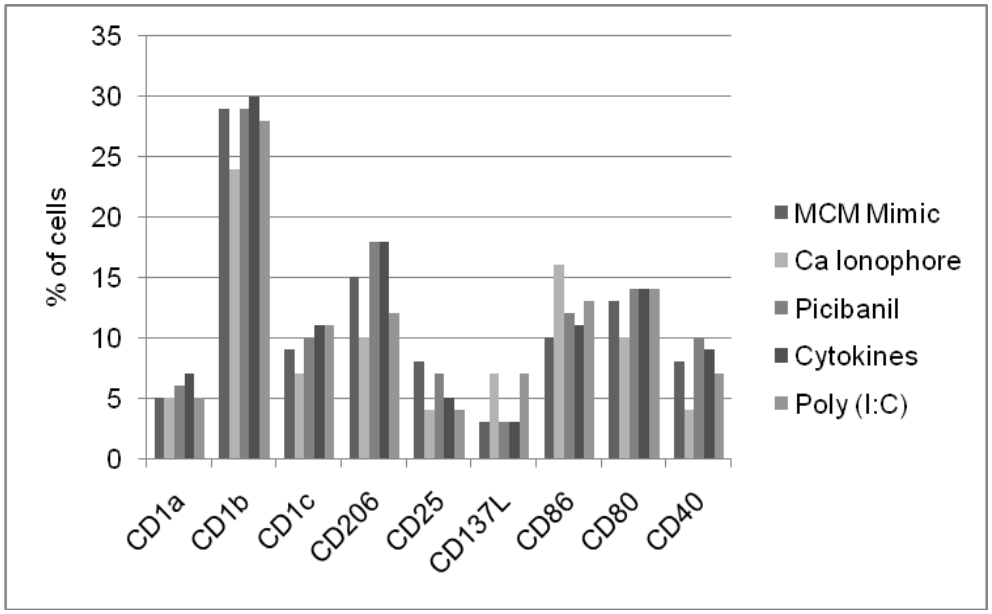
It could be shown that average counts of DC generated with different DC-generating methods were similar in AML samples (10% after culture with Ca Ionophore, 12% after culture with MCM Mimic and Poly I:C, and 13% after culture with Picibanil and Cytokines) (data not shown), whereas DCA were expressed in lower degrees on healthy samples (between 5% after culture with Ca Ionophore and 9% after culture with Cytokines) (data not shown). An analysis of average DCA expression rates on MDS samples was not possible due to a low case number.

Moreover, it is demonstrated in figure 16 that average expression rates of selected DCA in AML cases that were evaluated after parallel culture are similar, although individual variations of expressions occur. Average expression rates of <10% after culture with any of the 5 methods could be demonstrated for CD1a, CD25 and CD137L. Highest expression rates

were found for CD1b (between 24% after culture with Ca Ionophore and 30% after culture with Cytokines). No data were available for CD83.

Due to only 3 MDS cases and 6 healthy samples available for parallel comparison, a differential analysis of DCA expression rates under different culture conditions was not possible.

Figure 16: Average proportions of single DCA expressed on AML cells after culture; parallel comparison of 5 different methods: only AML (n=37) were included where all 5 methods (MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly (I:C)) had been tested in parallel to generate DC. Average expression rates (mean) of 10 selected DCA, depending on methods used, are given. Only cases were included with less than 7% DCA^+ cells before culture.



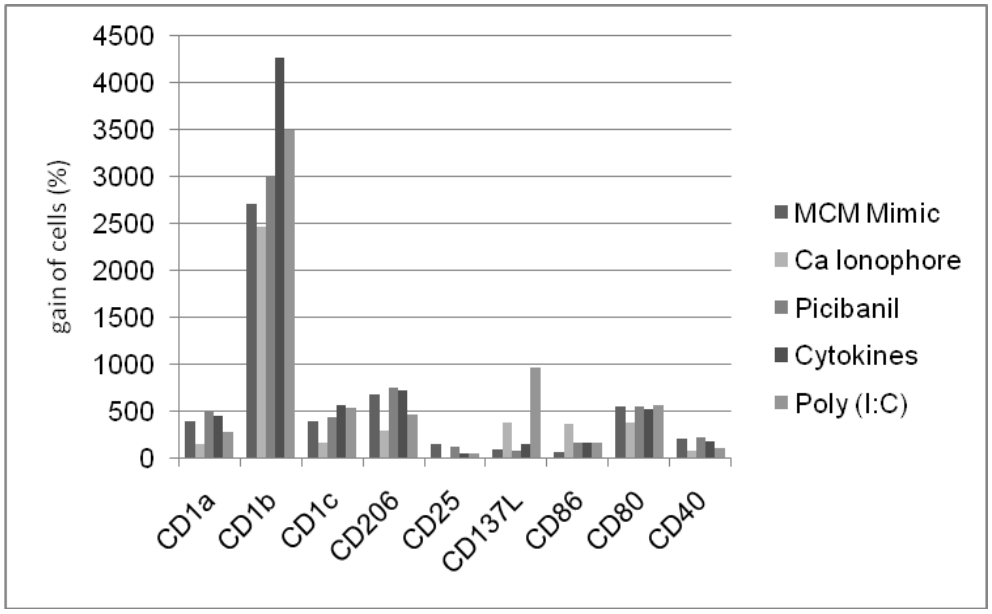
4.3.3 Average gain of DCA^+ cells on DC generated from AML, MDS and healthy MNC is similar under different parallel culture conditions

The gain of DCA^+ cells after culture in AML MNC after parallel culture in the 5 different media was compared. Again, cases with more than 7% DCA^+ cells before culture were excluded. Figure 17 shows comparable gains of DCA^+ cells in AML-samples after parallel culture in different DC-differentiating methods with highest gain of DCA^+ cells found for CD1b (between 2471% after culture with Ca Ionophore and 4269% after culture with Cytokines) (figure 17). It could be demonstrated that on average, all tested DCA are

upregulated after culture, independent from the methods used for DC generation; however, in some individual cases, a downregulation of one or more DCA could be found after culture. No data were available for CD83.

