Immunodiagnostics and immune surveillance in an era of evolving immunotherapeutic strategies in AML

Willemijn van den Ancker

Cover design & layout: Willemijn van den Ancker
Printed by: Off Page, Amsterdam

Financial support for printing this thesis is kindly provided by: Amgen, Becton Dickinson, Celgene, GlaxoSmithKline, Janssen Cilag, Novartis Oncology and Roche.

VRIJE UNIVERSITEIT

Immunodiagnostics and immune surveillance in an era of evolving immunotherapeutic strategies in AML

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecomissie
van de Faculteit der Geneeskunde
op vrijdag 29 november 2013 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Willemijn van den Ancker

geboren te Amsterdam

promotoren:	prof.dr. G.J. Ossenkoppele
	prof.dr. A.A. van de Loosdrecht
copromotoren:	dr. T.M. Westers
	prof.dr. T.D. de Gruijl

Voor mijn Ouders

Table of Contents

PART II

PART IV

List of abbreviations

7-AAD	7-aminoactinomycine-D
aBLL	acute bilinear leukemia
ACB	apoptotic cell bodies
ACR	apoptotic cell remnants
Ag	antigen
AIR-1	activator of immune response-1
AL	acute leukemia
ALAL	acute leukemia of ambigious lineage
ALL	acute lymphoid leukemia, subdivided in T cell lymofoblastic or B cell lymfoblastic leukemia
allo-SCT	allogeneic stem cell transplantation
AML	acute myeloid leukemia
APC	antigen presenting cell
APC	allophycocyanin
APL	acute promyelocytic leukemia
ARA-C	cytarabin
B/My	B cell / Myeloid
β2M	beta 2 microglobulin
BAL	biphenotypical acute leukemia
BCG	Bacillus Calmette-Guierin
BM	Bone marrow
BMDC	bone marrow-derived dendritic cell
βME	2-mercaptoethanol
CC	cytokine cocktail
cCD22	cytoplasmic CD22
cCD3	cytoplasmic CD3
cCD79a	cytoplasmic CD79a
CD	clusters of designation
CFSE	carboxyfluorescein succinimidyl ester
cIgM	cytoplamsic IgM
CIITA	class II transactivator
CIP	calf intestinal alkaline phosphatase
CLIP	class II associated Invariant chain pepide
CM	conditioned medium
CMA	chaperone-mediated autophagy
CML	chronic myeloid leukemia
cMPO	cytoplasmic myeloperoxidase
CP	citrate-phosphate
CpG	cytosine-phophate-guanosine
CR	complete remission
cTdT	cytoplasmic terminal deoxynucleotidyl transferase
CTL	cytotoxic T lymphocyte
DC	dendritic cell

DFS	disease free survival
DLI	donor lymphocyte infusion
DRIPs	defective ribosomal products
DTH	delayed-type hypersensitivity
EGIL	European group for the immunological classification of acute leukemia
ER	endoplasmatic reticulum
ERAAP	ER aminopeptidases associated with antigen processing
FAB	French-American-British
FCS	fetal calf serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate
FLT3-L	Fms-like tyrosine kinase 3-ligand
GILT	gamma interferon-inducible lysosomal thiol-reductase
GM-CSF	granulocyte-macrophage colony-stimulating factor
GvHD	graft versus host disease
GvL	graf versus leukemia effect
HDCAi	histone deacetylase inhibitors
HLA	human leukocyte antigen
HOVON	Dutch-Belgian hematology-oncology cooperative group
HSP	heat shock protein
hTERT	human telomerase reverse transcription
IDO	indoleamine 2,3-dioxygenase
Ii	Invariant chain
IL-10	interleukin-10
IL-1β	interleukin-1β
IL-3	interleukin-3
IL-6	interleukin-6
inv (16)	inversion 16
LAA	leukemia-associated antigens
LAP	leukemia-associated phenotype
LC	Langerhans cells
LF	leucapheresis
LIMMA	linear model for microarray analysis
LOH	loss of heterozygosity
LPS	lipopolysaccharide
LT-CR	long term complete remission
MCM	monocyte-conditioned medium
mDC	myeloid dendritic cells
MDS	myelodysplastic syndrome
MEC	medical ethical committee
MFI	mean fluorescence index
MGG	May-Grünwald Giemsa
mHAG	minor histocompatibility antigens
MHC	major histocompatibility complex
MIIC	MHC class II compartments
miRNA	microRNA

mLPA	monophosphoryl lipid A
MLR	mixed leukocyte reaction
moAb	monoclonal antibody
moDC	monocyte-derived dendritic cell
MPAL	mixed phenotype acute leukemia
MRD	minimal residual disaese
NGFR	nerve growth factor receptor
NK cell	natural killer cell
NSE	non specific esterase
OS	overall survival
PB	peripheral blood
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSCT	peripheral blood SCT
PCR	polymerase chain reaction
PD-L1	programmed death receptor ligand 1
PE	phyco-erythrin
PerCP	peridinin chlorophyll protein
PGE2	prostaglandin-E2
PGN	peptidoglycan
PHA	phytohemagglutin
PKC	protein kinase C
Poly(I:C)	polycyticylic acid potassium
PR1	proteinase 3 derived peptide
PRAME	preferentially expressed antigen of melanoma
PRR	pattern recognition receptors
Q-RT-PCR	quantative real-tIme polymerase chain reaction
RA	retinoic acid
RFS	relapse free survival
RHAMM	receptor for hyaluronic acid mediated motility
RT	room temperature
SAHA	HDACi suberoylanilide hydroxamic acid
SAM	significance analysis of microarrays
SBB	Sudan black B
sCD3	surface CD3
SCF	stem cell factor
SCT	stem cell transplantation
sIgM	surface immunoglobulin M
SOCS	suppressors of cytokine signaling
SSC	sideward scatter
T/My	T cell / Myeloid
TAA	tumor-associated antigens
T-ALL	T cell acute lymphoblastic leukemia
TAP	transporter for antigen presentation
TCR	T cell receptor

TFA	trifluoroacetic acid
TGF-β	tumor growth factor-beta
Th cell	T helper cell
TKI	tyrosine kinase inhibitors
TLR	Toll-like receptor
TLR-L	Toll-like receptor-ligand
Tm	tetramer
TNFα	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
UPN	unique patient number
VEGF	vascular endothelial growth factor
WBC	white blood cell
WHO	world health organisation
WT-1	Wilms' tumor-gene product 1

Part I: General introduction

W van den Ancker
TM Westers
GJ Ossenkoppele
AA van de Loosdrecht

Introduction

At the beginning of a new life, one totipotent stem cell gives rise to the development of all kinds of tissues which ultimately form the human being. Later in life, multipotent stem cells maintain the normal turnover of regenerative organs, such as blood, skin and intestinal tissues. The key features of stem cells are that they have the ability to differentiate into diverse specialized cell types and can self-renew to produce more stem cells. Hematopoietic stem cells reside in the bone marrow and are necessary for maintaining the cellular components of the blood. The myeloid stem cell is the precursor cell for the erythrocytes, neutrophils, monocytes and platelets, the lymphoid precursor cell gives rise to B, T and NK cells [1].

Acute myeloid leukemia (AML) is a cancer of the white blood cells (WBC) in which myeloid precursor or stem cells transform into leukemic cells that display uncontrolled growth and lack of differentiation into mature cells. In case of AML, leukemic cells accumulate in the bone marrow and hamper the production of normal blood cells. This will result in low numbers of erythrocytes (anemia) and low numbers of platelets (thrombopenia) and hence increased risk of bleeding. Furthermore, reduction in the numbers of functionally normal neutrophils (neutropenia) is associated with an increased risk of infection. AML is a rare disease (2-3 per 100.000 men and women per year in the Netherlands); however, since its incidence increases with age and due to aging of the population, the incidence is increasing. If left untreated AML will result in death within weeks to months [2]. This aggressive nature warrants intensive treatment with high dose chemotherapy, such as cytarabine and anthracyclins such as daunorubicin [3]. After obtaining complete remission, that is, no more leukemic cells are detectable by morphology, additional (chemo)therapy is needed to prevent relapse of the disease. This so-called post remission therapy can include allogeneic stem cell therapy in poor prognostic cases. Complete remission is achieved in 70-80% of patients below the age of 60. Unfortunately, despite intensive treatment, 30-40% overall survival rates are accomplished [4].

PART I: Classification of AML

Transformation of a normal myeloid progenitor cell into a malignant cell can happen at every stage of differentiation along the myeloid lineages. These differences in differentiation stages can be examined by morphology and define their subclasses, for example: minimally differentiated AML, acute myelomonoblastic leukemia and acute megakaryoblastic leukemia. In addition, classification based on chromosomal aberrancies indicates an acute leukemia as AML with recurrent genetic abnormalities. For instance, rearrangement of parts of chromosome 8 and chromosome 21 (called translocation t(8;21)) will result in the formation of a fusion protein: RUNX1-RUNX1T1 . Presence of RUNX1-RUNX1T1 classifies an acute leukemia as AML with recurrent genetic abnormalities and implicates a good chance for survival after treatment [5]. However, not all AML cases can be classified on morphology or chromosomal aberrancies. In that case classification is based on other cell specific features such as proteins present on the cell membrane or in the cytoplasm. Based on various combinations of these proteins, differentiation between maturation stage along the myeloid lineages can be made. Leukemic cells express different proteins compared with their non-

malignant counterparts, the so called aberrant markers. For instance myeloid leukemias can express aberrant lymphoid markers such as CD7 or CD19. Even more, sometimes it is not clear-cut whether a particular leukemia originates from the lymphoid or myeloid lineage; these cases are called mixed phenotype acute leukemia (MPAL) [6]. For therapy decision making discrimination between the predominant lineage, myeloid or lymphoid, is necessary: acute myeloid leukemia warrants different treatment schemes compared to B and T lymphoblastic leukemia. However, for MPAL the predominant lineage preference is unclear. In *PART I* we discuss the role of immunophenotyping in diagnosing MPAL cases and we further investigate how new immunodiagnostic approaches might help in deciphering the myeloid or lymphoid lineage predominance of an acute leukemia of ambiguous lineage.

PART II: The immune system and AML

In general, both myeloid and lymphoid cells protect the human being against pathogens and tumor cells. To do so, cells of the immune system have the capacity to distinguish non-self antigens from self antigens. Cells of the innate immune systems recognize these non-self antigens in a generic, relatively non-specific manner, but do not induce long-lived immunity. Cells responsible for innate immunity are mainly derived from the myeloid lineage, for instance neutrophils and macrophages. For long-lived immunity, activation of cells of the adaptive immune system is necessary and involves activation of T and B cells. After recognition of a specific antigen, naïve B and T lymphocytes will transform into activated B and T cells. B cells transform into plasma cells which secrete antigen-specific antibodies; these antibodies function as flags for the other cells of the immune system, such as Natural Killer (NK) cells and granulocytes to clear away these target cells. In contrast to B cells, activated cytotoxic ($CD8^+$) T cells, a subpopulation of T cells, are able to recognize and kill their target cells directly. Recognition of antigens by T cells also occurs in a specific manner: $CD8^+$ and $CD4^+$ T cells only recognize antigens presented by the Major Histocompatibility Complex (MHC) associated molecules which are found in most vertebrates; the human counterpart is called the human leukocyte antigen (HLA) molecule. HLA class I molecules are presented by all nucleated cells of the human body, HLA class II molecules can be found on antigen presenting cells (APC). Activation of T cells happens through APC, such as dendritic cells (DC), macrophages and B cells. APC are able to engulf infected cells (or part of cells) and present processed antigens onto their HLA class I and/or II molecules. Furthermore, APC have co-stimulatory molecules on their cell surface which ensure activation of $CD8^+$ T and $CD4^+$ T cells and subsequent outgrowth of memory/effector cytotoxic T cells or T helper cells, respectively. B cells and cytotoxic T cells receive help from a second group of T cells: $CD4^+$ T helper cells. T helper cells are activated in more or less the same manner as $CD8^+$ T cells; however, they recognize peptides presented in HLA class II molecules [7].

Presentation of antigens onto HLA class I molecules involves various steps: intracellular proteins are cleaved into peptides by the proteasome and these peptide fragments are transported by a transporter for antigen presentation (TAP) into the endoplasmatic reticulum (ER) where the peptide is bound to the HLA class I molecule. Via the Golgi apparatus the HLA-peptide complex is transported to the cell surface (*figure 1A*). Presentation of extracellular antigens onto MHC class I happens through the cross presentation pathway, summarized in *figure 1C*. Loading of antigens onto HLA class II molecules involves a different pathway:

extracellular molecules such as antibody or complement-coated complexes or (particles from) dead cells are engulfed by the APC and processed to peptides en route through the endosomal/lysosomal pathway. The Invariant chain protein is necessary for transport of the HLA class II molecule into the ER and MHC class II compartments (MIIC). In the MIIC the Invariant chain is cleaved and a small remnant called Class II-associated invariant chain peptide (CLIP) remains in the binding groove of the HLA molecule. Subsequently CLIP is exchanged for an immunogenic peptide and presented by the HLA class II molecule onto

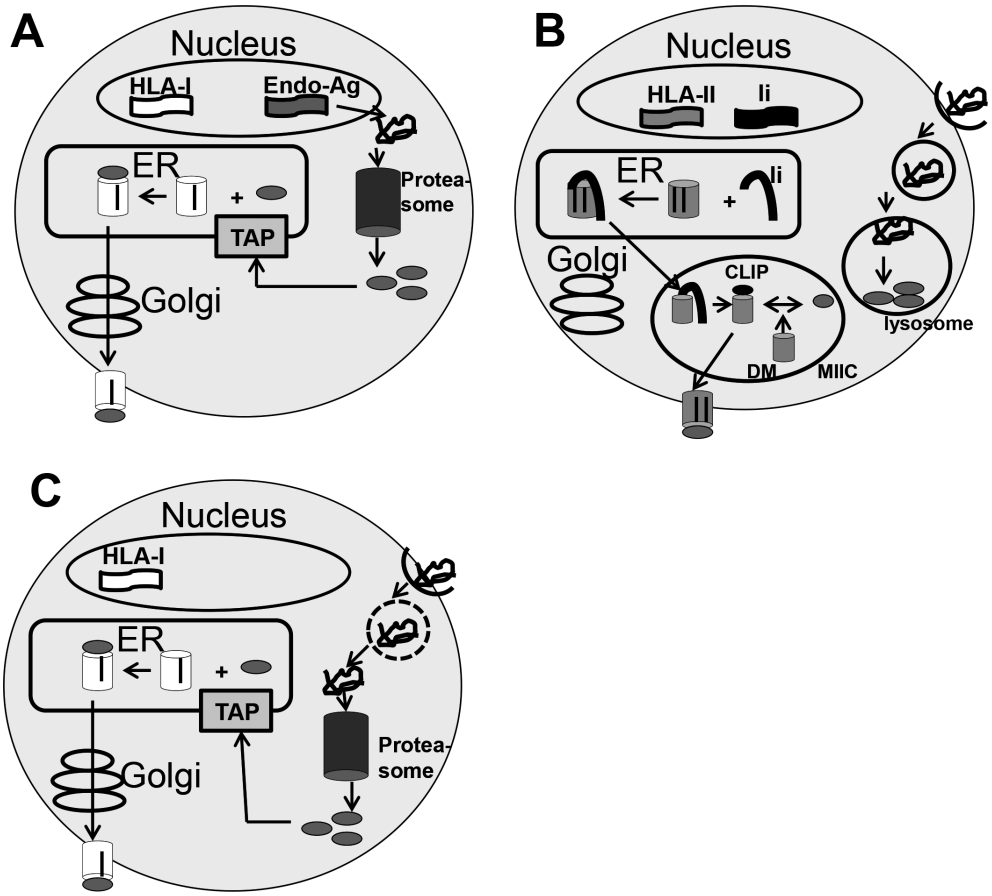


Figure 1. HLA class I and II antigen presentation. In (A) HLA class I antigen loading is presented. Intracellular peptides are cleaved by the proteasome and then transported via the transporter of antigen presentation (TAP) into the endoplasmic reticulum (ER) and subsequently antigens are presented onto HLA class I. Presentation of extracellular antigens onto HLA class II (B) involves a different pathway. Exogenous antigens are processed through the endosomal/lysosomal pathway. In the Major Histocompatibility Complex class II compartments (MIIC) compartment the Invariant chain (Ii) –derived Class II-associated Invariant chain peptide (CLIP) peptide is exchanged for an antigen under influence of HLA-DM and presented on the surface. In (C) cross-presentation of exogenoous antigens onto HLA-class I is depicted

surface the antigen presenting cell (summarized in *figure 1B*). However, CLIP can remain in the binding groove and thus prevents presentation of antigenic peptides. This results in escape of recognition by $CD4^+$ T helper cells; this immune escape mechanism and the role of CLIP on leukemic cells is discussed in *PART II* of this thesis and offers a target for immunotherapeutic strategies [8].

PART III: Cell based immunotherapy

Immunotherapy aims at generating an immune response directed against residual tumor cells which have escaped standard chemotherapy. Immunotherapy can be passive, e.g. antibody-based immunotherapy, or active, e.g. aimed at the *in vivo* induction of LAA-specific cytotoxic T cells and memory T and B cells. Activation of leukemia associated antigen (LAA)-specific T cells is achieved by whole tumor cell-based anti-leukemia vaccination. In the 1970s, the first cell-based vaccines were administered for the treatment of AML: patients received chemotherapy combined with a vaccine consisting of irradiated AML cells co-administered with Bacillus Calmette-Guierin (BCG). In relation to the control group, addition of immunotherapy prolonged overall survival (545 vs. 303 days) [9]. However, compared to APC leukemic cells are poor inducers of immune responses due to lack of costimulation and inadequate antigen presentation. In an attempt to enhance the immunogenicity of leukemic cells, these can be cultured into leukemic cell-derived DC. Another option is to culture monocytes into DC and load these with for instance LAA-derived peptides or lysates from leukemic cells. For the development of new immunotherapeutic strategies it is essential to optimize the AML vaccine preparation of choice. In *PART III* of this thesis we discuss the role of DC preparation, antigen loading and subsequently compare the function of various DC in order to identify the most efficacious DC preparation methodology for immunotherapeutic purposes.

Conclusion

In summary, in this thesis we exploit the additional value of immunologic methods for diagnosing AML and ALL with overlapping features i.e. MPAL. In addition, we investigate the biology of immune-escape mechanisms involved in antigen presentation by AML cells and exploit the role of immune modulation via DC for the immunotherapy of AML. These three pillars might be of importance in the understanding and subsequent further development of new emerging immunotherapeutic strategies for AML.

Reference List

1. Becker MW, Jordan CT. Leukemia stem cells in 2010: current understanding and future directions. Blood Rev. 2011;25:75-81.
2. Provan D, Singer CRJ, Baglin T, Lilleyman J. Acute myeloblastic leukaemia (AML). In: Provan D, Singer CRJ, Baglin T, Lilleyman J, eds. Oxford handbook of clinical haematology. Oxford, UK: Oxford University Press; 2004:149-157.

3. Dohner H, Estey EH, Amadori S et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 2010;115:453-474.
4. Lowenberg B, Ossenkoppele GJ, de Witte T, Boogaerts MA. Handboek Hematologie: de Tijdstroom; 2008.
5. Swerdlow SH, Campo E, Harris NL et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition.: IARC Press, Lyon; 2008.
6. Borowitz MJ, Béné M-C, Harris NL et al. Acute leukaemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition. 2008:
7. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245-252.
8. van Luijn MM, van den Ancker W, Chamuleau ME et al. Impaired antigen presentation in neoplasia: basic mechanisms and implications for acute myeloid leukemia. Immunotherapy 2010;2:85-97.
9. Powles RL, Crowther D, Bateman CJ et al. Immunotherapy for acute myelogenous leukaemia. Br.J.Cancer 1973;28:365-376.

Immunophenotyping of acute leukemia: introduction into the WHO2008

2

Adapted from: Immuno-fenotyperingsdiagnostiek van acute leukemie met lymfatische en myeloïde kenmerken: WHO 2008

M Terwijn
W van den Ancker
TM Westers
GJ Ossenkoppele
AA van de Loosdrecht

NTVH. 2009;6: 205-210

Abstract

Acute leukemias (AL) can be categorized as myeloid AL (AML), B lymphoblastic or T lymphoblastic leukemia (B- or T-ALL) based on predominance for the myeloid, B- or T- lymphoid lineage respectively. In about 4% of AL it is not clear whether an AL should be diagnosed as AML, B- or T-ALL. Those leukemias with both lymphoid and myeloid characteristics were classified as bilineal or bi-phenotypical AL according to the WHO criteria from 2001. In 2008 the WHO published new criteria for diagnosing a leukemia with a mixed phenotype. These changes contain some major changes in the classification of MPAL; the impact of the WHO2008 guidelines is reviewed here. Furthermore, we discuss some advances in recent diagnostic tools.

Introduction

Acute leukemias (AL) are classified according to commitment to the lymphoid or myeloid lineage. For differentiation between these two lineages immunophenotyping plays an important role. Using a flow cytometer cell specific features such as proteins present on the cell membrane or in the cytoplasm can be visualized. These proteins can be identified by monoclonal antibodies; the latter are grouped as so-called clusters of differentiation (or clusters of designation, CD) which is an internationally used nomenclature for antibodies. For instance, CD34 is an antibody that recognizes a cell surface glycoprotein which functions as a cell to cell adhesion factor and is found on progenitor cells. Some antibodies (more frequently called markers) are often used for identification of various stages of differentiation and are summarized in *figure 1* for the lymphoid lineage and in *figure 2* for the myeloid lineage. After labelling of cells in vitro with different fluorescent labelled antibodies, the phenotype of cells can be analyzed using a flow cytometer. This technique allows analysis of various different antigens on an individual cell.