Due to only 3 MDS cases and 6 healthy samples available for parallel comparison, a differential analysis of DCA upregulation under different culture conditions was not possible.

Figure 17: Gain of DCA^+ cells in AML cases after culture; parallel comparison of 5 different methods: only AML (n=37) were included where all 5 methods (MCM Mimic, Ca Ionophore, Picibanil, Cytokines and Poly (I:C)) had been tested in parallel to generate DC. Average gain (mean) of 10 selected DCA, depending on methods used, are given. Only cases were included with less than 7% DCA^+ cells before culture.



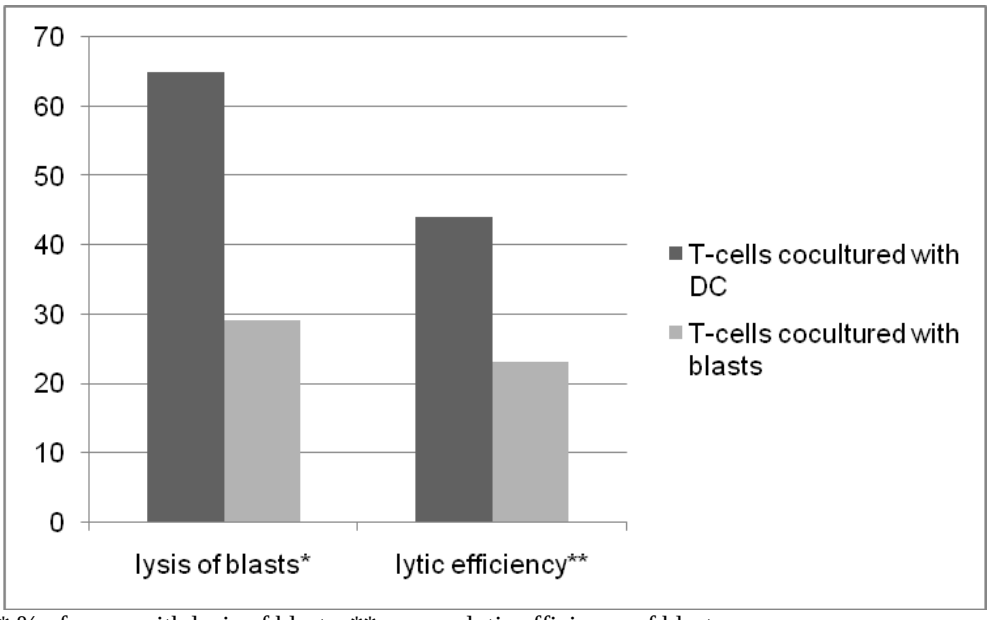
4.4 DC regularly contribute to prime T-cells against leukemic targets

For the proof of principle it has to be shown, that DC generated with different methods are able to prime T-cells, giving rise to specific anti-leukemia-directed T-cells. DC from 17 cases with AML and MDS blast containing MNC were generated and cocultured with T-cells for 10 days. T-cell sources were either autologous or allogeneic from the patients' stem cell donors. Naïve blasts in the MNC fractions added to the primed cells could be lysed in 11 of 17 cases (65%) (figure 18 left side) after 24 hours incubation with an average lytic efficiency of 44%

of blasts (range 10-83%) (figure 18 right side). In 6 cases (35%) no lysis or even a stimulation of blast proliferation was seen with an average increase of blasts of 70%. In the group with lytic activity, DC had been generated with MCM-Mimic (n= 9) or Ca-Ionophore (n=4). In cases with blast stimulatority activity, DC had been generated with MCM-Mimic (n=5) or Picibanil (n=2). Priming T-cells in parallel with blast-containing MNC instead of DC showed that a lytic activity of blast-containing MNC-primed T-cells could be achieved in only 5 of the 17 cases (29%) after 24 hours incubation with naïve blasts, with an average lytic efficiency of 23% of blasts (range 6%-69%). In 12 cases (71%), no lysis or even a stimulation of blast proliferation was seen with an average increase of blasts of 98%.

These data provide the proof of principle that in the majority of cases DC mediate a lytic activity of T-cells, although in some cases blast stimulation can be achieved, whereas a T-cell-priming with blast containing MNC results in a stimulation of blast proliferation in the majority of cases.

Figure 18: AML MNC (n=17) were cocultured with autologous DC and in parallel with autologous blasts. DC were generated from 17 cases with AML and MDS blast containing MNC and then cocultured with autologous or allogeneic T-cells for 10 days. In parallel, T-cells were cocultured with blast containing MNC instead of DC. Left side of the figure shows a comparison of the average rates of cases (mean results are given) with a documented lysis of blasts after coculturing T-cells either with DC or blast containing MNC.



* % of cases with lysis of blasts. **average lytic efficiency of blast

5 Discussion

5.1 Clinical course of AML and MDS and immunotherapeutic treatment options

Due to the high median age of MDS patients, allogeneic SCT is available only for a minority of patients. Thus, treatment options in MDS stay rather supportive (Hofmann and Koeffler, 2005;List, 2002). Although complete remission can be achieved in about 80% of AML patients by intensive chemotherapy-based treatments including SCT, long-term survival stays rather short (5-year overall survival 20%-25%) due to persistence of MRD in about 80% of AML patients (Houtenbos et al., 2006;Li et al., 2003;Venditti et al., 2000). Therefore, there is a tremendous need for new less intensive (post-remission) options both in AML and MDS.