In about 4% of AL it is unclear whether an AL should be classified as an acute myeloid leukemia (AML) or a precursor lymphoblastic leukemia (throughout this thesis abbreviated as ALL). The latter being devided in precursor B- and T-neoplasms. In the WHO criteria from 2001 AL with both lymphoid and myeloid characteristics are classified as bilinear or biphenotypical AL. Acute bilinear leukemia (aBLL) consist of AL with two (or more) separate blast populations with different cell lineage commitment. Biphenotypical AL (BAL) is classified when a single blast population is present with expression of antigens of different cell lineages. The prognosis of BAL or aBLL is worse compared with AML or ALL. This difference in prognosis can partly be explained by presence of the Philadelphia chromosome or complex chromosomal aberrancies [1]. Furthermore, extramedullary disease is more often present in aBLL or BAL compared to AML or ALL. As a consequence of the worse prognosis more intensive treatment might be necessary in case of BAL or aBLL. Furthermore, it is unclear whether BAL benefit from an AML or ALL-based (intensified) treatment protocol; this partly explains their unfavorable prognosis. [1-10] To unravel these questions randomised studies are warranted. Moreover, it is necessary that these studies are based on a uniform international classification system. Until 1995 no guidelines for classification of BAL or aBLL were available. With the development of the EGIL (European group for the immunological classification of AL) criteria clear guidelines were provided

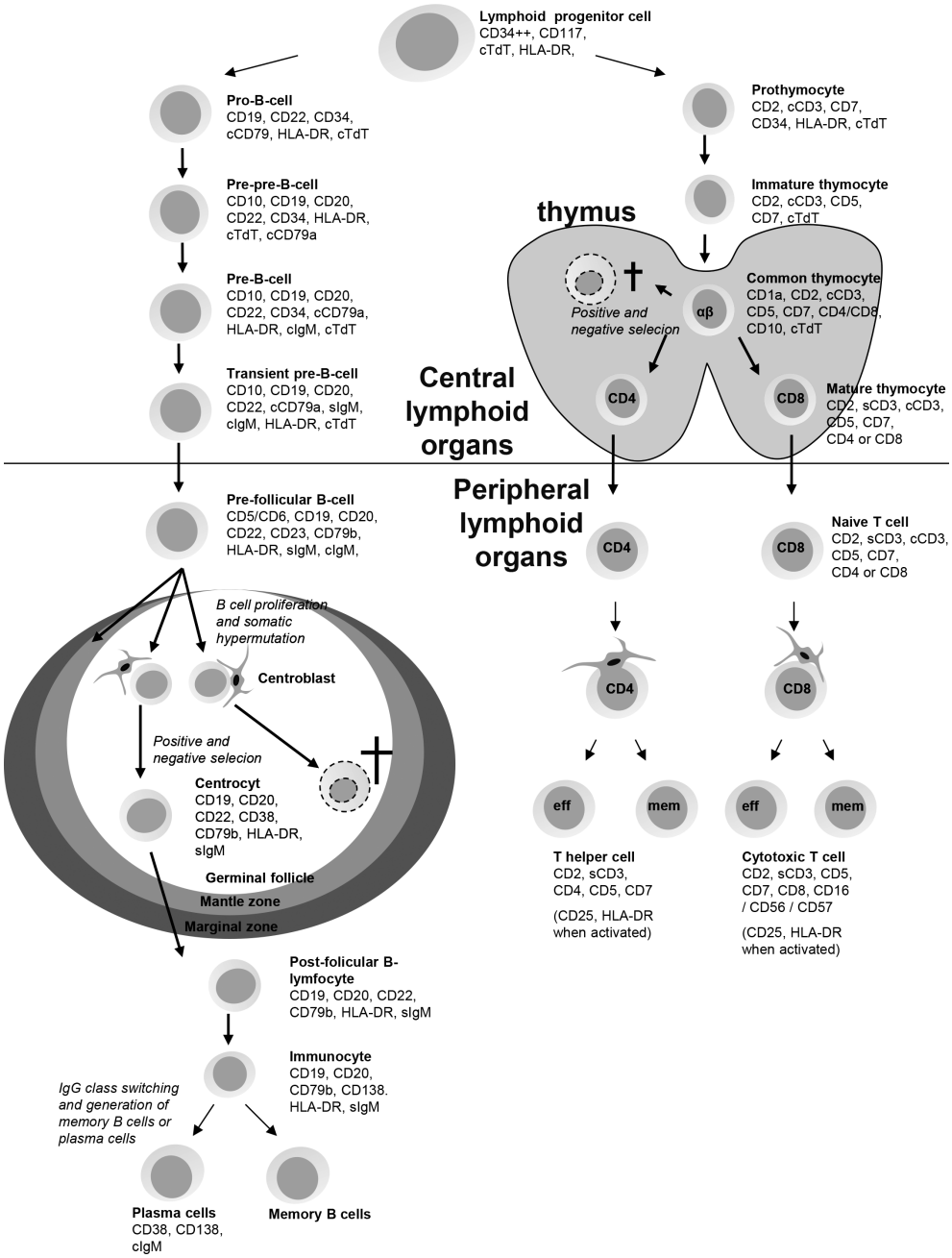


Figure 1. Hypothetic scheme of differentiation of the lymphoid lineage in central lymphoid organs, including the thymus and peripheral lymphoid organs. "++" strong expression, "c" cytoplasmic, "s" surface, eff: effector, mem: memory

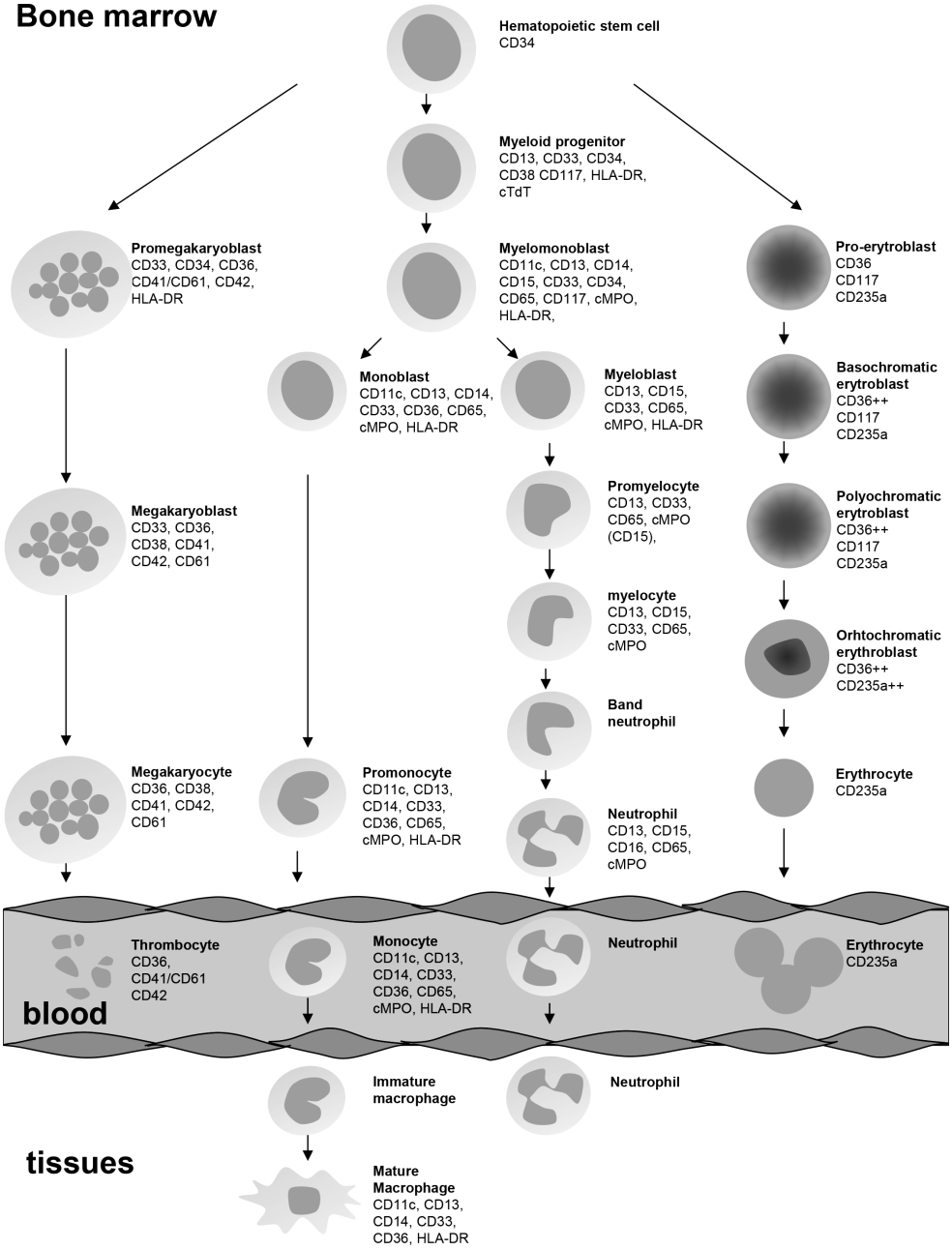


Figure 2. Hypothetic scheme of differentiation of the myeloid lineage in bone marrow, peripheral blood and tissues. "++" strong expression, "c" cytoplasmic, "s" surface, cMPO: cytoplasmic myeloperoxidase

for immunophenotypical classification of aBLL or BAL which were in 2001 adopted in the WHO2001 guidelines for the classification of haematopoietic malignancies [11;12]. In 2008 the adjusted WHO guidelines for diagnosis of haematopoietic and lymphoid malignancies were published [13]. The changes which these guidelines entail for diagnosis of AL with myeloid and lymphoid characteristics are reviewed here.

Biphenotypical acute leukemia according to EGIL 1995 and WHO 2001

Based on presence of immunological markers the EGIL group proposed criteria for differentiation between different cell lineages of AL [11]. Beside blast-like features such as presence of CD34 and diminished expression of CD45 (CD45dim), at least two early B cell lineage markers have to be present for classification of a B-ALL, for instance cytoplamsic IgM (cIgM), cytoplasmic CD22 (cCD22) or cytoplasmic CD79a (cCD79). A T-ALL is defined based on presence of cytoplasmic or surface CD3 (cCD3 or sCD3) expression. An AML is classified based on expression of at least two of the following markers: cytoplasmic myeloperoxidase (cMPO), CD13, CD33, CD65 or CD117; from these cMPO has the highest cell lineage specificity. For determining a marker as positive at least 20% of blasts are required to express a marker. For cCD79a, sCD3, cCD3, cMPO and cTdT a lower cut-off of 10% is used, when confirmed by light microscopy. However arbitrary, in clinical practice this cut-off of 10% is used without confirmation by light microscopy [8].

Table 1. Scoring system based on the criteria of the EGIL working party for the definition of biphenotypical acute leukemia (BAL)*

Points#	B cell lineage	T cell lineage	Myeloid lineage
2	Cytoplasmic CD79a	T cell receptor α/β	Cytoplasmic MPO
	Cytoplasmic IgM	T cell receptor γ/d	
	Cytoplasmic CD22	CD3 (cytoplasmic or surface expression)	
1	CD19	CD2	CD13
	CD10	CD5	CD33
	CD20	CD8	CD65
		CD10	CD117
0,5	Cytoplasmic TdT	Cytoplasmic TdT	CD14
	CD24	CD7	CD15
		CD1a	CD64

* A BAL is defined when more than 2 points for two cell lineages are present
# Every marker is scored according to the corresponding points when >20% positivity is found on the blast population; for cytoplasmic MPO, cytoplasmic CD79a, cytoplasmic TdT and cytoplasmic and surface CD3 a cut-off of 10% can be used when confirmed by light microscopy.

For diagnosis of aBLL, the WHO2001 proposed that at least two or more different populations should be present with expression of differentiation markers as defined for an AML or ALL. When one leukemic blast population expresses markers from different cell lineages differentiation between a BAL or AML or ALL with aberrant marker expression is necessary. The EGIL working party proposed a scoring system based on cell lineage specificity of various markers expressed on one blast population (*table 1*) [11;14]. An AL is considered a BAL when more than 2 points are scored for two different cell lineages.
Until 2000, CD117 was scored as 0.5 points, in that year it was changed to 1 point because of demonstrated higher specificity for the myeloid lineage[15;16]. The EGIL and WHO2001 criteria offered a clear and simple basis for the diagnosis of BAL and aBLL and thus offered a uniform basis for research of treatment and prognosis.

Limitations of the EGIL 1995 and WHO 2001 guidelines

Within the EGIL scoring system many different combinations of markers are possible and hence give rise to a heterogeneous group of BAL. Furthermore, some combinations of markers are less specific for BAL: a pro-B-ALL with MLL translocations often aberrantly express CD15, CD65, CD13, CD33. Furthermore, cCD79a expression is often observed in case of T-ALL. Another point of criticism is that the EGIL criteria have not implemented expression levels of various markers: for instance brighter expression of CD19 is considered more specific for the B cell lineage [8;17;18]. Moreover, various laboratories use different combinations of markers and often exclude some markers as proposed by EGIL, resulting in discrepancies in classification. Consequently, the WHO2008 guidelines provided revised criteria based on a shortened panel of markers and included new subgroups of acute leukemias based on cytogenetic changes. These WHO2008 guidelines provide the most recent criteria for classification of AL of unknown origin [19].

Biphenotypic acute leukemia according to the WHO2008: mixed phenotype acute leukemias

In the WHO2008 guidelines, no clear immunological criteria are provided for classification of a T-ALL, B-ALL or AML. Presence of myeloid markers combined with absence of lymphoid markers is sufficient for the diagnosis of an AML. Likewise, when lymphoid markers are present on blasts and myeloid markers are absent an ALL can be diagnosed. An AL unclassifiable can be considered when no cell lineage-specific markers are detected on blast cells (often CD34, CD38 and HLA-DR positive). The WHO2008 does not discriminate between bilinear leukemia and biphenotypic AL; both are classified as mixed phenotype AL (MPAL). When two different populations of leukemic cells are present with both clear different myeloid or lymphoid lineages (aBLL according to EGIL) this is sufficient for diagnosing a MPAL. When one population of blasts is present with combined expression of B-lymphoid, T-lymphoid or myeloid markers the WHO2008 has clear requirements for classification of MPAL (summarized in *table 2*). For the myeloid component of a MPAL expression of cMPO

is necessary; in contrast to the EGIL criteria, a combination of CD13, CD33 and CD117 is not sufficient to indicate myeloid restriction. Furthermore, when cMPO is absent but there is clear monocytoid differentiation (based on expression of two or more markers: being non specific esterase (NSE), CD11c, CD14, lysozyme or CD36) the myeloid component may be scored positive. For the T cell lineage expression of cytoplasmic or surface CD3 is sufficient when taking into account that the expression is as high as or higher than the non-malignant T cells of the patient. Whereas for the T cell and myeloid lineage, expression of solely CD3 and cMPO, respectively, is sufficient, for the B cell lineage expression of at least two markers is necessary. One of these markers should be CD19, combined with CD10 cCD22 and/or cCD79a. Only one exception is made: when cCD22, cCD79a and CD10 are present in combination with the myeloid/T cell markers, a MPAL may be considered [19].

Limitation of the WHO 2008 classification of acute leukemias of unknown origin.

Interestingly, the WHO2008 criteria did not include cut-off criteria for the various markers. Since intracellular markers such as cMPO and cCD3 are quite cell lineage-specific it can be hypothesized to maintain the 10% cut-off for these markers [8;11]. To diagnose a MPAL or AL unclassifiable, immunohistochemic and immunophenotypic analysis is necessary. Of note, NSE and lysozyme are essential to demonstrate commitment to the myeloid lineage but are not commonly used in flow cytometric analysis. Moreover, some phenotypes are not easily assigned to MPAL, AML or ALL.

Table 2. WHO 2008: requirements for diagnosing a mixed phenotype acute leukemia

B cell linage *	T cell lineage *	Myeloid lineage *
Strong expression[$] of CD19 combined with strong expression[$] of: Cytoplasmic CD22, CD10 or Cytoplasmic CD79a	Strong expression[$] of CD3 (cytoplasmic or surface expression)	Expression of MPO
or		or
weak expression[#] of CD19 with strong[$] expression of at least two of the following markers: cytoplasmic CD22, CD10 ór cytoplasmic CD79a		Presence of monocytic differentiation as defined by presence of at least two monocytoid markers: CD11c, CD14, CD36, CD64, lysozyme or non specific esterase (NSE)

* A MPAL is defined when the blasts meet the criteria of two or more lineages as described in the table.

[$] Strong expression is defined when expression of the marker is as high or higher than the non-malignant T or B cells in the patient's samples

[#] Weak expression is defined when expression of the marker is lower than the non-malignant B cells in the patient's samples.

Furthermore, an AL that does not meet all criteria for an undifferentiated AL but shows markers from different maturation lineages, are not easily classified by the WHO2008 criteria (these are by definition negative for cMPO, cCD3, cCD22, cCD79a and CD19). For instance, it is unclear how AL with expression of CD19 (diminished), cCD79a, CD33, CD13, CD11c, CD117 and absence of cMPO and CD34 should be classified (*table 4*). According to the EGIL criteria it is plausible to classify these AL as BAL.

Exceptions to immunological classification of MPAL by WHO 2008

By the WHO2008 all AL that can be classified as AML or ALL based on molecular, cytogenetic or clinical features are excluded from the diagnosis MPAL. For instance an AL with t(8;21); t(15;17) or inv(16) are classified as AML by definition and are thus excluded from MPAL [20]. Furthermore, AL with FGFR1 mutations, CML blast crises, AL with MDS prephase and therapy-related AL cannot be classified as MPAL.

Subclassification of MPAL

Next to immunophenotypic guidelines for AL with mixed phenotype, the WHO2008 provides further classification based on cytogenetics. Some translocations known to be commonly present constitute different subgroups of MPAL, for instance: MPAL with t (9;22)(q34;q11.2) (without CML pre-phase) and MPAL positive for the MLL gene. Besides these, many other genetic aberrancies have been described in combination with a MPAL phenotype as yet. However, these genetic aberrancies are not commonly present and cannot provide further classification of subclasses. The classification of MPAL according to the WHO2008 is summarized in *table 3*.

Table 3: Classification of MPAL

MPAL B cell/Myeloid (B/My), Not Otherwise Specified (NOS)
MPAL T cell/Myeloid (T/My), NOS
MPAL with t(9;22)(q34;q11.2), excluded are blast crises with presence of pre-phase of CML
MPAL with (11q23)MLL
MPAL NOS, rare subtypes:
> MPAL with T and B cell characteristics (MPAL T/B)
> MPAL with characteristics of three lineages (T/B/My)
> MPAL with megakaryocytic characteristics (B/ or T/megakaryocytic leukemia)
> MPAL with erytroblastoid characteristics (B/ or T/erytroleukemia)

Consequences of implemantation of the WHO2008

There are interesting differences between the BAL/aBLL according to WHO2001 and classification of a MPAL according to the WHO2008. A T/My or a B/MY BAL negative for (cytoplasmic) CD3, CD19 or cMPO cannot be classified as MPAL. Expression of cMPO or (cytoplasmic) CD3 is now sufficient for the myeloid or T cell lymphoid component of a MPAL. An AL with expression of cMPO and CD19 can be classified as a B/My MPAL depending on the expression of cCD22 cCD79a and CD10. In *table 4* some illustrating examples are shown of differences in classification of a BAL/aBLL or a MPAL in clinical practice [21].
Furthermore, the cut-off for marker positivity (20% according to the WHO2001) is left open for interpretation in the WHO2008. Recently, we demonstrated that a cut-off of 10% for cMPO could be used independently from results of sudan black B (SBB) staining in deteminating the myeloid or lymphoid predominance according to the WHO2008 guidelines [22]. These ambiguities in the WHO2008 might impede further research for the treatment and prognosis of MPAL.
Recently, a large retrospective study was published reviewing survival and different treatment schemes in 100 cases classified as MPAL. In these 100 MPAL the poor prognosis was confirmed however largely influenced by the Philadelphia positive subgroup of patients. Interestingly, treatment according to ALL protocols resulted in a favourable outcome, however these results were not correlated with different subgroups of MPAL as proposed by WHO2008 [23]. In conclusion, it would be highly favourable and a great challenge due to low frequency of AL with combined myeloid and lymphoid features, to prospectively validate the clinical impact of the WHO2008 compared with the WHO2001.

Table 4. Some examples of showing the differences in classification by the WHO 2001 and WHO2008

	WHO2001	WHO 2008
AL positive for : cMPO, CD13, CD33, cCD79a, CD10	BAL	AML
AL positive for: cMPO, CD33, CD13 and CD19strong, CD10	AML	MPAL
AL positive for: cMPO, CD13, CD19(weak), CD10, cTdT	BAL	AML
AL positive for: CD19(weak), cCD79a, CD33, CD13, CD117	BAL	Not classifiable
AL positive for: cCD3(strong), cMPO, cTdT	T-ALL	MPAL

Alternative classification strategies for acute leukemias of unknown origin

Classification of acute leukemias is based on different diagnostic tools: cytomorphology, flow cytometry, immunohistochemistry, cytogenetic analysis and molecular analysis. Yan et al. recently analyzed 117 MPAL cases for clinical, immunophenotypic, cytogenetic, and molecular genetic features and concluded that MPAL constitutes as a heterogenous group [24]. Thusfar, immunophenotypic analysis is the hallmark for identification of MPAL cases. However, the immunophenotype of acute leukemias is merely a limited reflection of the

genotypic state. Consequently, gene array or miRNA analysis might add to differentiating between AML, ALL or MPAL. Gene expression profiling on thirteen pediatric MPAL cases revealed that 5 cases clustered with AML; the rest constituted a separate entity, however, very close to B-ALL [25]. This suggest that some of the pediatric leukemias of ambiguous lineage assembly a separate entity. However, one could argue that these cases constitute a subtype within B-ALL since this separate group clustered very close to the B-ALL. In contrast to gene expression profiling, MiRNA expression profiling has shown to be more successful in the classification of tumors [26]. Furthermore, miRNA expression profiles can accurately discriminate AL of the lymphoid lineage from AL of the myeloid lineage [26-28]. Therefore, we recently evaluated the role of miRNA expression profiling for differentiation between MPAL, B-ALL, T-ALL and AML. Interestingly, the MPAL/BAL cases (n=15) did not segregate as a separate entity but showed microRNA expression profiles similar to that of either AML, B-ALL or T-ALL. This indicates the presence of a lineage specific genotype despite their mixed lineage immunophenotype.

Conclusion

Based on cytogenetic aberrancies risk profiles have been developed which determine whether or not a patient needs an intensified treatment scheme. Ultimately, a personalized treatment should be based on the features of the leukemic cells as assessed by for instance gene arrays and may differ per patient. These developments in diagnostic procedures might be applied onto leukemias of unknown origin: gene or miRNA arrays might offer a more accurate classification method and differentiate more precisely between ALL, AML or truly MPAL than currently applied diagnositic tools such as immunophenotypic analysis. These techniques might add to the classification of MPAL in the future and thereby aid treatment decision making.

Reference List

1. Xu XQ, Wang JM, Lu SQ et al. Clinical and biological characteristics of adult biphenotypic acute leukemia in comparison with that of acute myeloid leukemia and acute lymphoblastic leukemia: a case series of Chinese population. Haematologica 2009;94:919-927.
2. Aribi A, Bueso-Ramos C, Estey E et al. Biphenotypic acute leukaemia: a case series. Br.J.Haematol. 2007;138:213-216.
3. Buccheri V, Matutes E, Dyer MJ, Catovsky D. Lineage commitment in biphenotypic acute leukemia. Leukemia 1993;7:919-927.
4. Carbonell F, Swansbury J, Min T et al. Cytogenetic findings in acute biphenotypic leukaemia. Leukemia 1996;10:1283-1287.
5. Killick S, Matutes E, Powles RL et al. Outcome of biphenotypic acute leukemia. Haematologica 1999;84:699-706.
6. Lee JH, Min YH, Chung CW et al. Prognostic implications of the immunophenotype in biphenotypic acute leukemia. Leuk.Lymphoma 2008;49:700-709.

7. Legrand O, Perrot JY, Simonin G et al. Adult biphenotypic acute leukaemia: an entity with poor prognosis which is related to unfavourable cytogenetics and P-glycoprotein over-expression. Br.J.Haematol. 1998;100:147-155.
8. Owaidah TM, Al Beihany A, Iqbal MA, Elkum N, Roberts GT. Cytogenetics, molecular and ultrastructural characteristics of biphenotypic acute leukemia identified by the EGIL scoring system. Leukemia 2006;20:620-626.
9. Sulak LE, Clare CN, Morale BA, Hansen KL, Montiel MM. Biphenotypic acute leukemia in adults. Am.J.Clin.Pathol. 1990;94:54-58.
10. Weir EG, li Ansari-Lari M, Batista DA et al. Acute bilineal leukemia: a rare disease with poor outcome. Leukemia 2007;21:2264-2270.
11. Bene MC, Castoldi G, Knapp W et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995;9:1783-1786.
12. Brunning RD, Matutes E, Borowitz MJ et al. Acute leukaemias of ambiguous lineage. In: jaffe ES, Harris NL, Stein H, Vardiman JW, eds. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.: IARC Press, Lyon; 2001:106-107.
13. Swerdlow SH, Campo E, Harris NL et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition: IARC Press, Lyon; 2008:150-154.
14. Matutes E, Morilla R, Farahat N et al. Definition of acute biphenotypic leukemia. Haematologica 1997;82:64-66.
15. Bene MC, Bernier M, Casasnovas RO et al. The reliability and specificity of c-kit for the diagnosis of acute myeloid leukemias and undifferentiated leukemias. The European Group for the Immunological Classification of Leukemias (EGIL). Blood 1998;92:596-599.
16. Bene MC, Bernier M, Castoldi G et al. The value of c-kit in the diagnosis of biphenotypic acute leukemia. EGIL (European Group for the Immunological Classification of Leukaemias). Leukemia 1998;12:2038.
17. Kozlov I, Beason K, Yu C, Hughson M. CD79a expression in acute myeloid leukemia t(8;21) and the importance of cytogenetics in the diagnosis of leukemias with immunophenotypic ambiguity. Cancer Genet.Cytogenet. 2005;163:62-67.
18. Lai R, Juco J, Lee SF, Nahirniak S, Etches WS. Flow cytometric detection of CD79a expression in T-cell acute lymphoblastic leukemias. Am.J.Clin.Pathol. 2000;113:823-830.
19. Borowitz MJ, Béné M, Harris NL, Porwit A, McCann SR. Acute leukaemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. WHO Classification of Tumours of haematopoietic and Lymphoid Tissues, Fourth Edition.: IARC Press, Lyon; 2008:150-154.
20. Tiacci E, Pileri S, Orleth A et al. PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. Cancer Res. 2004;64:7399-7404.
21. van den Ancker W, Terwijn M, Westers TM et al. Acute leukemias of ambiguous lineage: diagnostic consequences of the WHO2008 classification. Leukemia 2010;24:1392-6
22. van den Ancker W, Westers TM, de Leeuw DC et al. A threshold of 10% for myeloperoxidase by flow cytometry is valid to classify acute leukemia of ambiguous and myeloid origin. Cytometry B Clin.Cytom. 2013;84:114-8
23. Matutes E, Pickl WF, Van't VM et al. Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. Blood 2011;117:3163-3171.

24. Yan L, Ping N, Zhu M et al. Clinical, immunophenotypic, cytogenetic, and molecular genetic features in 117 adult patients with mixed-phenotype acute leukemia defined by WHO-2008 classification. Haematologica2012;97:1708-12
25. Rubnitz JE, Onciu M, Pounds S et al. Acute mixed lineage leukemia in children: the experience of St Jude Children's Research Hospital. Blood 2009;113:5083-5089.
26. Lu J, Getz G, Miska EA et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834-838.
27. Wang Y, Li Z, He C et al. MicroRNAs expression signatures are associated with lineage and survival in acute leukemias. Blood Cells Mol.Dis. 2010;44:191-197.
28. Mi S, Lu J, Sun M et al. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. Proc.Natl.Acad.Sci.U.S.A 2007;104:19971-19976

The role of flow cytometry in diagnosing acute leukemias: two case reports

3

Adapted from: Uncommon lineage switch warrants immunophenotyping even in relapsing leukemia

W van den Ancker
M Terwijn
J Regelink
TM Westers
GJ Ossenkoppele
AA van de Loosdrecht

Leuk Res. 2009
Jul;33(7):e77-80

Adapted from: Paradox in immunophenotyping and morphology; a case report describing erythrophagocytosis in a mixed phenotype acute leukema

DC de Leeuw
W van den Ancker
TM Westers
AJ Loonen
SL Bhola
GJ Ossenkoppele
AA van de Loosdrecht

Leuk Res. 2011
May;35(5):693-6

Uncommon lineage switch warrants immunophenotyping even in relapsing leukemia

Introduction

Despite intensive treatment, 60% of adult patients with T-cell acute lymphoblastic leukemia (T-ALL), experience a relapse of the disease [1]. In the majority of cases this is due to the outgrowth of the initial T-ALL clone from minimal residual disease [2]. However, the induction of secondary acute myeloid leukemia (AML) by previous chemotherapeutic treatment, such as etoposide, has been described in about 5–7% of the cases at relapse, generally more than 2 years after the initial diagnosis. In most of the cases specific chromosomal abnormalities are present, which were not present at diagnosis [3-4]. Alternatively, relapses with complete lineage switches can occur. This has been proposed to be the result of the outgrowth of an undifferentiated leukemic stem cell, the result of the preferential outgrowth of a minor AML subclone present at diagnosis or the result of therapy [5-10]. In general, the morphology of the leukemic cells at relapse suggests a switch from ALL to AML. The patient described here showed a complete immunophenotypic switch, which was not considered because of similar morphology and chromosomal abnormalities at relapse and at diagnosis. In view of the increasing use of monoclonal antibodies in the treatment of acute leukemia, immunophenotypic investigation of leukemic cells at diagnosis and relapse will be of importance in clinical practice [11-12].

Case

A 20-year-old man was admitted to the hospital with recurring and antibiotic-resistant paronychia. He experienced a 25-kg weight loss in the preceding 3 months and complained of night sweats and fever. His medical history was unremarkable. Physical examination revealed paronychia of two toes and enlarged tonsils. There were no signs of lymphadenopathy, hepatosplenomegaly nor gingival hyperplasia. Laboratory investigations revealed an elevated lactate dehydrogenase (690 U/l; normal values 0–250 U/l) and a decreased hemoglobin level (6.8 mmol/l; normal values 8.5–10.5 mmol/l). The total leukocyte count and platelet count was normal. In the differential count, 70% blasts were found. By light microscopy, the blast cells were stained negative for Sudan black with many hand mirror cells (*figure 1A*). Flow cytometric analysis showed blasts ($CD34^+$) of lymphoid origin (cytoplasmic $CD3^+$ (cCD3), $CD5^+$, $CD2^+$, $cTdT^+$, $CD7^+$, $CD3^-$, $CD1a^-$, $CD10^-$, $cMPO^-$, $CD33^-$, $CD117^-$, $CD19^-$), with aberrant expression of CD56 and weak expression of CD13 (*figure 2A*). Based on morphology and immunophenotyping the diagnosis of a precursor T-cell acute lymphoblastic leukemia (T-ALL) was made according to WHO 2001 criteria. Cytogenetic analysis of bone marrow cells revealed a complex karyotype 52, XY, +?X, +8, +10, +11, +13, +19 [11]; 46, XY [9]. FISH

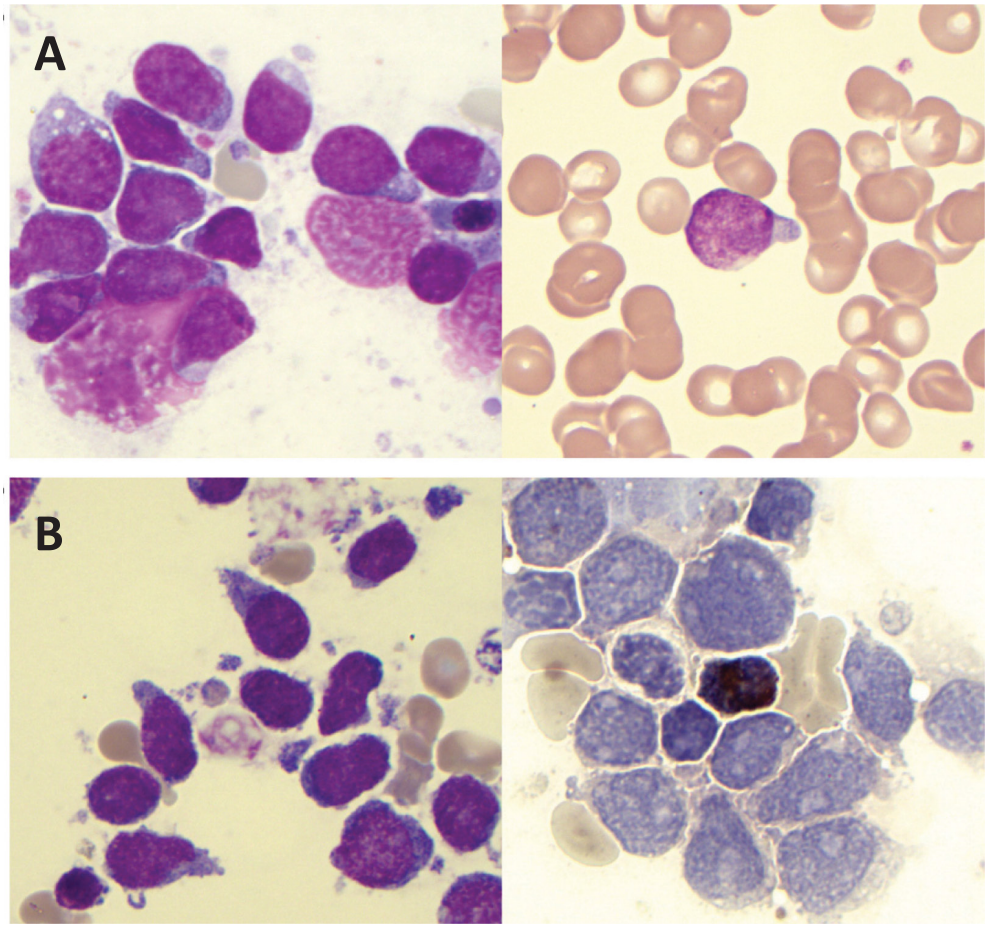


Figure 1. Morphology of leukemic cells at diagnosis and relapse. (A) Bone marrow and peripheral blood May-Gruenwald Giemsa (MGG) staining at diagnosis of T-ALL. (B) Bone marrow MGG and Sudan black staining of AML at relapse.

analysis performed with the LSI MLL-probe (Vysis) confirmed the presence of trisomy 11 by detecting 3 copies of the MLL-gene in 75% of 200 cells analysed. No molecular aberrancies could be detected by PCR. A lumbar puncture showed no localization of T-ALL.

The patient was treated according to a current Dutch ALL protocol (HOVON 70; *figure 3*). As no HLA-identical sibling was available he proceeded to maintenance therapy following induction and consolidation therapy, resulting in a complete remission. After nine months of maintenance therapy, 21 months after initial presentation, a relapse occurred. A bone marrow aspiration showed 91% Sudan black negative blasts with morphological features similar to diagnosis (*figure 1B*). Cytogenetic analysis showed an identical abnormal karyotype as compared at diagnosis. Molecular investigations (PCR) revealed no t(8;21) and FISH analysis showed trisomy 11 by MLL-gene analysis in 88% of the cells. Remarkably, the immunophenotype of the cells was completely switched from a lymphoid to a myeloid

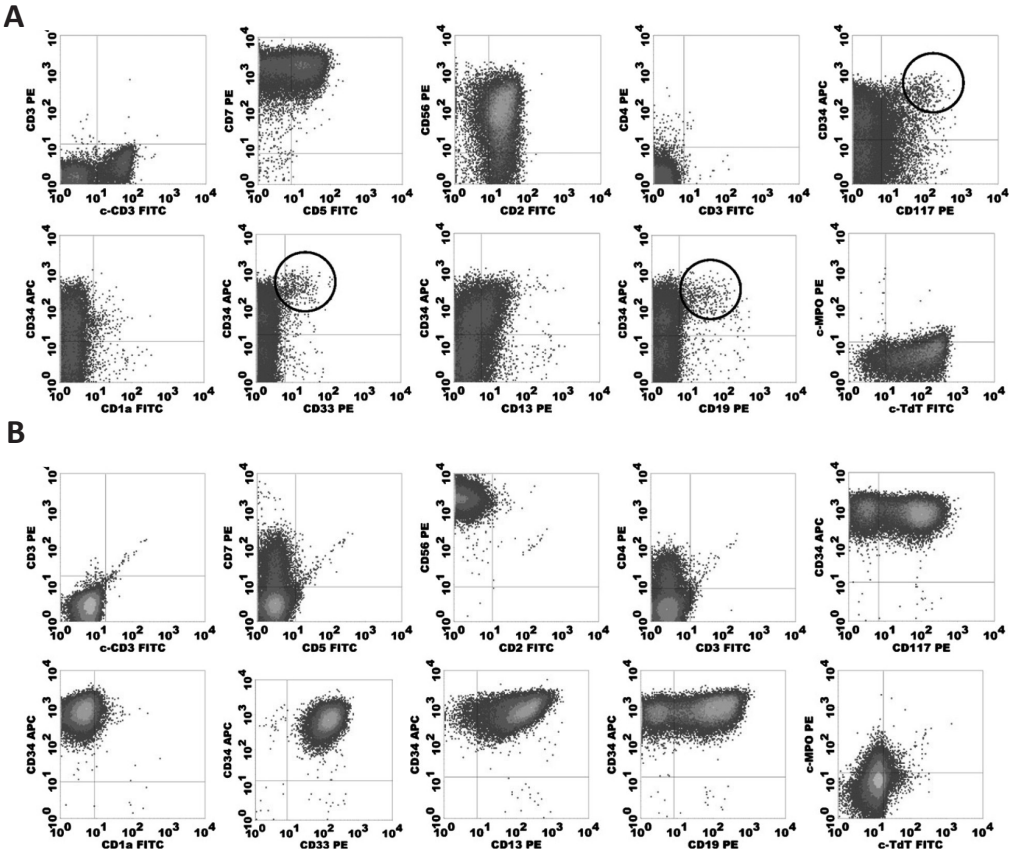


Figure 2. Immunophenotype of leukemic cells at diagnosis and relapse. (A) Immunophenotypic analysis of the T-ALL cells in bone marrow at diagnosis. A small myeloid subclone is encircled. (B) Immunophenotype of the myeloid cell population in peripheral blood at relapse.

phenotype ($CD117^+$, $CD33^+$, $CD13^+$, $CD56^+$, $cMPO^-$, $cTdT^-$, $CD7^-$, $cCD3^-$, $CD2^-$, $CD5^-$, $CD19^+$, *figure 2B*). AML was diagnosed following the WHO criteria. With the intention to perform an allogeneic stem cell transplantation with a matched unrelated donor, reinduction chemotherapy was initiated with high dose cytarabine (1000 mg/m2 tid for 4 days) and etoposide (120 mg/m2 oid for 4 days). Since no remission was reached, the patient subsequently underwent an autologous stem cell transplantation after treatment with high dose melphalan (200 mg/m2) [13]. Unfortunately, no remission was reached and he died 9 months after diagnosis of relapse due to the complications of longstanding pancytopenia.

Discussion

True lineage switches from T-ALL to AML in adolescents and adults are rare [5-7, 9, 14-16]. In a few of these cases, like in our case, both lymphoid and myeloid blast appeared to originate from one clone, as shown by an identical karyotype of the malignant cells [5-9].

Week 1	Pre-phase	Prednisone 60mg/m2
Week 2-5	Induction	Prednisone 40mg/m2; Vincristine 1.5mg/m2 (max 2.0); Daunorubicin 40mg/m2; Cyclophosphamide 1000mg/m2; Asparginase 6000IU/m2; Methotrexate intrathecal;
Week 5-9	Consolidation A	Thioguanine 60mg/m2; Cyclophosphamide 1000mg/m2; Cytarabine tid 30mg/m2; Methotraxate intrathecal
Week 10-13	Consolidation B	Prednisone 40mg/m2; 6-Mercaptopurine 50mg/m2; Vincristine 1.5mg/m2 (max 2.0); Methotrexate 5000mg/m2
Week 14-17	Intensification IA	Dexamethasone 10mg/m2; Vincristine 1.5mg/m2 (max2.0); Adriamycine 25mg/m2; Asparginase 6000IU/m2; Methotrexate intrathecale
Week 18-21	Intensification IB	Thioguanine 60mg/m2; Etoposide 150mg/m2; Cytarabine tid 30 mg/m2; Methotrexate intrathecale
Week 22-25	Interphase A	Predinsone 40mg/m2; Vincristine 1.5mg/m2 (max 2.0); 6-Mercapthopurine 50mg/m2; Methotrexate 5000mg/m2
Week 26-29	Interphase B	Prednisone 1dd 40mg/m2; 6-mercatopurine 50mg/m2; Vincristine 1.5mg/m2 (max 2.0); Methotrexate 5000mg/m2
Week 30-33	Intensification IIA	Prednisone 40mg/m2; Vincristine 1.5mg/m2 (max2.0); Daunorubicin 30mg/m2; Asparginase 6000IU/m2; Methotrexate intrathecal
Week 34-37	Intensification IIB	Thioguanin 60mg/m2; Cyclophosphamide 1000mg/m2; Cytarabine tid 30mg/m2; Methotrexate intrathecal
Week 38-104	Maintenance therapy	6-mercaptopurine 75mg/m2 (daily); Methotrexate 25mg/m2 (weekly)
Week 38-104	Re-induction	Prednixone 40mg/m2 (monthly); Vincristine 1.5mg/m2 (max2.0) (monthly)

Figure 3. ALL treatment according to the Dutch standard protocol HOVON 70.

Different hypotheses have been proposed for the occurrence of a lineage switch of clonally related cells in acute leukemia. It has been suggested that chemotherapy can induce changes in the differentiation program of the leukemic stem cell followed by switch of marker expression, while retaining its cytogenetic aberrancies [15, 17]. Interestingly, the group of Kurtzberg et al. described the occurrence of a lineage shift already 7 days after chemotherapy treatment with adenosine deaminase inhibitor 2´-deoxycoformycin. Because of the immature lymphoid phenotype ($CD7^+$, $CD4^-$ and $CD8^-$) and the rapid lineage switch, they hypothesized that the patient had a leukemia of a pluripotent stem cell that was capable of multilineage differentiation [8]. This outgrowth of an undifferentiated stem cell clone, could be confirmed in eight patients, from whom *in vitro* cultures of leukemic blasts gave rise to morphologically and immunophenotypically distinguishable progenitors of the myeloid, erythroid, megakaryocytoid, eosinophil, monocytoid, and lymphoid lineages independent of exposure to exogenous growth factors [18]. This is supported by the finding that in T cell leukemias with a $CD7^+$, $CD4^-$, $CD8^-$ and $CD1^-$ phenotype a stem cell phenotype can be determined defined as absence of both cCD3 and cMPO expression [19]. Additionally, in some of these patients a mixed lineage cell type could be determined, expressing both c CD3 and cMPO. Accordingly, more recently it has been described that early thymic progenitor cells possess both T-cell as well as myeloid differentiation potential [20-22]. After discovery of this early immature phenotype ($CD7^+$, $CD4^-$, $CD8^-$ and $CD1^-$) in ALL, these cases

were classified as immature T-ALL or T-stem cell leukemia [23]. Our patient also showed an immature T lymphoid phenotype: $CD7^{+}$, $CD4^{-}$, $CD8^{-}$ and $CD1a^{-}$ at initial diagnosis. At relapse this changed into an immature myeloid phenotype: $CD34^{+}$, $CD117^{+}$, $CD13^{+}$, $CD33^{+}$, $CD19^{+}$, $cMPO^{-}$, and $CD7^{-}$. Therefore, also in our case it can be hypothesised that under pressure of anti-lymphoid-leukemia directed therapy, an undifferentiated stem cell clone grew out as a myeloid relapse. On the other hand, in retrospect, we found a small population of $CD34^{high}$ cells of 1.2% in the $CD45^{dim}$ population at initial presentation (with possible co-expression of CD117, CD33 and CD19).