DC are the most potent antigen-presenting cells of the immune system. Besides, they play an important role in immune response against neoplastic cells (den Brok et al., 2005) and are able to stimulate naïve T-cells, which leads to an antigen-specific immune response (Banchereau et al., 2000). A defective host antitumor immune response is an important mechanism that allows tumors to evade the immune system (Almand et al., 2000). It has been shown that function and yields of cells of the innate and adaptive immune system can be reduced in various malignancies (Mohty et al., 2001;Ratta et al., 2002). Several studies focused on the defective function of DC in tumor-bearing mice and cancer patients (Almand et al., 2000;Mashino et al., 2002). Thus, in vivo targeting of DC seems not to be the best approach in leukemia (Houtenbos et al., 2006). Immunotherapeutic approaches based on ex vivo targeted DC addressing the role of T-cell immunity and the role of DC to elicit T-cell responses are regarded as belonging the most promising tools in the immunotherapy of hematological disorders (Fujii et al., 2009;Gilboa, 2007). The important role of T-cells in overcoming this therapeutic resistance has already been demonstrated by the use of DLI in relapsed AML, proving the anti-leukemia directed effect of cytotoxic T-cells – although not all patients respond to this therapy (Kolb et al., 1995;Kolb et al., 2003;Schmid et al., 2006;Venditti et al., 2000). Various animal models have demonstrated the ability of DC to prime antigen-specific T-cells which are capable of recognizing and killing tumor cells (Boon et al., 1994;Celluzzi et al., 1996). The anti-leukemia mediating activity of DC_{leu} could be previously demonstrated in vitro (Cella et al., 1996;Kufner et al., 2005a). First results of Phase I/II clinical trials vaccination with autologous DC in AML-patients showed that vaccinations with DC are feasible and safe, although not regularly clinically effective (Houtenbos et al., 2006;Roddie et al., 2006).

5.2 DC antigens (DCA)

5.2.1 Detection of DCA and their significance for vaccination strategies

DC for anti-tumor vaccination in patients with solid tumors or leukemia can be generated in different ways: either by pulsing DC with tumor-specific antigens or tumor lysates, by transfection of DC with RNA encoding for such antigens or by converting leukemic cells to DC (Gilboa, 2007;Soleimani et al., 2009;Toh et al., 2009). Moreover, TAA-pulsed DC (e.g.WT1-pulsed DC) were successful in generating anti-leukemia directed, specific cytotoxic T-lymphocytes (CTL) ex vivo that lysed specifically $CD34^+$ leukemic cells but not healthy $CD34^+$ progenitor cells. Thereby they yield the proof of principle that DC presenting a leukemic antigen are mediators of an anti-leukemia directed specific cytotoxic reaction (Gao et al., 2000). In addition, minor antigens like HA-1 and HA-2, restrictedly expressed on hematopoetic cells, are useful peptides to induce anti- HA-1/2 directed CTL after stimulation with donor –DC pulsed with these antigens (Mutis et al., 1999). Leukemic blasts from AML patients can also be converted to 'leukemia-derived DC' (‚DC_{leu}') in vitro by using different DC-generating methods based on cytokines, and giving rise to cells expressing ‚DC-typical markers' together with the patient's specific clonal or cell surface blast markers (Kufner et al., 2005a;Schmetzer et al., 2007). An advantage of this approach is the potential to develop a DC-vaccine that presents not only one but multiple leukemic antigens of the patient's individual AML, rather than constructing e.g. an artificial leukemic peptide-target that is only expressed in selected AML-subtypes (e.g. WT1 in HLA-A2-AML, cases after SCT with HA-1/HA-2 mismatch).

5.2.2 DCA expression profiles of 10 analyzed DCA

Only DCA that are not expressed on uncultured cells qualify for the detection of DC_{leu}. In previous examinations, we and others could already show that DCA can be expressed on uncultured AML and MDS MNC fractions – and even predict a worse prognosis for the patients (Graf et al., 2005) .

We postulated at least 10% generated DC and in addition 5% of DC_{leu} (DC that coexpressed blast markers) in the total cell fraction as 'successful' DC generation (Schmetzer et al., 2007).

Average expression rates were higher than 7% for CD1a, CD206, CD25, CD86 and CD40 in AML MNC, for CD1b, CD137L, CD86 and CD40 in uncultured MDS MNC and for CD137L, CD86 and CD40 in uncultured healthy MNC. Analyzing the number of DCA with positive expression rates for each individual sample, it was found that 28% of AML samples and 31% of MDS samples showed no expression of any of the tested DCA before culture. This means vice versa that about 70% of all patients did express at least 1 DCA on naïve MNC fractions, some of them even 3 or more DCA. High expression rates of DCA on naïve MNC fractions can be explained either by contamination of MNC fractions with non-leukemic cells expressing those markers (e.g. monocytes, lymphocytes) or by aberrant expression of those DCA on naïve blasts (e.g. CD137L, CD206 or CD25).

A detailed analysis for each DCA was performed to evaluate the frequency of positive expression rates of these markers on uncultured samples. More than one half of both AML and MDS samples presented with positive expression rates for CD86 before culture, about 30% of all samples presented with expression rates >7% for CD40. This means that those 2 DCA especially don't qualify for the detection of leukemia-derived DC in most cases. Less than 10% of all uncultured AML and MDS samples presented with positive expression rates for any of all other tested DCA. However, as there is huge interindividual variety of DCA expression profiles on naïve AML and MDS samples, it is important to pre-test a variety of DC-antigens in the analysis of DC expression profiles in every individual patient. Besides, the frequency of positive expression rates of DCA on naïve MNC fractions might be even higher as in results gained for this thesis, as not every DCA was tested in any given case. Data presented by other authors, who demonstrated a deficient expression of costimulatory molecules in leukemic blasts (especially CD40) that might hamper stimulation of T-cell responses (Li et al., 2003), could not be confirmed.