This population differed from the normal pre-B cells, which were brighter CD19 positive, lower in their CD34 expression and low in forward/side scatter compared to the $CD34^{high}$ population. Consequently, it can also be considered that a malignant population of myeloid blasts was already present at initial presentation (*figure. 2A*). Unfortunately, due to lack of material we were not able to classify this small population as myeloid malignant at diagnosis and therefore as bi-linear. In bi-lineage leukemias the therapy would have been directed towards the major population. Since the detected population in our patient covered only 1% of the total amount of blasts, the therapy would have been similar. However, in case of bi-linear leukemia, regular immunophenotyping to determine the immunophenotype of residual disease might indicate the need for therapy switches. A recent case of AML which relapsed as a T-ALL was described in which evidence was gained for a small T-cell clone at initial presentation with similar cytogenetic aberrancies.

In conclusion, we showed that a complete switch of immunophenotype can occur, while both morphology and cytogenetic features remain identical to the disease at diagnosis. In view of the increasing use of monoclonal antibodies this will be of importance in clinical practice. In this case anti-CD33 could have been an option in complementing standard high doses chemotherapy in conditioning treatment before unrelated matched stem cell transplantation. This case emphasizes the clinical importance of flow cytometric analysis at any relapse in patients with acute leukemias.

References

1. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet 2008;371:1030-1043.
2. Andersson A, Ritz C, Lindgren D et al. Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. Leukemia 2007;21:1198-1203.
3. Winick NJ, McKenna RW, Shuster JJ et al. Secondary acute myeloid leukemia in children with acute lymphoblastic leukemia treated with etoposide. J.Clin.Oncol. 1993;11:209-217.
4. Seedhouse C, Russell N. Advances in the understanding of susceptibility to treatment-related acute myeloid leukaemia. Br.J.Haematol. 2007;137:513-529.
5. Gagnon GA, Childs CC, LeMaistre A et al. Molecular heterogeneity in acute leukemia lineage switch. Blood 1989;74:2088-2095.
6. van der Velden V, van LK, Hoogeveen PG, Beverloo HB, Breems DA. Unusual immunophenotypic shift in a patient with T-cell acute lymphoblastic leukemia. Leukemia 2006;20:1626-1629.
7. Matsuda I, Nakamaki T, Amaya H et al. Acute myeloid leukemia originating from the same leukemia clone after the complete remission of acute lymphoid leukemia. Rinsho Ketsueki 2003;44:946-951.

8. Hershfield MS, Kurtzberg J, Harden E et al. Conversion of a stem cell leukemia from a T-lymphoid to a myeloid phenotype induced by the adenosine deaminase inhibitor 2'-deoxycoformycin. Proc.Natl.Acad.Sci.U.S.A 1984;81:253-257.
9. Saso R, Zomas A, Hamblin M et al. Sequential development of myelodysplasia and acute myeloid leukemia but with no karyotypic evolution after autografting in a patient with Philadelphia positive acute lymphoblastic leukemia. Leuk.Lymphoma 1997;26:625-628.
10. Mantadakis E, Danilatou V, Stiakaki E et al. T-cell acute lymphoblastic leukemia relapsing as acute myelogenous leukemia. Pediatr.Blood Cancer 2007;48:354-357.
11. Thomas DA, O'Brien S, Jorgensen JL et al. Prognostic significance of CD20 expression in adults with de novo precursor B-lineage acute lymphoblastic leukemia. Blood 2008
12. Castillo J, Winer E, Quesenberry P. Newer monoclonal antibodies for hematological malignancies. Exp.Hematol. 2008;36:755-768.
13. Bug G, Atta J, Klein SA et al. High-dose melphalan is an effective salvage therapy in acute myeloid leukaemia patients with refractory relapse and relapse after autologous stem cell transplantation. Ann.Hematol. 2005;84:748-754.
14. Hashimoto S, Toba K, Aoki S et al. Acute T-lymphoblastic leukemia relapsed with the character of myeloid/natural killer cell precursor phenotype: a case report. Leuk.Res. 2002;26:215-219.
15. Ihle R, Matthes H, Ihle H. Phenotype switch in acute leukemia patients after intensive chemotherapy. Haematol.Blood Transfus. 1989;32:101-103.
16. Pane F, Frigeri F, Camera A et al. Complete phenotypic and genotypic lineage switch in a Philadelphia chromosome-positive acute lymphoblastic leukemia. Leukemia 1996;10:741-745.
17. Stass S, Mirro J, Melvin S et al. Lineage switch in acute leukemia. Blood 1984;64:701-706.
18. Kurtzberg J, Denning SM, Nycum LM, Singer KH, Haynes BF. Immature human thymocytes can be driven to differentiate into nonlymphoid lineages by cytokines from thymic epithelial cells. Proc.Natl.Acad.Sci.U.S.A 1989;86:7575-7579.
19. Masuda M, Arai Y, Osawa M et al. Heterogeneity of $CD7^+$ 4- 8- 1- leukemia: usefulness of cytoplasmic antigen detection for subclassification. Leukemia 1993;7:1759-1765.
20. Bell JJ, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature 2008;452:764-767.
21. Wada H, Masuda K, Satoh R et al. Adult T-cell progenitors retain myeloid potential. Nature 2008;452:768-772.
22. Weerkamp F, Baert MR, Brugman MH et al. Human thymus contains multipotent progenitors with T/B lymphoid, myeloid, and erythroid lineage potential. Blood 2006;107:3131-3137.
23. Ferrara F, Cimino R, Antinolfi I et al. Clinical relevance of immunological dissection in T-ALL: a report on 20 cases with stem cell ($CD7^+$, $CD4^-$, $CD8^-$, $CD1^-$) phenotype. Am.J.Hematol. 1992;40:98-102.

Challenging diagnosis in a patient with clear lymphoid immunohistochemical features and myeloid morphology: mixed phenotype acute leukemia with erythrophagocytosis

Introduction

Acute leukemia (AL) is classified according to commitment towards either the myeloid or lymphoid cell lineage. In about 4% of acute leukemias it is unclear whether the blasts are of myeloid or lymphoid origin. These AL with both myeloid and lymphoid characteristics represent a worse prognostic subgroup and it is still a matter of debate whether patients may benefit from an acute lymphoid leukemia (ALL) or acute myeloid leukemia (AML) treatment. In the WHO2008 criteria for defining mixed phenotype acute leukemia (MPAL), important changes have been made in defining cell line specific and characterizing markers in AL as compared to European Group for the Immunological Characterization of Leukemias (EGIL) criteria incorporated in the WHO2001. In the WHO2001 criteria a weighted points system is used for defining a Biphenotypic Acute Leukemia (BAL) to be Myeloid, B- or T-cell. In this scoring system at least one additional lineage defining marker for both lineages was necessary to diagnose a BAL. In the WHO2008 an AL expressing both myeloperoxidase (MPO) and CD19, can be considered as a MPAL (B/Myeloid) depending on expression of either cCD79a, CD10 or cCD22. Furthermore, the WHO2008 excludes AL with well-defined cytogenetic or clinical presentations from MPAL. For example, AL with t(8;21); t(15;17) or inv(16) are classified as AML, independent of their immunophenotypic marker expression [1-3]. Here we describe a case in which cytochemistry showed Sudan Black B (SBB) negative, vacuole rich, leukemic blasts with a monocytoid appearance and erythrophagocytosis. Remarkably, flow cytometric analysis and immunohistochemistry showed clear expression of B cell markers and weak cytoplasmic (c)MPO. This unique case demonstrates the challenges in diagnosing an AL with ambiguous morphologic, immunohistochemical and flow cytometric characteristics.

Case

A 65-year-old woman was admitted to the hospital with general discomfort and a fever of 2-week duration. She experienced a weight loss of 9 kg in the preceding month and complained of night sweats. Her medical history revealed diabetes mellitus type II, atrial fibrillation, a psychosis and a former drug addiction. Physical examination did not show relevant findings. Laboratory investigations revealed an elevated lactate dehydrogenase (948 U/l; normal values 0–250 U/l), leucopenia (2.0×10^9/l; normal values $4.0–10 \times 10^9$/l), thrombocytopenia (18×10^9/l; normal values $150\text{-}400 \times 10^9$/l) and a decreased hemoglobin

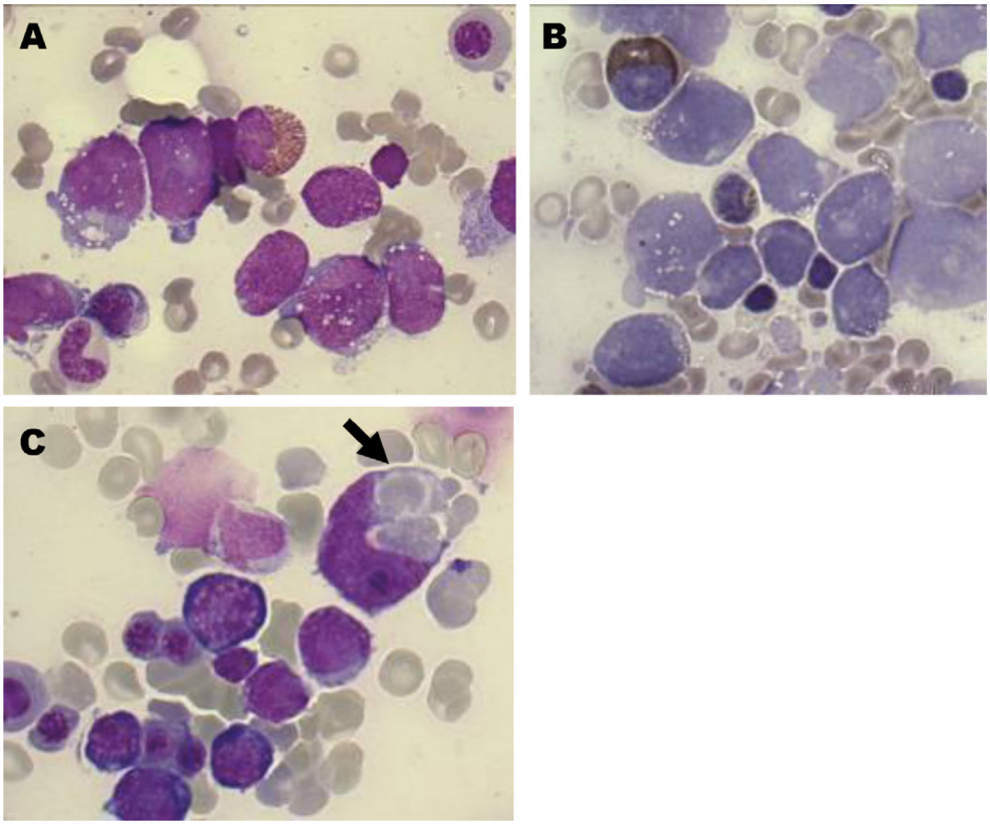


Figure 1. Morphology of leukemic cells at diagnosis (1000×). Bone marrow May-Grünwald Giemsa (MGG) staining of bone marrow smears at diagnosis: example of clear vacuole-rich blast cells (A), Sudan Black staining (B), arrow indicates erythrophagocytosis by blasts cells (C).

level (4.8 mmol/l or 7.7 g/dl; normal values 7.5–10.0 mmol/l or 12.1–16.1 g/dl).

Morphological peripheral blood (PB) examination revealed anisocytosis, polychromasia, dyserythropoiesis, dysgranulopoiesis and the presence of monocytoid leukemic blasts (60%) with pronounced vacuolization (*figure 1A*). The leukemic blasts stained negative for SBB (*figure 1B*). The bone marrow (BM) smear showed 64% blasts and a normal erythroid- and megakaryocytic lineage. Remarkably, erythrophagocytosis was observed in 4% of the leukemic blasts (*figure 1C*).

By flow cytometry two blast populations could be distinguished based on sideward scatter (SSC), CD45 and CD34 expression (*figure 2*). The largest population (97% of the total amount of blasts) was CD45 dim/neg, had a higher CD34 expression and an intermediate SSC. This population was positive for CD19, CD20, CD22, cCD79a and cTdT. Furthermore, these cells stained weakly for MPO and showed an over expression of CD90. The monocytic markers CD14 and CD36 were negative (data not shown). The smallest population (3% of the total amount of blasts) was CD45 positive, had a slightly lower CD34 expression and a low SSC. These blasts showed positivity for the following markers: CD13, CD117, CD33 and negativity for cMPO, CD19, CD10, CD20, CD22, cCD79a and cTdT. This population most likely resembled the normal myeloid blast compartment.

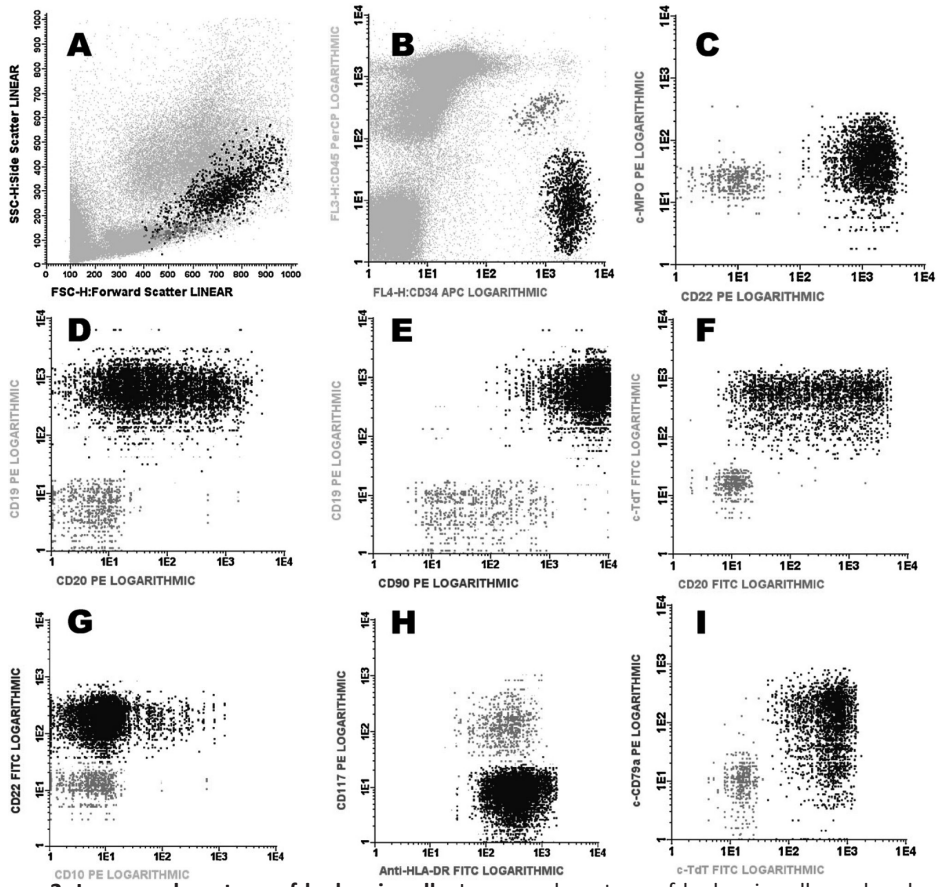


Figure 2. Immunophenotype of leukemic cells. Immunophenotype of leukemic cells analyzed with Infinicyt software (Cytognos, Salamanca, Spain). Plot A and B represent gating strategy for the two different blast populations. Plots C–I represent marker expression on the two populations.

Immunohistochemical analysis of the trephine biopsy revealed a hypercellular marrow with approximately 50% blast cells. The blasts were variable in size and showed irregular nuclear morphology with fine chromatin, small nucleoli and occasionally multi-lobulation. The cells stained positive for CD34, cTdT, CD20 and cCD79a, and also showed a weak staining for cMPO. A normal female karyotype (46,XX) in 20/20 cells was present while no molecular aberrancies could be detected (PCR or FISH for t(15;17), t(8;21), inv(16), t(9;22), t(10;17), MLL abnormalities, and FLT3-ITD were all negative).

To summarize, this patient presented with an AL with monocytoid morphology, erythrophagocytosis and a clear B-lymphocytic marker expression combined with positivity for cMPO in both flow cytometry and immunohistochemistry. Based on the combined marker expression of cCD79a, CD19, CD20 and cMPO, this patient was diagnosed as a MPAL (B/Myeloid) according to the WHO2008 criteria. She was treated with AML like chemotherapy combined with intrathecal methotrexate prophylaxis resulting in complete remission (CR). Unfortunately she relapsed after 11 months of follow up. Because she refused further therapy only maximal supportive care was provided.

Discussion

Overall, this case has a paradoxical appearance: morphology revealed a clear monocytoid picture with prominent vacuolization and erythrophagocytosis, both mostly seen in myeloid leukemia. In contrast, immunohistochemistry and immunophenotypical analysis showed clear expression of B cell lineage defining and specific markers combined with cMPO. Unlike other myeloid markers such as CD13 and CD33, cMPO expression is very rarely found in AL with a clear B-ALL phenotype. Although immunophenotypical and immunohistochemical cMPO expression was found to be of low intensity, it was clearly positive and considered specific. Strikingly, cytological examination did not stain positive for SBB in this case. Following the WHO2001 criteria, this AL would have been classified as an ALL. However, based on additional expression of solely cMPO, this case was classified as MPAL according to the WHO2008 guidelines.

The most paradoxal and unique feature in this case is the erythrophagocytosis. This rare finding is reported in less than 1% of AL cases and is most commonly associated with AML, especially those from a monoblastic or monocytic origin [4]. Erythrophagocytosis has also been associated with other subtypes such as acute undifferentiated AL, AML without maturation, AML with maturation and acute megakaryoblastic leukemia. Erythrophagocytosis as seen in AL is often associated with cytogenetic abnormalities on chromosome 8(p11) involving the C-MOZ gene [5]. Most frequently, the t(8;16)(p11;p13) translocation is found that gives rise to the CMOZ/CBP chimeric transcript. Recently, AML with t(8;16)(p11;p13) has been described as a distinct entity with unique features like erythrophagocytosis, positive cMPO with strong positive non-specific esterase staining and poor prognosis [6]. Furthermore, other translocations have been described such as t(16;21)(p11;q22), t(10;17)(p13;p12), inv(8)(p11q13), as well as 20q- deletion [5-9]. However, in the present case none of these cytogenetic aberrancies could be detected. In biphenotypic leukemia erythrophagocytosis is only described twice, both associated with a cytogenetic aberrancy; t(9;22) and inv(8)(p11q13) [5] and [8]. Considering the WHO2001 guidelines no cases of lymphoid AL with erythrophagocytosis have previously been described; the case presented here shows clear lymphoid marker expression and erythrophagocytosis. Moreover, based on the WHO2008 criteria, this is the first MPAL to show erythrophagocytosis without cytogenetic aberrancies. Although the introduction of the WHO2008 classification makes the diagnosis of a MPAL more clear and less frequent compared to the older EGIL criteria, diagnosing a MPAL remains difficult but important. Prospective trails are still needed to clearly understand its clinical impact. This case highlights the challenges in diagnosing an AL with ambiguous morphologic, immunohistochemical and flow cytometric characteristics.

References

1. van den Ancker W, Terwijn M, Westers TM et al. Acute leukemias of ambiguous lineage: diagnostic consequences of the WHO2008 classification. Leukemia 2010;24:1392-1396.
2. Bene MC, Castoldi G, Knapp W et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995;9:1783-1786.

3. Borowitz MJ, Béné M, Harris NL, Porwit A, McCann SR. Acute leukaemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. WHO Classification of Tumours of haematopoietic and Lymphoid Tissues, Fourth Edition.: IARC Press, Lyon; 2008:150-154.
4. La Starza R, Falini B, Amici A et al. T-lymphoid/myeloid biphenotypic leukemia morphologically resembling malignant histiocytosis. Immunological, cytogenetic and molecular studies. Haematologica 1993;78:187-191.
5. Liang J, Prouty L, Williams BJ, Dayton MA, Blanchard KL. Acute mixed lineage leukemia with an inv(8)(p11q13) resulting in fusion of the genes for MOZ and TIF2. Blood 1998;92:2118-2122.
6. Haferlach T, Kohlmann A, Klein HU et al. AML with translocation t(8;16)(p11;p13) demonstrates unique cytomorphological, cytogenetic, molecular and prognostic features. Leukemia 2009;23:934-943.
7. Mori H, Tawara M, Yoshida Y et al. Minimally differentiated acute myeloid leukemia (AML-M0) with extensive erythrophagocytosis and del(20)(q11) chromosome abnormality. Leuk.Res. 2000;24:87-90.
8. Ortuno FJ, Castilla C, Moreno MJ et al. Erythrophagocytosis in de novo-philadelphia-positive acute leukemia of ambiguous lineage. Haematologica 2006;91:ECR43.
9. Coulthard S, Chase A, Orchard K et al. Two cases of inv(8)(p11q13) in AML with erythrophagocytosis: a new cytogenetic variant. Br.J.Haematol. 1998;100:561-563.

Acute leukemias of ambiguous lineage: diagnostic consequences of the WHO2008 classification

4

W van den Ancker
M Terwijn
TM Westers
PA Merle
E van Beckhoven
AM Dräger
GJ Ossenkoppele
AA van de Loosdrecht

Leukemia. 2010
Jul;24(7):1392-6

Introduction

Acute leukemias (AL) are classified according to their commitment to either the myeloid or lymphoid lineage. Immunophenotyping for various intra- and extracellular cell lineage specific markers is an important feature in differentiating between acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). In about 4% of AL cases it is not clear whether blasts are derived from myeloid or lymphoid progenitors and are classified as AL of ambigious lineage (ALAL). AL with biphenotypic characteristics represent a subgroup with a worse prognosis which can be partly explained by a higher incidence of the Philadelphia chromosome or other complex cytogenetic aberrancies; these patients present more often with extramedullar localization of disease which urges for more intensive treatment protocols including central nervous system prophylaxis. The question whether these patients benefit from an (intensified) ALL or AML protocol is still unresolved due to lack of solid randomized treatment protocols [1-5].

In 1995 the European group for immunological characterizing of AL (EGIL) presented guidelines for classification of AL with biphenotypic marker expression. These criteria were incorporated in the WHO2001 guidelines for classifying AL of ambigious lineages. For many years the EGIL guidelines offered a platform for harmonizing diagnostic criteria important for prognosis and clinical outcome measurements [6;7]. In 2008, new WHO criteria were proposed for classification of acute leukemias of ambigious lineages [8]. For acute leukemias with a mixed phenotype (MPAL), these new criteria implicated significant modification with the EGIL scoring system with potential implications for treatment and clinical research [8]. For instance, in the WHO2008 both bilineal and biphenotypic AL are classified as a MPAL, whereas these were distinct entities in the EGIL. Furthermore, single expression of cytoplasmatic myeloperoxidase (cMPO) or CD3 (surface or intracellular) is now regarded as sufficient to determine whether blasts belong to the myeloid or T cell lineage, respectively. AL that express both cMPO and CD19 are now diagnosed as MPAL depending on expression of cCD79a, CD10 or cCD22. In contrast to the EGIL, the WHO2008 excludes AL with certain cytogenetic aberrancies or clinical presentations from MPAL: AL with t(8;21), t(15;17) or inv(16) are classified as AML with recurrent cytogenetic abnormalities despite their immunophenotypic marker expression. Furthermore, AL with FGFR1 mutations, CML-blast crises (CML-BC), AL with MDS pre-phase and therapy-related AL are separate entities according to WHO2008 criteria.

These new criteria are likely to change diagnosis and hence therapeutic considerations in various patients. To investigate this issue, we retrospectively analyzed a cohort of 517 patients admitted to our hospital with acute leukemia from 2000 to 2008 and compared diagnosis by applying both EGIL and WHO2008 criteria.

Material and Methods

Immunophenotyping of bone marrow samples was performed as part of regular clinical diagnostic procedures. Shortly, after bulk red cell lysis and antibody incubation at room temperature, expression levels of B cell, T cell and myeloid lineage specific and defining markers on blasts were determined by four-color flow cytometry on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Cells were characterized for the following

markers: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD19, CD20, CD22, CD25, CD33, CD34, CD36, CD42b, CD45, CD56, CD61 CD65, CD71, CD90, CD117, CD133 and HLA-DR. For analysis of cytoplasmic markers cells were fixed with paraformaldehyde and subsequently permeabilized with Facs Lyse (Becton Dickenson , San Jose, CA) followed by staining for CD3, IgM, CD79a, TdT and MPO. All antibodies were obtained from: Becton Dickenson, Zebra biosciences (Enschede, The Netherlands), Dako (Glostrup, Denmark) or Sanquin (Amsterdam, The Netherlands). Results were analyzed using CellQuest Pro software (Becton Dickinson). Blasts were determined by low side scatter (SSC), diminished or low CD45 expression and CD34 expression. In case of CD34 negative AL, blasts were gated using other immature markers such as CD117, CD133, CD10 and cIgM. In our analysis we used a cut off of 20% marker positivity, as specified n the EGIL criteria[6]. In the WHO2008, cell line specific markers CD19 and CD3 should be highly expressed on blasts (expression equal or higher than the normal T- or B-cells within the patient's sample) to assign a certain lineage positive in case of a MPAL. We considered blasts strongly positive ("s") for a certain marker if at least 20% of the population was positive as compared with the appropriate isotype control combined with a mean fluorescence intensity as high as, or higher than normal B or T cells within the same sample. Weak expression ("w") is defined when the expression level does not reach expression of corresponding normal cells. In cases where normal B or T cells were absent, we compared expression level with historical normal controls. Molecular diagnostics procedures were performed as part of regular clinical diagnostic procedures. Quick frozen cell pellets were used for determination of presence of translocations t(9;22), t(8;21), t(15;17), MLL, FLIT3-ITD by PCR following institutional guidelines (www.modhem.nl).

Results and discussion

Out of 517 AML patients 26 (5.0%) could be classified as either BAL (EGIL), MPAL (WHO2008) or both with a B cell /myeloid (B/My) combined phenotype (summarized in *table 1A*). Of those, 7 patients (UPN 1-4 and 6-8) showed an expression profile which allowed classification for both MPAL and BAL. Since AML with t(8;21) frequently show coexpression of CD19 and cCD79a next to the myeloid markers CD13, CD33 and MPO, UPN 6-8 are classified as AML with t(8;21) considering WHO2001 and as AML with recurrent cytogenetic abnormality according the WHO2008 criteria.

Based on medical history UPN 11 and 12 were classified as AML with myelodysplasia-related changes and UPN 9 and 10 as a CML-BC, excluding these patients as possible MPAL. Despite presence of t(9;22), UPN 1 and 5 were not classified as CML-BC because of absence of a CML pre-phase; these ALAL substitute a subgroup of MPAL. There are only two patients, UPN 5 and 27, who are considered a MPAL in WHO2008 guidelines based on expression of solely cMPO; these were classified as a B- and T-ALL, respectively, following EGIL criteria. In our cohort we did not find any cases with monocytoid differentiation (based on expression of CD14, CD11c, CD45 bright, HLA-DR and side and forward light scatter properties) combined with expression of lymphoid markers; especially not on those patients previously diagnosed as BAL. Although we did not include CD36 and CD64 in our analysis, detection of CD14 and CD11c is considered as adequate in assigning the monocytoid lineage.

One argument to change the criteria in the WHO2008 classification was to distinguish the

Table 1A. Comparison of EGIL and WHO2008 in classification of acute leukemias with combined expression of B-cell and myeloid lineage markers

UPN	Age (years)	EGIL	WHO 08	cMPO	CD 13	CD 33	CD 117	CD 15	CD 65	CD 19	cIgM	cCD79a
1	49	BAL	MPAL	65	67w	35w	1	27w	1	99s	35	95s
2	9	BAL	MPAL	38	21w	2	0	0	1	99s	0	80s
3	40	BAL	MPAL	80	94	91	3	1	0	85s	0	80s
4	57	BAL	MPAL	98	35	37	2	35	29	61s	1	52s
5	65	ALL	MPAL	69	18w	3	1	1	0	98s	0	90s
6	46	AML*	AML	98	80	95	82	20	2	63h	0	25h, s
7	16	AML*	AML	98	45w	26w	98	11	1	95s	0	22h, s
8	15	AML*	AML	96	74	97	98	14	9	97s	1	50w
9	52	BAL	CML BC	1	99	26	67	0	5	10	0	30w
10	28	BAL	CML BC	58	98	67	72	4	13	78w	0	56h, s
11	68	BAL	AL*	29	98	99	98	3	10	7	0	43w
12	59	BAL	AL*	96	91	99	92	19	23	3	85h	0
13	23	BAL	AL**	1	94	98	82	1	1	0	12	52w
14	23	BAL	AL**	7	97	99	71	13	39	73s	0	25w
15	52	BAL	AML	73	98	97	91	22w	6	25w	ND	52w
16	54	BAL	AML	85	92	99	94	82	6	40h	4	53w
17	72	BAL	AML	99	99	99	90	18	6	46w	ND	50w
18	41	BAL	AML	94	91	93	85	19	0	28w	0	22w
19	58	BAL	AML	51	99	12	80	2	ND	45h	1	18
20	18	BAL	AML	84	97	87	90	16	15	10	0	35w
21	69	BAL	AML	37	99	21	94	41	21	4	0	30h, s
22	56	BAL	AML	50	99	57	89	15	59	29w	1	31w
23	1	BAL	AML	34	90	99	98	0	0	88w	2	59w
24	63	BAL	AML	67	99	98	96	7	1	1	0	45w
25	36	BAL	ALL	6	65w	82w	3	58w	2	96s	73	80s
26	15	BAL	ALL	0	42w	60w	38w	9	1	97	0	80

Abbreviations: AML, acute myeloid leukemia; AL, acute leukemia; ALL, acute lymphoblastic leukemia; BAL, biphenotypic acute leukemia; cMPO, cytoplasmic myeloperoxidase; cTdT, cytoplasmic terminal deoxynucleotidyl transferase; CML-BC, chronic myleoid leukemia blast crisis; MPAL, mixed phenotype acute leukemia; NSE, Non-specific esterase; pos, positive; neg, negative; ND, not determined; xUPN, unique patient number. Results are shown for those samples, which are scored positive for a biphenotypic acute leukemia corresponding to the EGIL criteria and/or for a mixed phenotype acute leukemia (MPAL) corresponding to the WHO2008 criteria. Discrimination is made between weak, heterogeneous or strong expression. Strong expression ('s') is defined as higher or as high as

UPN	CD 20	CD 22	CD 10	cTdT	CD11c and/ or CD14	t(9;22)	t(8;21)	t(15;17)	MLL	FLIT-ITD
1	84	99	98	96	Neg	pos	neg	neg	neg	ND
2	3	36	95s	78	Neg	neg	ND	ND	neg	ND
3	1	48	85s	85	Neg	neg	neg	neg	neg	Normal
4	63	55	79	86	Neg	neg	ND	ND	neg	ND
5	74	98	99s	99	Neg	pos	ND	ND	neg	ND
6	1	0	ND	1	Neg	ND	pos	neg	neg	ND
7	1	6	1	37	Neg	neg	pos	neg	neg	Normal
8	1	1	1	31	Neg	neg	pos	neg	neg	Normal
9	0	0	0	87	Neg	pos	ND	ND	ND	ND
10	1	2	1	91	Neg	pos	neg	neg	neg	Normal
11	3	2	3	25	Neg	neg	neg	neg	neg	Normal
12	2	0	0	21	Neg	neg	neg	neg	neg	Heterozygous
13	1	55	2	46	Neg	neg	neg	neg	neg	ND
14	0	5	0	1	Neg	neg	neg	neg	neg	Normal
15	34	5	42	2	Neg	neg	neg	neg	neg	ND
16	1	13	ND	1	Neg	neg	neg	neg	neg	ND
17	0	0	ND	85	Neg	ND	ND	ND	ND	ND
18	0	1	3	37	Neg	neg	neg	neg	neg	Heterozygous
19	2	4	50h	82	Neg	neg	neg	neg	neg	Normal
20	3	5	4	54	Neg	neg	neg	neg	neg	Normal
21	1	21	1	53	Neg	neg	neg	neg	pos	Normal
22	1	3	5	44	Neg	neg	neg	neg	neg	Normal
23	0	1	0	72	Neg	neg	neg	neg	neg	Normal
24	1	81	24w	0	Neg	neg	neg	neg	neg	Normal
25	94	95	93	71	Neg	ND	ND	ND	ND	ND
26	47	97	99	99	Neg	ND	ND	ND	ND	ND

the expression on nonmalignant cells in the same sample or, known from healthy donors. Weak expression ('w') is defined when the expression level does not reach expression of corresponding normal cells. Heterogeneous and strong expression ('h, s') is defined when, next to a heterogenous expression, the cells with highest expression are as high or higher as the expression of non-malignant cells. Monoblastic appearance is defined by expression of at least CD14 or CD11c. AML*, UPN 6, 7 and 8 are classified as AML with t(8;21) considering EGIL criteria; AL*, UPN 11, 12 & 28 are excluded from MPAL based on the classification as an MDS-related acute leukemia; AL**, for UPN 13, 14 and 29 it is not clear whether these should be classified as AML, ALL or MPAL in the WHO2008.

Table 1B. Comparison of EGIL and WHO2008 in classification of acute leukemias with combined expression of T-cell and myeloid lineage markers.

UPN	Age	EGIL	WHO 08 T/ My	CD3	cCD3	CD4	CD8	CD2	CD5	CD7	CD 10	cTdT	cMPO
27	23	ALL	MPAL	2	48s	3	65	89	98	98	3	75	57
28	2	BAL	MPAL	2	38	41	72	5	95	98	98	70	pos
29	67	BAL	AL**	0	0	80	0	31w	61	93	23	1	1
30	10	BAL	ALL	1	37s	13	4	92	81	97	68	97	9
31	59	BAL	AML	2	0	40	2	56	7	46	31	21w	69
32	75	BAL	AML	0	5	26	3	7	34 w	25w	33w	74	16
33	72	BAL	AML	1	0	58	1	24w	23w	77w	16	2	25

Table 1C. Comparison of EGIL and WHO2008 in classification of acute leukemias with combined expression of B-cell , myeloid and T-cell lineage markers

UPN	CD 15	CD 65	CD 34	CD 19	cIgM	cCD 79a	CD 20	CD 22	t(9;22)	CD11c and/or CD14	t(8;21)	t(15;7)	MLL	FLIT3-ITD
34	13	19	2	8	0	41w	7	4	Neg	Neg	Neg	Neg	Neg	Normal
35	35	3	87	1	0	24w	0	5	Neg	Neg	ND	ND	Neg	ND

Abbreviations: AML, acute myeloid leukemia; AL, acute leukemia; ALL, acute lymphatic leukemia; BAL, biphenotypic acute leukemia; cMPO, cytoplasmic myeloperoxidase; cTdT, cytoplasmic terminal deoxynucleotidyl transferase; CML-BC, chronic myleoid leukemia blast crisis; MPAL, mixed phenotype acute leukemia; NSE, Non-specific esterase; pos, positive; neg, negative; ND, not determined; xUPN, unique patient number. Results are shown for those samples, which are scored positive for a biphenotypic acute leukemia corresponding to the EGIL criteria and/or for a mixed phenotype acute leukemia (MPAL) corresponding to the WHO2008 criteria. Discrimination is made between weak, heterogeneous or strong expression. Strong expression ('s') is defined as higher or as high as

UPN	CD 13	CD 33	CD 117	CD 15	CD 65	CD11c and/or CD14	CD 34	t(9;22)	t(8;21)	t(15;17)	MLL	FLIT-ITD
27	5	0	0	0	0	Neg	39	Neg	ND	ND	Neg	ND
28	76		2			Neg	1	neg	neg	neg	neg	Normal
29	99	76	92	3	4	Neg	94	Neg	Neg	Neg	Neg	Normal
30	47	90	62	2	1	Neg	89	Neg	ND	ND	Neg	Normal
31	92	88	83	9	0	Neg	88	Neg	Neg	Neg	Neg	Normal
32	95	91	93	2	4	Neg	93	ND	ND	ND	ND	ND
33	99	97	94	23	77	Neg	93	Neg	Neg	Neg	Neg	Normal

UPN	Age	EGIL	WHO 2008	CD 3	cCD 3	CD 4	CD 8	CD 2	CD 5	CD 7	CD 10	c TdT	c MPO	CD 13	CD 33	CD 117
34	75	BAL B/T/My	MPAL T/my	4	39s	98	94	98	98	98	70	93	70	31	3	9
35	16	BAL B/T/My	T-ALL	0	56s	0	0	99	0	99	0	91	1	99	11	92

the expression on nonmalignant cells in the same sample or, known from healthy donors. Weak expression ('w') is defined when the expression level does not reach expression of corresponding normal cells. Heterogeneous and strong expression ('h, s') is defined when, next to a heterogenous expression, the cells with highest expression are as high or higher as the expression of non-malignant cells. Monoblastic appearance is defined by expression of at least CD14 or CD11c. AML*, UPN 6, 7 and 8 are classified as AML with t(8;21) considering EGIL criteria; AL*, UPN 11, 12 & 28 are excluded from MPAL based on the classification as an MDS-related acute leukemia; AL**, for UPN 13, 14 and 29 it is not clear whether these should be classified as AML, ALL or MPAL in the WHO2008.

lineage markers expressed on leukemic blasts that truly reflect the biphenotypic nature of cells from the markers which are commonly seen on subtypes of AL. For instance pro-B-ALL and pre-pre-B-ALL often show expression of myeloid markers; UPN 25 and 26 showed, in absence of cMPO, high expression of CD19 and cCD79a combined with low expression of CD33, CD13 and CD15. Considering the EGIL guidelines these patients were classified as BAL, whereas in WHO2008 these patients have to be classified as B-ALL (*table 1A*).

In the EGIL and WHO2001 acute bilineal leukemias were classified as a distinct entity, whereas in the WHO2008 these are joined with biphenotypic AL as MPAL. In case of MPAL of bilineal origin (WHO2008) or bilineal AL (EGIL) there should be two or more different populations in which at least one of these meets the immuphenotypic criteria for AML (with the exception that the population does not need to comprise at least 20%). In our cohort, five AL (1%) with a bilineal phenotype were found. Since criteria for bilineal origin are the same for EGIL and WHO2008, data are not shown.

Seven patients (1.4%) could be classified as BAL, MPAL or both with T cell / myeloid (T/My) lineage (*table 1B*). Remarkably, only UPN 28 is considered a BAL and MPAL based on immunologic marker expression; nevertheless this patient was excluded from classification because of a known history of MDS.

UPN 34 and 35 showed positivity for three lineages and can be considered as a B/T/My AL following the EGIL criteria (*table 1C*). In the EGIL classification system, discrimination between T and B cell lineage is difficult to define since CD10 and cTdT are cell lineage defining markers for both lineages. When CD10 and cTdT are only scored once for either T or B lineage according to EGIL criteria instead of double scored for both lineages, no B/T mixed phenotype AL could be found in our cohort.

Sometimes, current criteria fail to classify AL as a T-ALL, B-ALL or AML, i.e. in cases were CD3, CD19 and cMPO are negative and a BAL might be considered based on other cell lineage defining markers. For example, UPN 29 is negative for cMPO, cCD3 and CD3, but expresses CD5, CD7, CD2, CD13, CD33 and CD117; WHO2008 guidelines do not elucidate whether this case should be considered T-ALL or AML. Similarly, UPN 13 and 14 are indecisive for B-ALL or AML. In these cases, former EGIL criteria might give further directions.

Overall, we can conclude that the WHO2008 classification has a clear diagnostic impact for AL that express different cell lineage-specific and defining markers. Considering EGIL criteria 30/517 AL (5.8%) were classified as BAL, whereas in WHO2008 only 7 cases (1.3%) would have been classified as MPAL. BAL and MPAL cases did not fully coincide with each other, only 5/517 cases (0.9%) would have been classified as BAL as well as MPAL by EGIL and WHO2008 criteria, respectively. Based on our data almost all AL with combined myeloid and B or T characteristics are re-classified as ALL or AML in the new WHO2008 classification which in the future will have impact on therapeutic decision making. Therefore, the clinical significance of the WHO2008 criteria needs to be shown in prospective clinical studies.

References

1. Zheng C, Wu J, Liu X et al. What is the optimal treatment for biphenotypic acute leukemia? Haematologica 2009;94:1778-1780.

2. Owaidah TM, Al BA, Iqbal MA, Elkum N, Roberts GT. Cytogenetics, molecular and ultrastructural characteristics of biphenotypic acute leukemia identified by the EGIL scoring system. Leukemia 2006;20:620-626.
3. Xu XQ, Wang JM, Lu SQ et al. Clinical and biological characteristics of adult biphenotypic acute leukemia in comparison with that of acute myeloid leukemia and acute lymphoblastic leukemia: a case series of Chinese population. Haematologica 2009;94:919-927.
4. Legrand O, Perrot JY, Simonin G et al. Adult biphenotypic acute leukaemia: an entity with poor prognosis which is related to unfavourable cytogenetics and P-glycoprotein over-expression. Br.J.Haematol. 1998;100:147-155.
5. Weir EG, li Ansari-Lari M, Batista DA et al. Acute bilineal leukemia: a rare disease with poor outcome. Leukemia 2007;21:2264-2270.
6. Bene MC, Castoldi G, Knapp W et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995;9:1783-1786.
7. Brunning RD, Matutes E, Borowitz MJ et al. Acute leukaemias of ambiguous lineage. In: jaffe ES, Harris NL, Stein H, Vardiman JW, eds. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.: IARC Press, Lyon; 2001:106-107.
8. Borowitz MJ, Béné M, Harris NL, Porwit A, McCann SR. Acute leukaemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. WHO Classification of Tumours of haematopoietic and Lymphoid Tissues, Fourth Edition: IARC Press, Lyon; 2008:150-154.

A threshold of 10% for myeloperoxidase by flow cytometry is valid to classify acute leukemia of ambiguous and myeloid origin

5

W van den Ancker
TM Westers
DC de Leeuw
FCM van der Veeken
AA Loonen
E van Beckhoven
GJ Ossenkoppele
AA van de Loosdrecht

Cytometry B Clin Cytom.
2013 Jan 16

Abstract

Background
According to WHO2008 guidelines an important role is designated for cytoplasmic myeloperoxidase (cMPO) as measured by flow cytometry for classifying acute leukemia of myeloid or ambiguous origin (AML or MPAL). However, no threshold with respect to expression level and percentage positive cells is provided. Since the expression of solely cMPO can change the diagnosis from acute lymphoid leukemia into MPAL in the current WHO2008, a consensus is needed for the cut-off for cMPO.
Methods
In this study, we investigated whether or not a cut-off of 10% positivity for cMPO equally defines an acute leukemia as AML or MPAL as compared to a cut-off for cMPO of 20% and compared this with results obtained for Sudan Black B (SBB) staining by cytomorphology.
Results
Cell lineage-defining markers and SBB staining were analyzed retrospectively in a cohort of 198 patients who presented with acute leukemia. Eight patients were positive for SBB (>3%), but were considered negative for cMPO (<10%); six patients were negative for SBB (≤3%) and positive for cMPO (≥10%) staining. In six patients, we found 10-20% cMPO positive leukemic cells. Five of these cases were SBB positive; the sixth patient showed a clear myeloid phenotype without positivity of any lymphoid marker. Using a 10% cut-off instead of 20% would have changed diagnosis from ALL into MPAL in two patients; both cases were SBB positive by morphology.
Conclusion
We conclude that a 10% cut-off is a secure lower limit for cMPO expression and can be used independently from SBB expression.