CD1 antigens are expressed on DC, monocytes, B-cells and cortical thymocytes and are essential for antigen presentation on DC and for T-cell activation (Banchereau et al., 2000;Bell et al., 1999;Brigl and Brenner, 2004;Sloma et al., 2004). We found relatively low average expression rates of CD1 antigens in uncultured MNC fractions and an upregulation with any of the tested DC-differentiating methods. Only M3 cases presented with a loss of $CD1a^+$ cells (results of all methods pooled). Athanasas-Platsis et al. could show that GM-CSF, TNFα, IL-6 and IL-1β can induce CD1a expression (Athanasas-Platsis et al., 1995). However, we found similar expression rates after culture in Ca Ionophore that bypass the

cytokine-driven differentiation. We can confirm the observation of Pietschmann et al. that, compared to other DCA, CD1a is expressed to a lower degree due to serum free culture (Pietschmann et al., 2000). These findings indicate that CD antigens qualify as useful markers for the quantification of DC_{leu} in most cases, however, positive expression of CD1a, CD1b and CD1c can be found in individual cases on naïve MNC fractions.

CD83 is one of the most important antigens to identify mature DC and is essential for antigen presentation to T-cells (Hirano et al., 2006;Tobiasova-Czetoova et al., 2005). We found low average expression rates for CD83 in uncultured AML and MDS fractions, but also relatively low expression rates on cultured AML MNC samples thereby again confirming the observation of Pietschmann et al. (Pietschmann et al., 2000).

Average CD206 expression rates on uncultured MNC fractions were 11% for AML and 4% for MDS samples. After culture, we detected higher expression rates of CD206 in MDS compared to AML samples and also in AML cases with poor cytogenetic risk, but a relative low expression of CD206 on cells after culture with Ca Ionophore. As it is known that antigen-uptake by DC via CD206 results in a multiplied antigen-presentation and thus a more efficient activation of T-cells (Engering et al., 1997), this could mean that DC cultured with Ca Ionophore have a lower capacity of antigen presentation compared to other methods.

CD25 is expressed on DC precursors, but also on mature DC (Cella et al., 1997;von Bergwelt-Baildon et al., 2006). Since CD25 is not specific for DC, but also a T-cell-proliferation marker (Chen et al., 2006), expression rates of up to 12% $CD25^+$ cells in uncultured AML MNC can be expected. We found relatively low expression rates of CD25 in uncultured MNC (11% in AML cases and 4 % in MDS cases). In general, CD25 was upregulated in low degrees. Highest upregulation of CD25 was found after culture in MCM Mimic and Picibanil, both media containing PGE2, which is known to promote CD25 expression on DC (von Bergwelt-Baildon et al., 2006). These findings indicate that CD25 is not a useful marker for quantification of leukemia-derived DC in most cases.

An aberrant expression of CD137L on carcinoma cells and in leukemia has been reported (Salih et al., 2001;Scholl et al., 2009). Moreover, CD137L is known to be upregulated during DC differentiation, resulting in T-cell activation (Mittler et al., 2004). This means, that 16% $CD137L^+$ cells being found in uncultured blast containing samples can be expected as well as an upregulation of CD137L expression. All in all it thus can be said that CD137L doesn't qualify as a marker to detect DC in most cases. We found relatively low average expression

rates of CD137L in uncultured AML and MDS samples (6% and 16%), compared to healthy probands and an average upregulation in both AML and MDS samples. A loss of $CD137L^+$ cells could be demonstrated for M3 and RAEBt cases.

CD80, CD86 and CD40 are important costimulatory molecules on DC to activate T-cells triggering T-cell responses towards either Th1- or Th2-cells (Banchereau et al., 2000;Nicod et al., 2005;Rogers et al., 2005). We could show high expression rates for CD86 and CD40 on uncultured AML and MDS samples, especially in cases with poor cytogenetic risk, thereby confirming previously published data (Graf et al., 2005). This means that an expression of those three markers after DC culture, as recommended by some authors, cannot be used as a reliable factor to estimate DC without previous testing of their expression on uncultured MNC (Roddie et al., 2006;Schmetzer et al., 2007). Extremely high average expression rates of CD86 in CMML cases (n=2) (58%) might be caused by an aberrant expression of CD86 on one CMML sample (90% $CD86^+$ cells) or by contamination with $CD86^+$ cells. Confirming data of other authors (Li et al., 2005), CD80, CD86 and CD40 were upregulated after culture.

Summing up, none of the analyzed 10 DCA qualifies as a single suitable marker (no expression on uncultured cells, high upregulation after culture) for a reliable detection and quantification of leukemia-derived DC in all cases of AML and MDS: although a low average expression on uncultured cells and average upregulation could be demonstrated for some DCA, a big interindividual variety in DCA expression profiles was found in AML and MDS cases. This means that: 1. there is a need for new, more specific DCA. 2., DCA expression profiles have to be evaluated before and after culture in every individual case to find those markers in every given patient that qualify as DC markers (no expression on uncultured cells, high upregulation after culture). With regard to the 10 analyzed DCA, CD1a, CD1b and CD1c seem to be the most suitable DCA for a quantification of DC in most cases.