Introduction

Acute leukemias (AL) are classified by flow cytometry according to their commitment to either the myeloid or lymphoid lineage [1]. In about 4% of AL, blast cells express both myeloid and lymphoid markers directing the diagnosis towards a mixed phenotype AL (MPAL) rather than acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) [2]. Based on WHO2008, a MPAL has to fulfill criteria based on expression of immunological markers that assign a blast cell to at least two lineages [1]. In case no directive conclusions can be drawn from molecular diagnostics, cytogenetics and morphology, flow cytometry has a decisive role when leukemic cells have both lymphoid and myeloid features. Expression of solely CD3 and flow cytometrically detected cytoplasmic myeloperoxidase (cMPO) is sufficient to assign a blast cell to the T cell and the myeloid lineage, respectively; for the B cell lineage one particular B cell marker in addition to expression of CD19 is required [3]. Cell lineage specific markers CD19 and CD3 should be highly expressed on blasts; however, for cMPO no thresholds with respect to expression level or percentage positive cells are provided. Since the expression of cMPO as an isolated marker can change the diagnosis from ALL into MPAL in the current WHO2008, a consensus is needed for the cut-off for cMPO.
The former WHO2001 criteria clearly described a cut-off for positivity of a marker of 20% of the whole blast population as compared to the appropriate isotype control. The 20%

threshold was formerly defined based on one-color flow cytometry; this higher cut-off was necessary to exclude false positivity due to e.g. contamination of non-leukemic cells. With the development of multicolor flow cytometry and because of the high degree of specificity of cMPO, cTdT and cCD79a, a lower cut-off of 10% was implemented when positivity was confirmed by light microscopy (EGIL criteria); in clinical practice, this is usually assessed by Sudan Black B (SBB) staining on blood and bone marrow smears. In cytomorphology, SBB representing cMPO expression is considered the hallmark of the myeloid lineage; the presence of 3% or more SBB positive leukemic progenitor cells is diagnostic for myeloid leukemias [4].

In this study, we investigated whether or not a cut-off of ≥10% positivity for cMPO by flow cytometry equally defines an AL as AML or MPAL as compared to a cut-off for cMPO of ≥20%; these results were compared with results obtained with SBB staining.

Material and methods

Patient samples and standard diagnostic procedures

Cell lineage-defining markers and SBB staining were analyzed retrospectively in a cohort of 198 patients who presented with AL in our hospital between 2001 and 2009; part of these patients were discussed in reference 2. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki and institutional guidelines. Only those patients were selected in which cytochemistry and immunophenotyping were performed on the same samples drawn at equal time points. Diagnostic procedures were performed according to standard operating procedures for classifying AL as described previously [2]. For bone marrow aspirates, first needle aspiration was used for morphology; second and third needle aspirations were used for immunophenotypic, molecular and cytogenetic analysis. SBB and May-Grünwald-Giemsa (MGG) staining were performed on blood and bone marrow smears by standard morphologic and cytochemical procedures. All patient samples were screened for t(9:22), t(8:21), inv(16), t(15;17) and FLT-3ITD by routine molecular and cytogenetic diagnostics (6).

Flow cytometric analysis

Blasts cells were analyzed for expression levels of B cell, T cell and myeloid defining- and specific-markers with FITC or PE-conjugated antibodies with ubiquitous CD45-PerCP and CD34-APC by performing a four-color flow cytometric analysis on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA), as described earlier [2]. Regarding cMPO expression we compared the impact of previously described cut-offs levels of 10 and 20%. Results were analyzed using CellQuestPro software (BD Biosciences). Percentage of marker positivity of blast cells and mean fluorescence index (MFI) were calculated as compared to appropriate isotype controls.

Statistical analysis

By construction of receiver operating characteristic (ROC) curves using the SPSS 15.0 software the specificity and sensitivity of various cut-offs for cMPO were compared with SBB results.

Table 1. Comparison of Sudan Black B and flow cytometric myeloperoxidase staining.

	SBB 1-3	SBB 4-100
MPO 0-9	63	8*
MPO 10-19	1†	5†
MPO 20-100	5#	116

Note: Total number of cases is shown subdivided in Sudan Black B (SBB) and cytoplasmic myeloperoxidase (cMPO) positive and negative groups. For SBB a cut-off of 3% of blasts is used. SBB positive and negative groups are organized into three groups, those expressing cMPO below 10%, those with 10-19% and those with 20% or more cMPO positive blasts as compared to the appropriate isotype control.
† Detailed information concerning these cases are shown in *table 2A*
* Detailed information concerning these cases are shown in *table 2B*
# Detailed information concerning these cases are shown in *table 2C*

Results

In 121 out of 198 (61%) of AL in our cohort cMPO expression was found to be higher than 20% (*table 1*). These cases were classified as AML (n=115) or MPAL (n=6), the latter depending on co-expression of lymphoid markers. The vast majority of patients (116/121, (96%) showed no discrepancies in SBB and cMPO staining (*table 1*). By applying a cut-off of ≥10%, six more cases are recognized as cMPO positive (UPN 1-6, 3% of cases, *table 2A*). Two of these patients should be reclassified as MPAL instead of B-ALL or T-ALL according to their immunophenotypic profile (UPN1 and 2; simultaneous expression of lymphoid markers and cMPO≥10%). Notably, both patients scored positive for SBB and were thereby classified as AML by morphology. Cytomorphology classified UPN 3-6 as myelomonocytic or monoblastic and monocytic leukemias (former FAB M4 and M5, respectively). Of note, UPN4 was classified as AML with inv(16) which is classified as an AML with genetic abnormalities according to the WHO2008. These leukemias often show a low to intermediate SBB and cMPO expression; thus, changing cMPO threshold did not affect the diagnosis.

Regarding discordant results, eight patients (UPN7-14, *table 2B*) were positive for SBB (>3%), but negative for cMPO (<10%). In only a minority of patients (n=6) we found negative SBB (≤3%) and positive cMPO (≥10%) (UPN3 and UPN15-19, 3% of the cohort). These discrepancies did not affect the overall classification. Two examples of SBB and cMPO discordance are shown in supplementary figure 1.

Receiver operating curve was used to compare cMPO results with SBB positivity or negativity (either >3% or below); When choosing a 20% cut-off a sensitivity of 87% with a specificity of 92% was reached. However, when choosing a cut-off of 10% a slightly more favourable ratio is found: a sensitivity of 95% and specificity of 87% (data not shown); the area under the curve was 0.959 ($p<0.001$).

Discussion

Within the current WHO2008 classification of hematopoietic neoplasms, cMPO is considered the hallmark protein for defining the myeloid lineage. In this regard, we compared the effect of a cut-off of 10 and 20% for cMPO in diagnosing AL and compared the results with SBB

Table 2A. Marker expression of acute leukemias with 10-20% cMPO

UPN	1	2	3	4	5	6
B cell markers						
cCD79a	1	70	5	1	20	0
CD19	0	98	1	0	1	1
CD20	1	16	0	2	2	1
CD22	7	24	3	9	5	1
CD10	1	80	7	ND	0	ND
cIgM	0	0	0	15	2	0
T cell markers						
CD2	92	3	5	23	2	1
CD3	0	0	0	0	1	1
cCD3	54	0	0	0	0	0
CD4	2	9	88	50	75	90
CD5	0	0	4	0	2	1
CD7	95	52	3	6	3	2
CD8	0	0	0	ND	10	ND
cTdT	79	65	49	1	17	0
Myeloid markers						
CD117	96	13	91	68	94	6
CD13	99	97	99	98	89	90
CD14	0	0	4	25	1	39
CD15	17	2	9	54	25	52
CD33	2	91	80	99	99	99
CD65	14	2	4	5	19	7
cMPO	12	15	19	19s	18	10s
MFI cMPO	9.5	4	8.9	9.3	3.1	5.1
Cytohistochemical MPO	ND	pos	neg	ND	ND	ND
Histomorphological SBB	27	24	2	100	28	29
WHO2008 10% cMPO	MPAL T/ myeloid NOS	MPAL with t(9;22) (b2a2)	Acute monoblastic and monocytic leukemia	AML with inv(16)	Acute monoblastic and monocytic leukemia	Acute monoblastic and monocytic leukemia
WHO2008 20% cMPO	T-ALL	B-ALL	Acute monoblastic and monocytic leukemia	AML with inv(16)	Acute monoblastic and monocytic leukemia	Acute monoblastic and monocytic leukemia

Table 2B. Marker expression of acute leukemias with positive SBB and negative cMPO

UPN	7	8	9	10	11	12	13	14
B cell markers								
CD79a	20	0	5	0	8	2		2
CD19	5	1	8	0	3	0	3	12
CD20	6	5	1	14	4	17	1	1
CD22	43	6	1	0	7	31	1	1
CD10	6	ND	0	ND	ND	ND	1	ND
cIgM	30	5	0	4	33	ND	1	0
T cell markers								
CD2	0	4	4	16	3	4	1	0
CD3	0	2	0	1	2	1	1	0
cCD3	0	0	0	1	9	0	0	0
CD4	90	87	60	97	42	52	20	57
CD5	7	4	0	1	1	0	3	0
CD7	7	12	79	4	7	35	4	89
CD8	0	ND	0	ND	ND	ND	1	ND
cTdT	30	1	24	0	9	4	27	0
Myeloid markers								
CD117	40	12	93	1	94	95	96	96
CD13	90	37	99	92	93	95	98	98
CD14	3	21	3	46	2	0	2	0
CD15	8	93	20	98	34	45	1	2
CD33	95	98	83	99	91	99	91	99
CD65	40	47	10	50	3	11	1	3
cMPO	9s	9	7	6	5	1	4s	3s
Cytohisto-chemical MPO	ND	neg	neg	neg*	ND	neg*	partly	ND
Histomorpho-logical SBB	19	22	31	50	26	80	7	4
WHO2008 10% cMPO	AL with (9;22) (b2a2)	Acute Myelo-monocytic Leukemia	Acute mono-blastic and monocytic leukemia	Acute mono-blastic and monocytic leukemia	AML with minimal differen-tiation	AML with differen-tiation	Acute erythroid leukemia	Acute mono-blastic and monocytic leukemia
WHO2008 20% cMPO	AL*	Acute Myelo-monocytic Leukemia	Acute mono-blastic and monocytic leukemia	Acute mono-blastic and monocytic leukemia	AML with minimal differen-tiation	AML with differen-tiation	Acute erythroid leukemia	Acute mono-blastic and monocytic leukemia

Table 2C. Marker expression of acute leukemia with negative SBB and positive cMPO

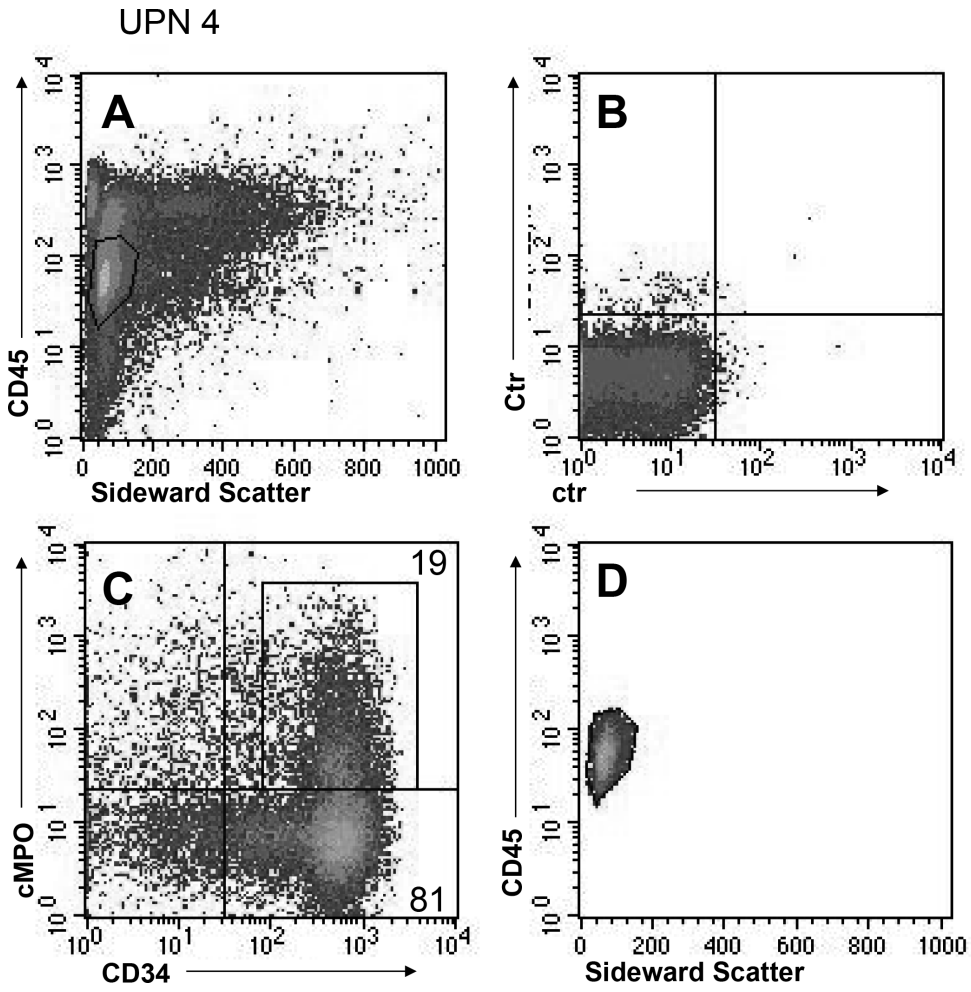
UPN	15	16	17	18	19
B cell markers					
CD79a	41	2	95	59	0
CD19	8	1	99	88	0
CD20	7	1	84	0	3
CD22	4	1	99	1	0
CD10	70	3	98	0	ND
cIgM	8	0	35	2	ND
T cell markers					
CD2	98	89	2	2	0
CD3	4	2	15	0	4
cCD3	39	48		2	0
CD4	98	3	6	1	15
CD5	98	98	1	0	0
CD7	98	98	2	2	2
CD8	94	65	ND	0	ND
cTdT	93	75	96	72	13
Myeloid markers					
CD117	9	0	1	98	99
CD13	31	5	67	90	99
CD14	2	0	0	1	0
CD15	13	0	27	0	0
CD33	3	0	35	99	2
CD65	19	0	1	0	0
cMPO	70s	57s	85	34	65
MFI cMPO	12.6	4.7	17.4	3	15.7
Histomorpho-logical SBB	0	0	0	1	1
WHO2008 10% cMPO	MPAL T/myeloid, NOS	MPAL T/myeloid, NOS	MPAL with t(9;22)e1a2	MPAL B/myeloid, NOS	AML with MDS-related changes
WHO2008 20% cMPO	MPAL T/myeloid, NOS	MPAL T/myeloid, NOS	MPAL with t(9;22)e1a2	MPAL B/myeloid, NOS	AML with MDS-related changes

percentages. By applying a cut-off of 10% for cMPO two patients would have been reclassified from ALL to MPAL based on expression of solely cMPO. Interestingly, both patients scored positive for SBB and were thereby classified as AML by morphology. It might be more appropriate to use comparable cut-offs for cMPO and SBB. This cut-off should be high enough to ensure that the subpopulation is of malignant origin, since normal myeloid progenitor cells ($CD34^+$ and $cMPO^+$) might reside in the same $CD45^{dim}$ blast compartment. In $CD34^-$ AML it has been shown that up to 1.0% non-malignant $CD34^+$ progenitors can be found [5]. For other AL the percentage of normal myeloid progenitor cells has to be revealed by further studies and should direct to the SBB and cMPO cut-off of choice. Overall, our data support to a cut-off of 10% for cMPO by flow cytometric analysis, independently of results found for SBB staining. Furthermore, by applying a cut-off of 10% a sensitivity of 95% and specificity of 87% is reached, compared with 87 and 92% when using a 20% cut-off. Rarely in cases of ALL, SBB can stain intracellular lipoid inclusions not containing cMPO [6]. In our cohort, eight patients were positive for SBB and negative for cMPO (<10%). Of note, these AL were considered to be of myeloid origin according to morphology and their immunophenotypic profile. Thus, discussion on cMPO positivity would not have had diagnostic consequences in these cases. In 3% of patients discrepancies caused by negative SBB (3≤%) and positive cMPO (≥10%) were seen. However, cMPO was expressed at a very low intensity. It is known that in case of AML without and AML with minimal differentiation SBB can be below 3% by light microscopy while cMPO may be positive by flow cytometry [1;7]. Yet, four out of five of these particular cases were classified as MPAL based on their immunophenotypic profile, one patient (UPN19) was classified as AML with MDS-related changes. In summary, a cut-off of 10% for cMPO expression would have changed diagnostic classification in only a minority of cases (1%); both were SBB positive by morphology. Therefore, we conclude that a 10% cut-off is a secure lower limit for cMPO expression and can be used independently from SBB expression. Clinical consequences have to be prospectively investigated.

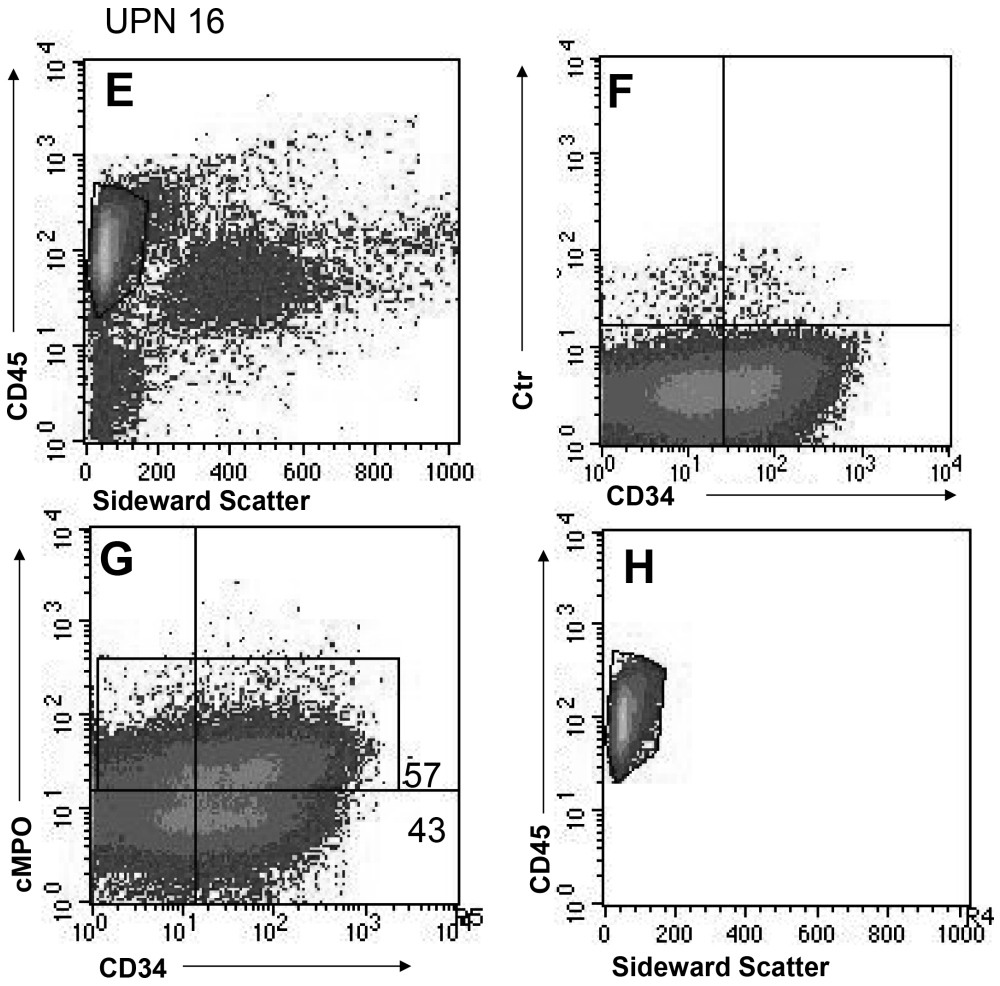
References

1. Swerdlow SH, Campo E, Harris NL et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition.: IARC Press, Lyon; 2008.
2. van den Ancker W, Terwijn M, Westers TM et al. Acute leukemias of ambiguous lineage: diagnostic consequences of the WHO2008 classification. Leukemia 2010;24:1392-1396.
3. Borowitz MJ, Béné M, Harris NL, Porwit A, McCann SR. Acute leukaemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. WHO Classification of Tumours of haematopoietic and Lymphoid Tissues, Fourth Edition.: IARC Press, Lyon; 2008:150-154.
4. Bennett JM, Catovsky D, Daniel MT et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br.J.Haematol. 1976;33:451-458.
5. van der Pol MA, Feller N, Roseboom M et al. Assessment of the normal or leukemic nature of $CD34^+$ cells in acute myeloid leukemia with low percentages of CD34 cells. Haematologica 2003;88:983-993.
6. Tricot G, Broeckaert-Van OA, Van HA, Verwilghen RL. Sudan Black B positivity in acute lymphoblastic leukaemia. Br.J.Haematol. 1982;51:615-621.
7. Bennett JM, Catovsky D, Daniel MT et al. Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). Br.J.Haematol. 1991;78:325-329.

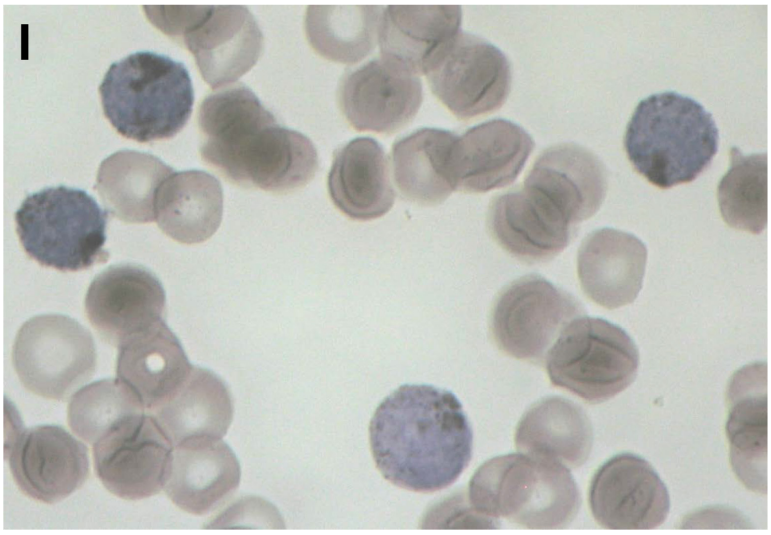
Supplemental material



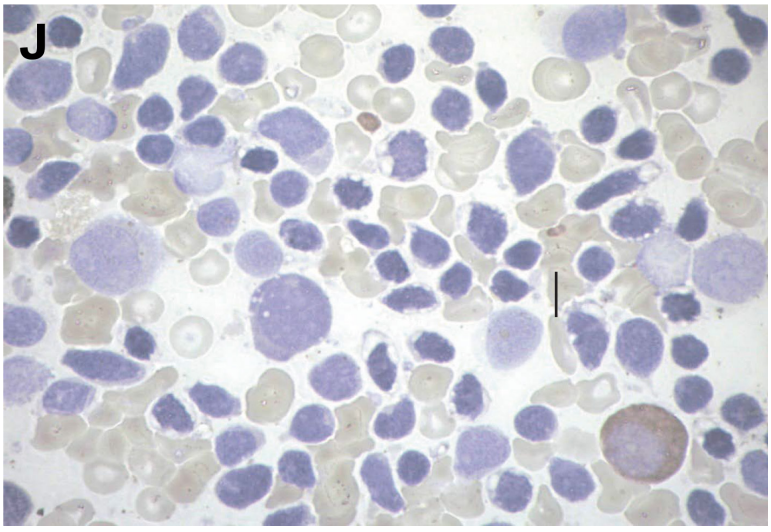
Supplementary figure 1. Examples of Sudan Black B and flow cytometric myeloperoxidase discordance. FACS density plots are shown. In plot A, CD45 diminished blast cells from UPN4 were selected and subsequently shown in plot B. Plot B represents the cytoplasmic control and plot C the cytoplasmic MPO staining (cMPO). In plot C, percentages represent the right lower quadrant for cMPO negative cells and right upper quadrant for cMPO positive cells. In plot D $cMPO^{+}$ cells are backgated in the CD45/sideward scatter plot. For UPN16, the same gating strategy was applied and results are shown in plot E, F, G and H. In Plot I and J, results of Sudan Black B staining by cytomorphology are depicted UPN4 and UPN16; total number of SBB positive cells per 100 counted leukemic cells is displayed. Complete immunophenotypic profile of these two patients is shown in table 2a and 2c, respectively.
In UPN16, cytomorphology would consider SBB as negative, in contrast, flow cytometry would state cMPO positivity; otherwise, in UPN4, SBB staining is clearly positive while cMPO at a flow cytometry cut-off of 20% would be considered negative. Of note, a cut-off of ≥10% would convert cMPO to positive.


UPN 16
E
CD45
Sideward Scatter
F
Ctr
CD34
G
cMPO
CD34
57
43
R5
H
CD45
Sideward Scatter
R4

UPN 4; SBB 100/100



UPN 16; SBB 0/100



MicroRNA profiling can classify acute leukemias of ambiguous lineage as either acute myeloid leukemia or acute lymphoid leukemia

6

DC de Leeuw
W van den Ancker
F Denkers
GJ Ossenkoppele
AA van de Loosdrecht
L Smit

Clin Cancer Res. 2013
Apr 15;19(8):2187-96.

Abstract

Purpose: Classification of acute leukemia (AL) is based on commitment of leukemic cells to either the myeloid or the lymphoid lineage. However, a small percentage of AL cases lack immunophenotypical lineage commitment. These leukemias of ambiguous lineage represent a heterogeneous category of AL that cannot be classified as either myeloid AL (AML) or lymphoid AL (ALL). he lack of clear classification of acute leukemias of ambiguous lineage as either AML or ALL is a hurdle in treatment choice for these patients.
Experimental design: Here, we compared the microRNA (miRNA) expression profiles of 17 cases with acute leukemia of ambiguous lineage and 16 cases of AML, B-cell acute lymphoid leukemia (B-ALL), and T-cell acute lymphoid leukemia (T-ALL).
Results: We show that leukemias of ambiguous lineage do not segregate as a separate entity but exhibit microRNA expression profiles similar to either AML, B-ALL or T-ALL. We show that by using only five of the most lineage discriminative microRNAs we are able to define AL of ambiguous lineage as either AML or ALL.
Conclusion: Our results indicate the presence of a myeloid or lymphoid lineage-specific genotype, as reflected by miRNA expression, in these acute leukemias despite their ambiguous immunophenotype. MiRNA-based classification of acute leukemia of ambiguous lineage might be of additional value in therapeutic decision making.

Introduction

Acute leukemias (AL) are classified as myeloid or lymphoid by using immunological markers that determine their lineage commitment. Four percent of AL cases cannot be classified as acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) due to co-expression of both lymphoid and myeloid lineage markers [1-6]. The prognosis of leukemias of ambiguous lineage is worse compared to AML or ALL and no specific treatment programs exist for these leukemias. To date, it is unsettled whether patients benefit from AML, ALL or combination therapy which might cause their mistreatment and poor prognosis [7-12]. The therapeutic strategies used for treatment of AML or ALL are considerably different, indicating the importance of classification of AL cases at diagnosis as either myeloid or lymphoid.
The diagnostic criteria for leukemias of ambiguous lineage were previously defined by the WHO 2001 classification and are based on the presence of immunological markers detected by flow cytometrie cytometry [1]. According to the WHO2001 scoring system many different combinations of immunophenotypic markers can define an AL as one with an ambiguous lineage and consequently this results in a heterogeneous group of biphenotypic acute leukemias (BAL). In 2008 these diagnostic criteria were refined: AML cases with recurrent chromosomal abnormalities and blast crises chronic myeloid leukemia (CML) cases were excluded from mixed phenotype AL (MPAL) [2]. The major immunophenotypic markers used by the WHO2008 to determine the lineage for these AL cases are cytoplasmic myeloperoxidase (cMPO), CD19, and cytoplasmic CD3 (cCD3). However, the current WHO2008 classification is difficult to interpret due to the implementation of expression intensities of B- and T-cell makers and the absence of clear cut-offs.
The discrimination between ALL and AML has been shown to be possible by morphologic, immunohistochemical, and immunological methods; however, also by gene expression

profiling [13,14] and microRNA (miRNA) expression profiling [15,16]. MiRNAs are small single stranded RNA molecules which regulate gene expression by promoting degradation of mRNAs or repressing their translation [17]. Cancer, including leukemia, is characterized by globally aberrant miRNA expression patterns which are highly informative for tumor classification. Remarkably, in poorly differentiated tumors, miRNA expression profiles can successfully classify these tumors in contrast to mRNA expression profiles [18]. In AML, genome-wide, large-scale miRNA expression profiles showed that miRNAs are associated with specific AML subtypes and may serve as biomarkers for classification and diagnosis of AML subtypes [19-23]. Several of those miRNA expression profiling studies showed the association of miRNA expression profiles with the outcome of AML patients [22-25]. Like in AML, miRNA expression signatures have been shown to be associated with prognosis of ALL patients [15]. Moreover, miRNA expression patterns showed to reflect the lineage state of acute leukemia [15,16,18]. Interestingly, from the 27 miRNAs differentially expressed between ALL and AML two were sufficient to accurately (97-99%) identify the myeloid or lymphoid identity of these AL [16]. In another study, 16 miRNAs (mir-23a, mir-27a/b, mir-128a, mir-128b, mir-221, mir-222, mir-223, let-7b, mir-17, mir-20a, mir-29a/c, mir-29b, mir-146a, mir-150, mir-155, and mir-196b) were found to be differentially expressed between AML and ALL [15].

Because miRNA expression profiles can classify AL cases as either AML or ALL [15,16,18] we decided to investigate the miRNA expression profile of AL cases that do not have a clear lineage commitment based on immunophenotypical marker expression. We compared the miRNA expression profiles of leukemia cases of ambiguous lineage with those of ALL and AML cases and showed that the cases with an ambiguous lineage do not segregate as a separate entity but have miRNA expression profiles similar to either AML, B-ALL or T-ALL. Thus, AL without clear immunophenotypic lineage commitment can be classified as either AML or ALL based on their miRNA expression profile. This classification might help the diagnosis of AL of ambiguous lineage as either AML or ALL and therefore in treatment decision making.

Material and Methods

Patient samples and cell lines

Sixteen patients with a leukemia of ambiguous lineage and 12 patients with AML, B-ALL or T-ALL were selected by database review of all patients with acute leukemia treated at the VU University Medical Center, Amsterdam, The Netherlands from 2000 until 2012. The 12 AML, B-ALL and T-ALL samples were selected based on a clear immunophenotypic myeloid or lymphoid lineage commitment. Immunophenotypical analysis was performed on bone marrow (BM) samples or peripheral blood (PB) samples in case no BM samples were available. The use of AML patient material for this study and the informed consent procedure has been approved by the medical ethical committee (MEC) of our institute. Bone marrow or peripheral blood samples from 16 patients diagnosed with a leukemia of ambiguous lineage and 17 patients with AML (n=7), B-ALL (n=6) or T-ALL (n=4) were used for miRNA expression analysis. Cell lines HL60 (CCL-240) and CCRF-CEM (CCL-119) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA).

Immunophenotypic analysis

Analysis was carried out as part of regular diagnostic investigations. In short, red blood cells were lysed using Pharm Lyse lysing solution (BD Biosciences, San Jose, CA), washed with phosphate buffered saline (PBS) and incubated with antibodies defining or specific for the B cell, T cell and myeloid lineage. Samples were analyzed with four-color flow cytometry on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). The following Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin chlorophyll protein (PerCP) or Allophycocyanin (APC) antibodies were used: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD19, CD20, CD22, CD25, CD33, CD34, CD36, CD42b, CD45, CD56, CD61, CD64, CD65, CD71, CD90, CD117, CD133 and anti-HLA-DR. Cytoplasmic markers CD3, CD22, IgM, cCD79a, cTdT and cMPO were evaluated after fixation with paraformaldehyde and subsequently permeabilization with Facs Lyse (Becton Dickenson, San Jose, CA). Antibodies were purchased from: BD Biosciences, Zebra biosciences (Enschede, The Netherlands), Dako (Glostrup, Denmark) or Sanquin (Amsterdam, The Netherlands). Data analysis was performed using CellQuest Pro software (BD Biosciences). Blasts were defined by low side scatter (SSC), diminished or low CD45 expression and CD34 expression. In case of CD34 negative AL, blasts were selected using other immature markers such as CD117, CD133, CD10 and cIgM. For defining the B-lymphoid lineage of a MPAL according to the WHO2008 criteria, CD19 should be highly expressed on blasts. We considered blasts strongly positive for CD19 if at least 20% of the population was positive as compared with the appropriate isotype control combined with a mean fluorescence intensity as high as, or higher than normal B cells within the same sample. When normal B cells were absent, we compared expression levels with historical normal controls [1-3,5].

Molecular diagnostics and cytogenetic analysis

Mononuclear cells were isolated using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and subsequently snap frozen. DNA and/or total RNA was isolated from the cell pellets and used for determination of presence of translocations; t(9;22), t(8;21), t(15;17), MLL and FLT3-ITD by PCR following institutional standard operating procedures (www.modhem.nl). Chromosomal analysis was performed on pretreatment bone marrow at diagnosis. Specimens were processed using short term unstimulated cultures. Chromosome banding was performed by the use of standard techniques, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature.

Cell purification

BM or PB cells derived from AL patients and stored in liquid nitrogen were thawed, washed and labeled with fluorochrome-conjugated antibodies. In general, blasts were stained with CD45, CD34 and various patient specific markers, mostly CD3, CD7, CD19, CD10, CD13 and CD33. The blast population from all AL cases were sorted by flow cytometry using FACS ARIA Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA)

RNA isolation and miRNA expression analysis

Total RNA was isolated with the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol. RNA was concentrated using a vacuum concentrator

(SPD111V, Thermo Savant) and the concentration was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Wilmington, DE). Human miRNA microarrays (Agilent Technologies; Palo Alto, CA), containing 60.000 probes representing 1205 human and 144 human viral miRNAs were used for miRNA expression profiling according to the manufacturers protocol. In short, 100ng of total RNA from each sample was dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIP) and then ligated with a Cyanine3-pCp molecule using Agilent miRNA Complete Labeling and Hyb Kit (Agilent Technologies; Palo Alto, CA). Labeled RNA was hybridized to Human miRNA microarray for 20 hours at 55°C. Microarray slides were scanned using a High-Resolution C Scanner (Agilent Technologies) and images were analyzed with Feature Extraction TM software, version 10.5.1.1 (Agilent). Microarray data are available on Array Express (accession no. E-MTAB-1459). The expression of several miRNAs was confirmed by Quantitative Real-Time Polymerase Chain reaction (Q-RT-PCR).

Quantitative Real-Time Polymerase Chain Reaction

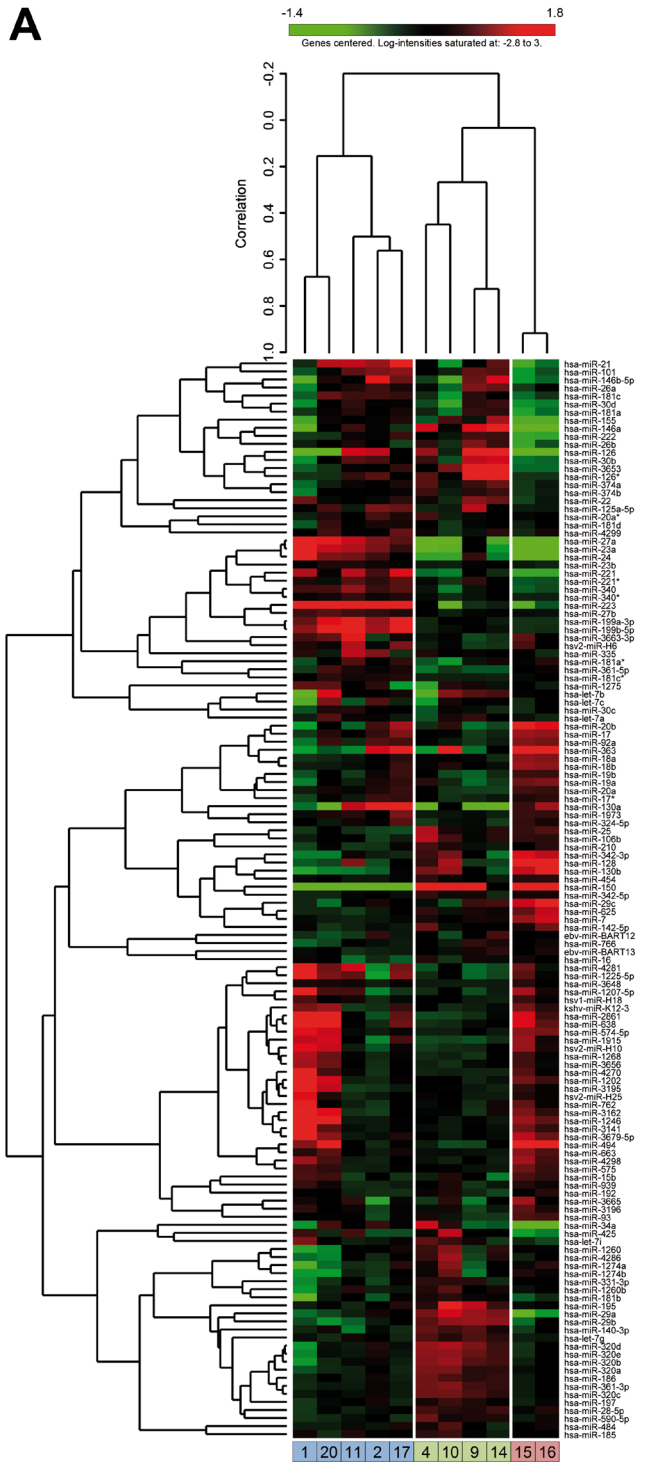
Selection of the most differentiating miRNAs in our cohort was performed using Significance Analysis of Microarrays (SAM) on the myeloid and lymphoid cluster as was formed by unsupervised clustering of all samples (controls combined with AL of ambiguous lineage). The five miRNAs with the highest observed relative difference were selected. This signature included; miR-23a, miR-27a, miR-221, miR-223 and miR-199b-5p (*supplemental table 4*). Specific primers (Applied Biosystems) for these five lineage discriminating miRNAs were used for Q-RT-PCR validation. RNU48 and miR-378, a miRNA which is highly expressed, has minimal variation in expression between samples and is not discriminative between AML and ALL, were used as control. PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems). All experiments were performed in duplicate and Ct-values were averaged. Expression was calculated using 2^-ΔCT method, log2-transformed and analyzed with BRB-ArrayTools.

Data analysis

Green median signal values from the microarray data were normalized using the quantile method [26], and log2-transformed. As the arrays included probes mapping a set of 1347 miRNAs spotted multiple times, we averaged the probes representing the same miRNA. The comparisons of the individual miRNAs between samples of the different lineage groups were performed using a linear regression model. This model takes advantage of the large number of miRNAs simultaneously studied to improve upon the individual estimates by means of empirical Bayes [27]. P-value lists for each comparison were corrected by multiple testing using the step-up false discovery rate (FDR) procedure of Benjamini-Hochberg [28]. Each miRNA was considered as differentially expressed between groups if its corresponding FDR was up to 0.05, unless otherwise stated.

Hierarchical clustering was used to produce false-color heatmaps illustrating the data patterns. The clustering used average linkage and one-minus-correlation as distance measure.

Analyses were performed using the statistical package R (version 2.14.0) [29], the package limma [30] for empirical Bayes linear regression, and BRB-ArrayTools (version 4.2.0) for producing the heatmaps.


A
-1.4
1.8
Genes centered. Log-intensities saturated at: -2.8 to 3.
Correlation
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
hsa-miR-21
hsa-miR-101
hsa-miR-146b-5p
hsa-miR-26a
hsa-miR-181c
hsa-miR-30d
hsa-miR-181a
hsa-miR-155
hsa-miR-146a
hsa-miR-222
hsa-miR-26b
hsa-miR-126
hsa-miR-30b
hsa-miR-3653
hsa-miR-126*
hsa-miR-374a
hsa-miR-374b
hsa-miR-22
hsa-miR-125a-5p
hsa-miR-20a*
hsa-miR-181d
hsa-miR-4299
hsa-miR-27a
hsa-miR-23a
hsa-miR-24
hsa-miR-23b
hsa-miR-221
hsa-miR-221*
hsa-miR-340
hsa-miR-340*
hsa-miR-223
hsa-miR-27b
hsa-miR-199a-3p
hsa-miR-199b-5p
hsa-miR-3663-3p
hsv2-miR-H6
hsa-miR-335
hsa-miR-181a*
hsa-miR-361-5p
hsa-miR-181c*
hsa-miR-1275
hsa-let-7b
hsa-let-7c
hsa-miR-30c
hsa-let-7a
hsa-miR-20b
hsa-miR-17
hsa-miR-92a
hsa-miR-363
hsa-miR-18a
hsa-miR-18b
hsa-miR-19b
hsa-miR-19a
hsa-miR-20a
hsa-miR-17*
hsa-miR-130a
hsa-miR-1973
hsa-miR-324-5p
hsa-miR-25
hsa-miR-106b
hsa-miR-210
hsa-miR-342-3p
hsa-miR-128
hsa-miR-130b
hsa-miR-454
hsa-miR-150
hsa-miR-342-5p
hsa-miR-29c
hsa-miR-625
hsa-miR-7
hsa-miR-142-5p
ebv-miR-BART12
hsa-miR-766
ebv-miR-BART13
hsa-miR-16
hsa-miR-4281
hsa-miR-1225-5p
hsa-miR-3648
hsa-miR-1207-5p
hsv1-miR-H18
kshv-miR-K12-3
hsa-miR-2861
hsa-miR-638
hsa-miR-574-5p
hsa-miR-1915
hsv2-miR-H10
hsa-miR-1268
hsa-miR-3656
hsa-miR-4270
hsa-miR-1202
hsa-miR-3195
hsv2-miR-H25
hsa-miR-762
hsa-miR-3162
hsa-miR-1246
hsa-miR-3141
hsa-miR-3679-5p
hsa-miR-494
hsa-miR-663
hsa-miR-4298
hsa-miR-675
hsa-miR-15b
hsa-miR-939
hsa-miR-192
hsa-miR-3665
hsa-miR-3196
hsa-miR-93
hsa-miR-34a
hsa-miR-425
hsa-let-7i
hsa-miR-1260
hsa-miR-4286
hsa-miR-1274a
hsa-miR-1274b
hsa-miR-331-3p
hsa-miR-1260b
hsa-miR-181b
hsa-miR-195
hsa-miR-29a
hsa-miR-29b
hsa-miR-140-3p
hsa-let-7g
hsa-miR-320d
hsa-miR-320a
hsa-miR-320b
hsa-miR-320a
hsa-miR-186
hsa-miR-361-3p
hsa-miR-320c
hsa-miR-197
hsa-miR-28-5p
hsa-miR-660-5p
hsa-miR-484
hsa-miR-185
1
20
11
2
17
4
10
9
14
15
16