5.3 Value of different DC-differentiating methods for generation of leukemia-derived DC (DC_{leu})

Various methods, characterized by different modes of action, can induce DC-differentiation. Most authors only chose one single method for DC generation, resulting in an insufficient DC

generation in many cases, as described (Kharfan-Dabaja et al., 2005;Kufner et al., 2005a;Roddie et al., 2006).

It has already been demonstrated that DC can be generated successfully in vitro from $CD14^+$ monocytes or from $CD34^+$ progenitor cells or leukemic cells in the presence of cytokines (Lee et al., 2002;Westers et al., 2003;Woiciechowsky et al., 2001), Calcium ionophores (Houtenbos et al., 2003), nucleic acid fragments (Rouas et al., 2004) or bacterial lysates (Sato et al., 2003), with different mechanisms of inducing DC differentiation. The addition of Flt-ligand increases the harvest of DC (Kufner et al., 2005b;Woiciechowsky et al., 2001). The use of foetal calf serum (FCS)-free media for DC-generation should be preferred to avoid immune reactions against FCS-peptide-associated, xenogeneic antigens or anaphylactic reactions (Mackensen et al., 2000a).

In this thesis, 6 different media were compared: a Basic method (Woiciechowsky et al., 2001), MCM Mimic (Lee et al., 2002), Cytokines (Westers et al., 2003) using Cytokine-based ways of DC-differentiation, Picibanil as a bacterial lysate combined with PGE2 (Sato et al., 2003), double-stranded RNA in Poly I:C (Rouas et al., 2004) and Ca Ionophores (Houtenbos et al., 2003).

Previous studies tried to generate leukemia-derived DC via receptor-mediated extracellular pathways, using different cytokine combinations (Westers et al., 2003;Woiciechowsky et al., 2001). The 'Basic method' used by us and described and established by Woiciechowsky et al. 2001 contains GM-CSF, IL-4, TNFα and FLT3-ligand. FLT3-ligand has been shown to act synergistically with other cytokines and increases yields of DC after culture. Besides, DC-generation from $CD34^+$ cells is increased by the addition of FLT3-ligand (Woiciechowsky et al., 2001). Using the described method, Woiciechowsky et al. were able to differentiate DC from 77% of AML patients, regardless of FAB classification and clinical status (Woiciechowsky et al., 2001). MCM Mimic is another cytokine-based DC-differentiation method mimicking the components of monocyte-conditioned medium ('MCM mimic') including TNFα, IL-1β, IL-6. MCM Mimic also contains PGE_2 which is known to improve yields of DC, especially mature DC, and function of DC, e.g. migration capacity (Lee et al., 2002). The use of MCM Mimic, first described by Jonuleit et al. 1997, improves the yield and function of DC, including the skewing of T-cells to a TH1 phenotype (Jonuleit et al., 1997). Lee et al. could show that the use of MCM Mimic in the differentiation of monocyte-derived DC results in a uniformly mature phenotype and a high immunostimulatory capacity (Lee et

al., 2002). The third cytokine-based medium used by us to generate leukemia-derived DC contains a conventional cocktail of cytokines for DC differentiation: GM-CSF, TNFα, IL-3, SCF, FLT3-ligand and IL-4. Westers et al. could demonstrate that the generation of APC was possible in the majority of AML patients by the use of this cytokine cocktail. However, when cells where cocultured with Ca Ionophores, AML-APC were at a more mature stage after culture with Ca Ionophores (Westers et al., 2003).

Picibanil (OK-432) is a biological response modifier (BRM) derived from the Su strain of Streptococcus pyogenes. This bacterial lysate stimulates immature DC towards maturation and can improve production of Th-1-type cytokines, e.g. IL-12 , especially in combination with PGE_2 (Sato et al., 2003). Sato et al. could demonstrate that DC derived from cancer patients in the presence of OK-432 showed a mature phenotype. By a combination of OK-432 and PGE_2, CCR7 expression and migratory capacity of the generated DC were significantly higher compared to OK-432 alone, without inhibiting other immunostimulatory functions (Sato et al., 2003).

Poly(I:C) is a synthetically fabricated double-stranded RNA acting through TLR3 expressed on DC. Poly(I:C) functions as a 'danger signal' and acts through TLR3 expressed by DC. Rouas et al demonstrated that Poly(I:C), compared to other media such as cytokine cocktails, might be one of the most appropriate agents to generate stable mature DC, as these DC are still able to secrete high levels of IL-12 and might generate effective in vivo immune responses (Rouas et al., 2004).

The Calcium Ionophore A23187 is a DC-differentiating substance bypassing the cytokine-driven DC-differentiation. Houtenbos et al. demonstrated that serum-free generation of leukemia-derived DC using Ca Ionophore is feasible, and could observe a trend towards a more mature phenotype of DC after culture with Ca Ionophore compared to a cytokine mix (Houtenbos et al., 2003).

Data presented in this thesis show that there is no specific method to create DC for a single patient and that different methods qualified better or worse for a single patient. This might be due to the heterogeneity and state of maturation.

DC generation was defined as successful if > 10% DC using the DC_{opt} marker and in addition at least 5% DC_{leu} could be generated. This marker (no expression on uncultured cells, highest

upregulation after culture) was evaluated for every individual patient. However, actual total amounts of generated DC might be even higher than evaluated by DC_{opt}, as not all generated DC might express this specific marker, but other DCA.

None of the used media was a reliable tool to generate DC successfully in every patient. However, it could be shown that a successful DC generation was possible in every given case by a parallel pretesting of 3 different methods. Every method failed to generate DC in some cases, but we could show that a successful DC generation was possible in every given case by a pretesting of 3 different methods and a selection of the best method. Every method failed to generate DC in some cases, but the combination of MCM mimic, Ca Ionophore and Picibanil tested in parallel yielded to a successful generation of DC with at least one method. Consequently, we can overcome the resistance of blasts to be converted to DC, as described by Roddie 2002 and 2006 (Roddie et al., 2006;Roddie et al., 2002).

On average, no significant differences were found in the expression of different DCA, the average generation of DC_{opt}, DC_{leu}, mature, migratory or viable DC in AML or MDS samples depending on used method. On average, more than 50% of DC were 'leukemia-derived' with respect to the total MNC fraction. In consequence this means that high proportions of unconverted blasts were found in the suspension, confirming the observation of Houtenbos et al. that high blast counts (about 50%) remain unconverted in the culture (Houtenbos et al., 2003). Observations of Houtenbos et al. that DC generated in the presence of Ca Ionophores are more mature but less viable compared to DC generation with Cytokines, could not be confirmed (Houtenbos et al., 2006).

5.4 Value of FACS analysis for quantification of leukemia-derived DC

Recently we have published a method that allows a sensitive, reliable and reproducible strategy to quantify DC as well as to estimate their quality with respect to different DC-subtypes and especially their leukemic derivation (Schmetzer et al., 2007). Using a refined 2-step gating strategy that considers different scatter profiles of blasts and DC, amounts of DC_{leu}, DC without proof of leukemic origin and nonconverted blasts is possible. Especially, the convertibility of blasts to DC_{leu} can be estimated without knowledge of a clonal chromosomal marker, which is very practical due to the fact that only about 50% of AML

patients show chromosomal aberrations (Frohling et al., 2002). On the other hand, counts of DC_{leu} may be underestimated, because only those DC were accepted as 'leukemia-derived', that coexpressed a patient-specific blast marker and a DC marker, whereas blasts that didn't express this blast marker (but may belong to the blast population) were not accepted as DC_{leu}. However, the leukemic origin of DC cannot be proven with this method in cases without clonal aberration by cytogenetic/FISH methods.

Moreover, a gain of migratory capacity, necessary for the migration of DC from the injection site to lymph nodes can be evaluated, as well as the maturity of DC, responsible for TH_1 activation or a combined antigen presentation (Lee et al., 2002;Sallusto and Lanzavecchia, 2002;Westers et al., 2003)

5.5 DC regularly and specifically prime T-cells, however not always successfully

The adoptive transfer of selected tumor-reactive T-cells, especially of DC-primed CTL, could be an effective therapeutic option for patients after non-myeloablative chemotherapy (Choudhury et al., 1999;Spisek et al., 2002). It has been shown that costimulatory molecules are often downregulated on leukemic cells, which impedes contact to effector cells (Vollmer et al., 2003). However, we and others found that costimulatory antigens like CD80 and CD86 can be expressed on naïve AML and MDS blasts in varying degrees (Graf et al., 2005). These data indicate that the expression of costimulatory antigens on naïve leukemic cells alone is not sufficient to induce anti-leukemic T-cell responses and affirm the common assumption that a simultaneous expression of several costimulatory antigens and leukemic markers on professional APC is needed to activate specific T-cells. We could demonstrate that DC generated from AML and MDS MNC fractions with different methods are able to prime T-cells, giving rise to specific anti-leukemia-directed T-cells. This confirms results of other DC studies (Choudhury et al., 1999;Kufner et al., 2005a). Data provide the proof of principle that in the majority of cases, DC mediate a lytic activity of T-cells, although in some cases blast stimulation can be achieved, whereas a T-cell-priming with blast containing MNC results in a stimulation of blast proliferation in the majority of cases. Critical points remain to be discussed: our functional data show, that a successful priming of T-cells, giving rise to specific anti-leukemia directed cytotoxic T-cells, is not possible in every case. Therefore, the

role and influence of the chosen DC-media (with/without immunogenic stimuli) on the quality of DC (e.g. mature DC, DC_{leu}) or a possible inhibitory effect of unconverted blasts or soluble factors released by blasts, DC or T-cells in the mixed lymphocyte culture for a successful ex vivo T-cell priming has to be evaluated.

Detailed analyses of the role of cellular (e.g., DC, T-cells) and soluble partners (e.g. chemokines, cytokines) are in the focus of the ongoing research of our group. Moreover, the focus is on strategies to increase DC_{leu} counts and their T-cell priming efficiency. New results of our group show that not only the maturation stage of DC, but especially the proportions of leukemia-derived DC are predictive for the induction of anti-leukemic T-cells (Schuster et al., 2008) (thesis of Christine Grabrucker, Med III, Klinikum Großhadern: Qualität von Dendritischen Zellen (DC), die aus leukämischen Blutproben von Patienten mit AML und MDS generiert wurden, als prädiktiver Faktor für das lytische Potenzial DC-geprimter leukämiespezifischer T-Zellen). Moreover, the quality and composition of T-cells is predictive for the antileukemic efficiency of DC-primed cells (data not yet published; data gained in the course of thesis of Anja Liepert, Med III, Klinikum Großhadern: Qualität von Blasten- bzw DC-geprimten T-zellen als prädiktiver Faktor für deren lytisches, leukemiespezifisches Potenzial bei Patienten mit AML und MDS).

Moreover, a possible correlation of ex vivo results with the clinical course of the disease, e.g. after an immunotherapy like SCT, could contribute to understanding biological antileukemia-directed mechanisms involved in the specific (DC-mediated) T-cell priming in vivo. Vice versa, it could contribute to developing strategies to overcome the blasts' resistances to apoptosis by specifically adapted anti-leukemia directed immunotherapies. A positive selection of DC_{leu} by double staining with a DC and a blast marker could help to enrich DC_{leu} for vaccination.

5.6 Previous and current DC-generation studies

Deeb et al. could demonstrate the efficacy of DC-based vaccination in leukemia for eradication of MRD and prevention of relapse in a mouse model (Deeb et al., 2006). Leukemia-derived DC-generation studies and clinical trials were first carried out in CML patients, e.g. Choudhury et al were one of the first who could show that in vitro-generated DC

can be effectively used as APC for the ex vivo expansion of antileukemic T-cells (Choudhury et al., 1997). Dietz et al treated CML patients with subcutaneous injection of autologous leukemia-derived DC (Dietz et al., 2001). First clinical trials with leukemia-derived DC as a T-cell stimulating vaccine were carried out in 5 patients with CML (Claxton et al., 2001). First results of Phase I/II clinical vaccination trials with autologous DC in AML-patients showed that vaccinations with DC are feasible and safe, although not regularly clinically effective (Houtenbos et al., 2006;Roddie et al., 2006). Lee et al. vaccinated relapsed AML patients with DC pulsed with leukemic lysates and could show immunological responses with positive delayed-type hypersensitivity skin reaction and increasing autologous T-cells stimulatory, but no improvement of the BM blast proportion (Lee et al., 2004). Li et al could also demonstrate an enhanced and specific response of cytotoxic T-cells in 5 relapsed AML patients who had been treated with subcutaneous injections of autologous AML-DC without any severe side effects. Three patients remained in a stable condition for up to13 months, two patients died from rapidly progressive AML (Li et al., 2006a). These data prove that DC-based therapies lead to T-cell activation in vivo. However, a reduction of the blast threshold was not yet observed. This means that DC-based therapies might be of special value for AML and MDS patients in remission to fight MRD and improve long-time survival.

5.7 Challenges in DC vaccination

In order to evaluate the value of DC vaccination in AML and MDS patients, some consensus on quality criteria and immune monitoring is necessary. Protocols for DC preparation and quality controls should include criteria like cell source, cell purity, cell viability, markers of DC activation, sterility control, veiled appearance, and in vitro T-lymphocyte response. Cytokines, growth factors etc. should be manufactured under good manufacturing practice (GMP) (Nestle et al., 2001). There is still much debate about selection of culture methods. We could demonstrate that DC generation is possible in every case of AML and MDS by a pretesting of three different media and selection of the best one. However, no single established method for DC generation is known yet that would allow successful generation of DC in every given case. Besides, long culture times of the most established media for generation of leukemia-derived DC provide another problem (Lee et al., 2002;Rouas et al., 2004;Woiciechowsky et al., 2001). This problem, of which Ca Ionophores (2-3 days culture

time) (Houtenbos et al., 2003) is the exception, brings risks of contamination with bacteria etc. and cause high costs.

Reproduction of DC for vaccination reflects a balance between antigen presenting capacity and antigen loading. The optimal DC maturation state is still a critical parameter for the use of DC in active immunization. Dhodapkar et al. could demonstrate that the injection of antigen-loaded immature DC can lead to immune tolerance by inducing IL-10 producing regulatory T-cells as well as to antigen-specific inhibition of preexisting $CD8^+$ cell immunity (Dhodapkar et al., 2001). In contrast, mature DC are able to induce functionally superior $CD4^+$ and $CD8^+$ T-cells (Banchereau et al., 2001). This means that the quality could be predictive for the induction of antileukemic responses.

The amount of injected DC that is necessary to evoke anti-leukemic responses in AML and MDS patients is not yet known and may depend on different factors, such as immune status of the patient, residual leukemic burden and antigenic density (Houtenbos et al., 2006;van de Loosdrecht et al., 2009). Consequently, immune responses have to be monitored with reliable tools that can predict clinical efficacy. One critical point is that the induction of immune responses is not necessarily correlated with a clinical response (Nestle et al., 2001). However, it has been shown that the clinical outcome of melanoma patients vaccinated with antigen-pulsed mature DC correlates with the presence of specific T-cells in delayed-type hypersensitivity responses (de Vries et al., 2005). Ossenkoppele et al. demonstrated that four vaccines, each with $10x10^6$ DC, resulted in strong delayed type hypersensitivity responses in CML patients vaccinated with autologous DC (Ossenkoppele et al., 2003). For the generation of DC in AML patients, an amount of $4x10^8$ viable AML blasts is needed at diagnosis, based on the assumption that an effective vaccination regimen requires four vaccinations with each $10x10^6$ cells and that the average AML-DC yield is 25% (Houtenbos et al., 2006).

Injection schedules have to be optimized as they are mostly derived from animal models or human vaccination studies in infectious diseases and vaccination with overestimated doses might be harmful. Most DC vaccination trials could show highest tumor-specific T-cell activation when DC vaccines were applied one or two times a month (Nestle et al., 2001).

Another unresolved question is the optimal route of administration of DC vaccines. Intradermal or subcutaneous injections may lead to better T-cell responses than those following intravenous administration (Butterfield et al., 2003;Fong et al., 2001). However, these routes of administration rely on the capacity of the injected DC to migrate towards the

lymph nodes. Intranodal injections of DC circumvent this problem and allow the delivery of a defined amount of DC to the desired anatomic region, which might lead to an increased T-cell immunity (Mullins et al., 2003). Fong et al. found Ag-specific T-cell responses in all patients following immunization regardless of the route of administration. However, intradermal and intralymphatic injections were superior to intravenous injections for the induction of IFN-gamma production (Fong et al., 2001).

As most antigens used in DC vaccination protocols are not only tumor-specific antigens, but also self-antigens, the induction of strong immune responses against these antigens might lead to severe autoimmune disorders. Interestingly, recent studies on DC vaccination that could show clinical responses never observed severe side effects or autoimmune complications (Avigan et al., 2004;Lee et al., 2004;Li et al., 2006b;Nestle et al., 2001). One possible explanation for the lack of autoimmune responses could be that the stimulation of (auto-directed) T-cell responses is too weak (Dannull et al., 2000). Another explanation could be that transient autoantibody responses in vaccinated patients may simply be undetected. Data obtained in a mouse model suggest that DC vaccination consistently triggers autoimmune responses, but only susceptible subjects develop clinical autoimmune reactions (Bondanza et al., 2003). Homma et al. could show that serum levels of antinuclear antibodies (ANA) can be elevated in cancer patients after DC vaccination, which might be associated with anti-tumour immune response induced by vaccination. Elevation of ANA was correlated with better clinical response (Homma et al., 2006).

Last but not least, there is a need for reliable tools for monitoring of immune responses after DC vaccination. At the moment, the focus is mostly on the monitoring of cytotoxic T-cell activity, however the induction of T-cell responses is not necessarily correlated with the clinical outcome. Qualitative assays of immune effector mechanisms as surrogate markers for clinical responses of patients after DC vaccination have to be established (Nestle et al., 2001).

5.8 Conclusion

DC-based immunotherapy might be a promising new approach to stabilize remissions before or after SCT in AML and MDS. Data provided by recent human pilot studies indicate that DC vaccination can induce immunological and clinical responses in AML patients. However, many problems concerning protocols for successful DC generation, clinical settings of

possible DC vaccination trials and monitoring of immune and clinical responses remain to be discussed. Hopefully this thesis could contribute to the improvement of DC generation and to the establishment of quality-controls for future DC-based immunotherapies. Improved quality control, implementation and study reporting may provide a basis for choosing the best DC vaccination strategy and give AML and MDS patients a new therapeutic option.

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Erklärung

Erklärung gemäß §2 Absatz 1 Punkt 2 und 4 der Promotionsordnung für die Medizinische Fakultät der Ludwig-Maximilians-Universität München vom 01. Juni 1983 in der Fassung der siebten Änderungssatzung vom 01. Juni 2005:

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe und keine anderen Hilfsmittel als die angegebenen angewandt wurden. Alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, habe ich als solche kenntlich gemacht und nach ihrer Herkunft unter der Bezeichnung der Fundstelle einzeln nachgewiesen.

Die vorliegende Dissertation wurde weder in gleicher noch in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht.

München, den 26.02.2010

Julia Dreyßig

Eigene Publikationen

Schmetzer HM, Kremser A, Loibl J, Kroell T, Kolb HJ. Quantification of ex vivo generated dendritic cells (DC) and leukemia-derived DC contributes to estimate the quality of DC, to detect optimal DC-generating methods or to optimize DC-mediated T-cell-activation-procedures ex vivo or in vivo. Leukemia. 2007 Jun;21(6):1338-41.

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Loibl J, Kremser A, Kroell T, Kufner S, Schmid C, Schweiger C, de Valle F, Kern W, Schoch C, Doehner C, Salih H, Kolb HJ, Schmetzer H. .Dendritic cell (DC) marker expression profiles have to be evaluated before and after the generation of DC from blasts in acute myeloid leukemia and myelodysplastic syndromes to characterize and quantify DC in experimental settings. [Abstract eingereicht für die gemeinsame Jahrestagung der DGHO im Oktober 2005 in Hannover sowie für das wissenschaftliche Treffen der Medizinischen Klinik III des Klinikums Großhaderns im Kloster Irsee im Juli 2005]

Kremser A, Loibl J, Kroell T, Kufner S, Salih H, Schmid C, Kern W, Schoch C, Schweiger C, de Valle F, Doehner C, Kolb HJ, Schmetzer H. Leukemia-derived DC can be generated and quantified in every patient with AML or MDS using 3 alternative methods in combination. [Abstract eingereicht für die gemeinsame Jahrestagung der DGHO im Oktober 2005 in Hannover]

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Schmetzer HM, Kremser A, Loibl J, Hentschel N, Steiner K, Schmid C, Kroell T, Kufner S, Salih H, del Valle F, Doehner C, Schweiger C, Schwartz C, Hiddemann W, Kolb HJ. Effective Ex Vivo-Generation and Quantification of Leukemia-Derived DC Has To Precede a Specific T-Cell Stimulation for Adoptive Immunotherapy in AML and MDS. Blood (ASH Annual Meeting Abstracts) 2005 106: Abstract 5241

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Lebenslauf

Persönliche Daten

Name	Dreyßig
Geburtsname	Loibl
Vornamen	Julia Theresa
Geburtsdatum/-ort	04.04.1981, Starnberg
Anschrift	Loristr. 7
	80335 München
Telefon	0176-70055692
Familienstand	verheiratet
Kinder	eine Tochter

Schulbildung

1987 – 1991	Grundschule Friedrichshofen/ Ingolstadt
1991 – 2000	Katharinen-Gymnasium Ingolstadt
	Abschluss: Allgemeine Hochschulreife

Hochschulbildung

2000 – 2006	Studium der Humanmedizin an der Ludwig-Maximilians-Universität München
2002	Physikum
2003	1. Abschnitt der ärztlichen Prüfung
2005	2. Abschnitt der ärztlichen Prüfung
2005 – 2006	Praktisches Jahr
	Chirurgie (Klinikum München-Schwabing)
	Innere Medizin (Krankenhaus München-

	Harlaching)
	Gynäkologie/Geburtshilfe (Krankenhaus München-Harlaching)
November 2006	3. Abschnitt der ärztlichen Prüfung

Ärztliche Tätigkeit

seit Januar 2007	Assistenzärztin in der Frauenklinik des Krankenhauses München-Harlaching

München, den 26.02.2010

Julia Dreyßig