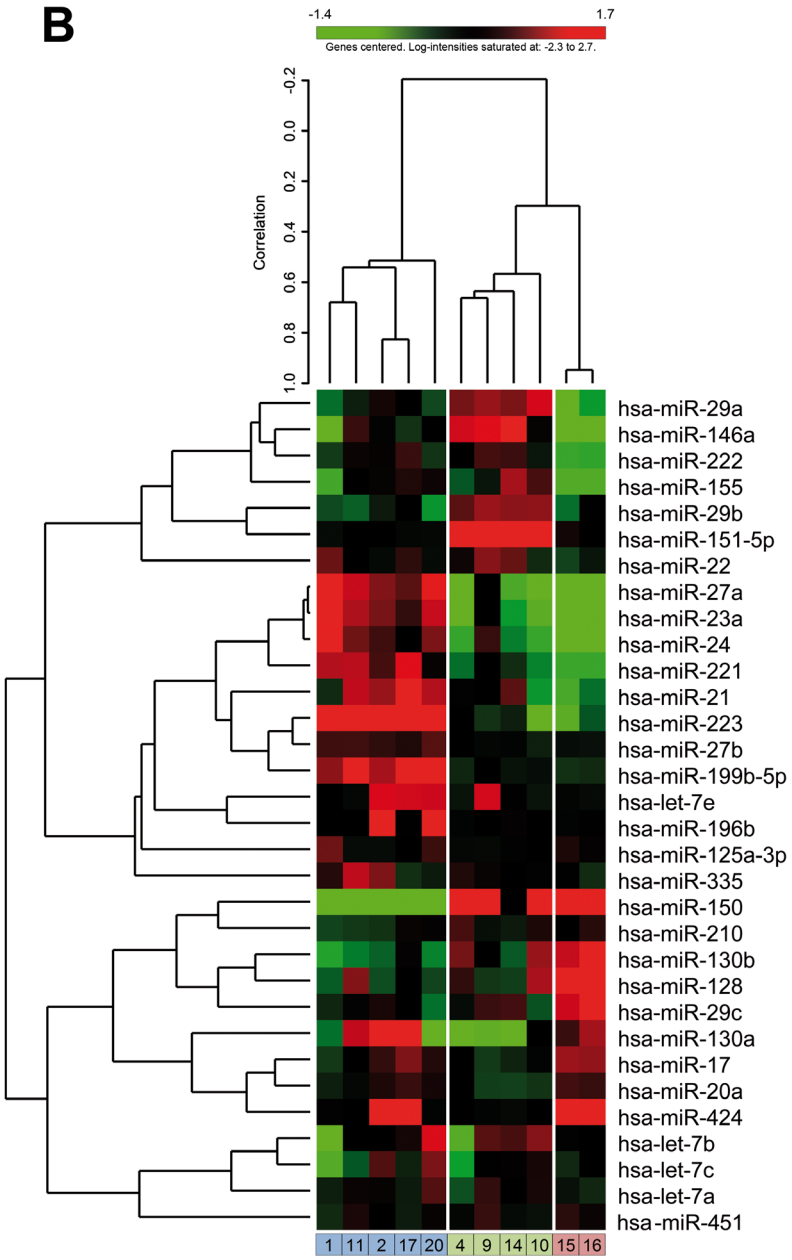


Figure 1: MiRNA expression signatures discriminate between AML, B-ALL and T-ALL. (A) The top 10 percent of most variable miRNAs were selected (n=134) for unsupervised clustering analysis of the AL samples. AML samples cluster separately from ALL samples. Within the ALL cluster, B-ALL and T-ALL samples can be discriminated. (B) Unsupervised clustering of the AL samples using the 32 miRNA literature based signature. AML and ALL can be discriminated using previously reported lineage specific miRNA expression. In both A and B the columns represent patients; rows represent miRNAs; blue, AML samples; red, T-ALL samples; green, B-ALL samples;

Table 1. miRNAs are differentially expressed between AML and ALL

Gene	AMLvsALL t	AMLvsALL p	AMLvsALL FDR	Max expression in dataset (selected >6)
hsa-miR-199b-5p	-8,602706	0,000006	0,003259	7,61
hsa-miR-27b	-8,449291	0,000007	0,003259	6,94
hsa-miR-150	8,393688	0,000007	0,003259	10,94
hsa-miR-199a-3p	-7,008514	0,000035	0,011802	7,40
hsa-miR-223	-6,165671	0,000102	0,024114	13,21
hsa-miR-27a	-6,126263	0,000107	0,024114	10,06
hsa-miR-340*	-5,858288	0,000154	0,028143	6,68
hsa-miR-340	-5,731613	0,000183	0,028143	6,68
hsa-miR-23a	-5,481022	0,000260	0,033675	10,26
hsa-miR-221	-5,293199	0,000340	0,038177	8,26

NOTE: Ten miRNAs were found to be differentially expressed betweenAMLand ALL (B-ALL and T-ALL) by LIMMA. “Max. expression in dataset” refers to the highest expression found in all samples for that particular miRNA. MiRNAs were considered expressed when median signal values after log2 transformation and normalization were higher than 6.0. “AML versus ALL t” represents moderated t statistic values, “AML versus ALL P” represents P values. “AML versus ALL FDR” represents false discovery rate.

Results

MicroRNAs are differentially expressed between ALL and AML

To confirm the already observed differential expression of miRNAs in AML, B-ALL and T-ALL we analyzed the miRNA expression profiles of several AML, T-ALL and B-ALL cases. Blasts were purified by flow cytometry, RNA was isolated and hybridized with miRNA-arrays (Agilent). The microarray data was normalized and the miRNAs with the highest mean absolute deviation (highest variation in expression between samples) were selected for further analysis. The subsequent unsupervised clustering analysis of these AL control samples generated two separate clusters. One cluster that contained all AML samples while the other contained all the ALL samples. This lymphoid cluster could be further separated in a T-ALL and B-ALL cluster (*figure 1A*). Next, we performed a Linear Model for Microarray Analysis (LIMMA) on these AML and ALL samples to see whether we could identify a similar miRNA profile as previously described that discriminates between the myeloid and lymphoid lineage [15,16]. The ten most significant miRNAs that are able to discriminate between AML and ALL are shown in *table 1*. One of these miRNAs (mir-150) showed enhanced expression in ALL as compared to AML, whereas the other nine miRNAs are higher expressed in AML as compared to ALL (miR-223, miR-199b-5p, miR-199a-3p, miR-27a, miR-27b, miR-23a, miR-340, miR-340* and miR-221). Seven of these 10 lineage discriminating miRNAs were described by others to discriminate between ALL and AML [15,16].

Since previous reports have described lineage discriminating miRNA signatures we used these discriminative miRNAs on our AML and ALL samples. In the study by Mi et al. [16] twenty-seven miRNAs were found to be significantly differentially expressed between the two subtypes of AL. In the study by Wang et al. [15], 16 miRNAs were differentially

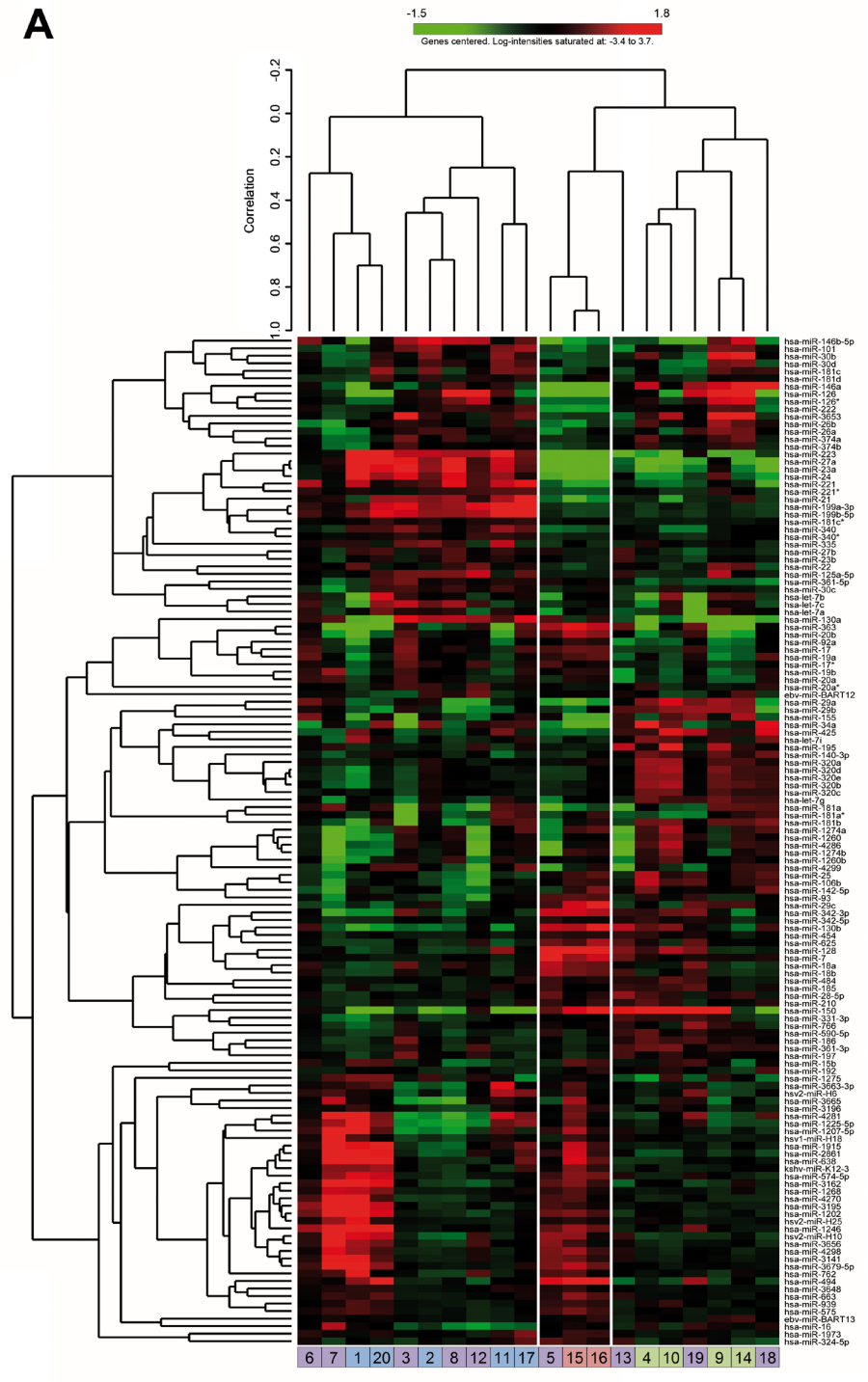
expressed between AML and ALL. To generate a robust literature based miRNA signature we integrated the miRNA lists from these two studies which resulted in a miRNA signature containing 32 miRNAs (*supplemental table 1*). Using these 32 miRNAs in an unsupervised clustering analysis of our miRNA expression results we were able to define our control AL samples as either AML or ALL in the correct way (*figure 1B*). Moreover, using the expression of these 32 miRNAs, even the segregation of the lymphoid cluster in a B-ALL and T-ALL group was seen. Thus, our miRNA expression profiles can discriminate between AML and ALL in an unsupervised clustering approach by using either the most variable 10% expressed miRNAs or the 32 miRNA signature that was published before. These results confirm the potential of miRNA expression profiling in classification of AL as either belonging to the myeloid or lymphoid lineage.

Leukemias of ambiguous lineage are not a distinctive entity.

Because the immunophenotype of cells is a limited reflection of the genotype and, as shown before, miRNA expression profiling can identify AL cases as either AML or ALL we analyzed the miRNA expression profiles of 9 AL cases of ambiguous lineage and compared these profiles with the AML, B-ALL and T-ALL samples. Using LIMMA we analyzed the expression of all the miRNAs in the four morphological and immunophenotypical defined groups of AL cases (AML, B-ALL, T-ALL and AL of ambiguous lineage) (*supplemental table 4A-C*). First, this showed that there is no significant difference in overall expression pattern observed in AL cases of ambiguous lineage as compared to AML samples (lowest false discovery rate, FDR >0.26), indicating that AL of ambiguous lineage was not significantly different from AML. However, when comparing the overall miRNA expression of the AL of ambiguous lineage with B-ALL cases, the miR-320 family (miR-320a/b/c/d/e) was found to be differentially expressed. Furthermore, the ambiguous lineage samples showed differential expression of miR-29c and miR-513a-5p when compared to T-ALL. This differential expression of miR-29c, miR-513a-5p and the miR-320 family is also observed between B-ALL and T-ALL samples *(supplemental table 4D*), likely indicating that the differential expression of these miRNAs is a direct result of intrinsic differential expression between B-ALL and T-ALL. Thus, we did not observe a distinctive pattern of overall miRNA expression between AL of ambiguous lineage and AML or ALL cases, suggesting that AL cases of ambiguous lineage are not a separate entity.

Leukemias of ambiguous lineage can be assigned to the lymphoid or myeloid lineage by miRNA expression profiling.

To study whether miRNA expression analysis is able to identify the myeloid or lymphoid origin of the leukemias of ambiguous lineage, we performed unsupervised clustering of the miRNA expression of the AL cases with ambiguous lineage as well as the AML and ALL cases (11 AL cases and 9 AL cases with ambiguous lineage). This resulted in the identification of three AL groups, a myeloid group containing all AML samples and a lymphoid group that could be further divided in B-ALL and T-ALL (*figure 2A*). The miRNA expression signatures of the leukemias with ambiguous lineage showed great resemblance with the B-ALL, the T-ALL or the AML miRNA signature, resulting in grouping of these leukemias with either one of the AL groups (*figure 2A*). Using the 32 miRNA literature based signature in an unsupervised way on all our samples (*figure 2B*) showed as well grouping of leukemias with ambiguous lineage with either AML, T-ALL or B-ALL.


A
-1.5
1.8
Genes centered. Log-intensities saturated at: -3.4 to 3.7.
Correlation
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
hsa-miR-146b-5p
hsa-miR-101
hsa-miR-30b
hsa-miR-30d
hsa-miR-181c
hsa-miR-181d
hsa-miR-146a
hsa-miR-126
hsa-miR-126*
hsa-miR-222
hsa-miR-3653
hsa-miR-26b
hsa-miR-26a
hsa-miR-374a
hsa-miR-374b
hsa-miR-223
hsa-miR-27a
hsa-miR-23a
hsa-miR-24
hsa-miR-221
hsa-miR-221*
hsa-miR-21
hsa-miR-199a-3p
hsa-miR-199b-5p
hsa-miR-181c*
hsa-miR-340
hsa-miR-340*
hsa-miR-335
hsa-miR-27b
hsa-miR-23b
hsa-miR-22
hsa-miR-125a-5p
hsa-miR-361-5p
hsa-miR-30c
hsa-let-7b
hsa-let-7c
hsa-let-7a
hsa-miR-130a
hsa-miR-363
hsa-miR-20b
hsa-miR-92a
hsa-miR-17
hsa-miR-19a
hsa-miR-17*
hsa-miR-19b
hsa-miR-20a
hsa-miR-20a*
ebv-miR-BART12
hsa-miR-29a
hsa-miR-29b
hsa-miR-155
hsa-miR-34a
hsa-miR-425
hsa-let-7i
hsa-miR-195
hsa-miR-140-3p
hsa-miR-320a
hsa-miR-320d
hsa-miR-320e
hsa-miR-320b
hsa-miR-320c
hsa-let-7g
hsa-miR-181a
hsa-miR-181a*
hsa-miR-181b
hsa-miR-1274a
hsa-miR-1260
hsa-miR-4286
hsa-miR-1274b
hsa-miR-1260b
hsa-miR-4299
hsa-miR-25
hsa-miR-106b
hsa-miR-142-5p
hsa-miR-93
hsa-miR-29c
hsa-miR-342-3p
hsa-miR-342-5p
hsa-miR-130b
hsa-miR-454
hsa-miR-625
hsa-miR-128
hsa-miR-7
hsa-miR-18a
hsa-miR-18b
hsa-miR-484
hsa-miR-185
hsa-miR-28-5p
hsa-miR-210
hsa-miR-150
hsa-miR-342-3p
hsa-miR-766
hsa-miR-590-5p
hsa-miR-186
hsa-miR-361-3p
hsa-miR-197
hsa-miR-15b
hsa-miR-192
hsa-miR-1275
hsa-miR-3663-3p
hsv2-miR-H6
hsa-miR-3665
hsa-miR-3196
hsa-miR-4281
hsa-miR-1225-5p
hsa-miR-1207-5p
hsv1-miR-H15
hsa-miR-1915
hsa-miR-2861
hsa-miR-638
kshv-miR-K12-3
hsa-miR-574-5p
hsa-miR-3162
hsa-miR-1268
hsa-miR-4270
hsa-miR-3195
hsa-miR-1202
hsv2-miR-H25
hsa-miR-1246
hsv2-miR-H10
hsa-miR-556
hsa-miR-4298
hsa-miR-3141
hsa-miR-3679-5p
hsa-miR-762
hsa-miR-494
hsa-miR-3648
hsa-miR-663
hsa-miR-939
hsa-miR-675
ebv-miR-BART13
hsa-miR-16
hsa-miR-1973
hsa-miR-324-5p
6 7 1 20 3 2 8 12 11 17 5 15 16 13 4 10 19 9 14 18

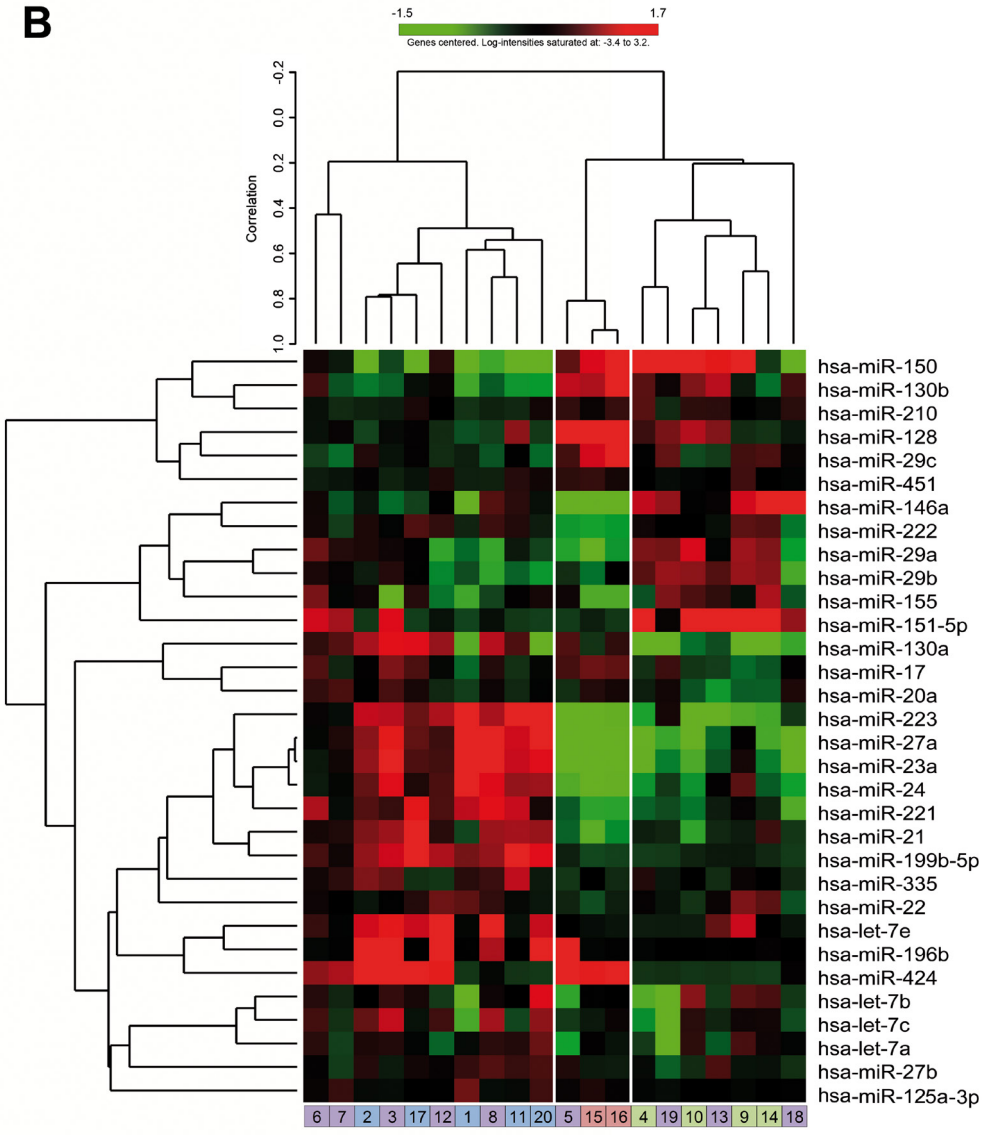


Figure 2. Leukemias of ambiguous lineage show similar expression profiles as either AML, B-ALL or T-ALL cases. (A) Unsupervised hierarchical clustering was performed using all AL samples. Leukemias of ambiguous lineage show similar expression profiles as AML, B-ALL or T-ALL. (B) Unsupervised clustering using the 32 miRNA literature based signature. Columns represent patients; rows represent miRNAs; blue, AML samples; red, T-ALL samples; green, B-ALL samples; purple, AL of ambiguous lineage.

All together, our results show that leukemias without a clear immunophenotypic lineage commitment do not display a distinct miRNA expression profile but one that can classify these leukemias as either of the myeloid or the lymphoid lineage.

Acute leukemia of ambiguous lineage can be classified as ALL or AML by Q-RT-PCR expression analysis of a small selection of miRNAs.

AL patients are either treated with an ALL or an AML treatment protocol and the assignment of AL cases of ambiguous lineage as either one of those AL subtypes is therefore of most relevance. To investigate the feasibility of classification of AL cases of ambiguous lineage as either AML or ALL by the expression of only a limited set of miRNAs, we selected the top five significantly differentially expressed miRNAs that could distinguish between AML and ALL (*supplemental table 3*). These five miRNAs, miR-23a, miR-27a, miR-223, miR-221 and miR-199b, are as well present in the top list of differentially expressed miRNAs between AML and ALL in two published studies (15,16). We analyzed the expression of these miRNAs by Q-RT-PCR in 19 AL cases that were used for the array hybridization (9 AL of ambiguous lineage, 5 ALL and 5 AML cases) as well as an additional 8 AL samples of ambiguous lineage, three ALL cases (two T-ALL, one B-ALL), two AML cases, the AML cell line HL60 and the T-ALL cell line CCRF-CEM. The expression profile of miR-23a, miR-27a, miR-221, miR-223 and miR-199b could discriminate two groups of AL cases, a myeloid and lymphoid group (*figure 3*). The nine AL of ambiguous lineage cases (unique patient number; UPN3, 5, 6, 7, 8, 12, 13, 18, 19) grouped in a similar fashion with either the AML or ALL cases by this Q-RT-PCR analysis as by array analysis. Moreover, the additional 8 AL of ambiguous lineage cases analyzed by Q-RT-PCR grouped either with the AML group or with the ALL group. Thus, the sole expression of five specific miRNAs using Q-RT-PCR can assign AL cases of ambiguous lineage as either from the lymphoid or myeloid lineage.

Correlation of immunophenotypic marker expression and miRNA classification.

The diagnosis of AL as either AML or ALL according to their miRNA expression profile might correlate with specific expression of one or more immunophenotypic markers already used to classify these leukemias. MiRNA profiling diagnosed 8 out of 17 leukemias of ambiguous lineage as AML and 9 cases as ALL. Overall the diagnosis according to the WHO2008 showed a more similar assignment to AML or ALL with the miRNA expression profiles than the WHO2001 criteria. This was most clear in samples that showed a myeloid miRNA expression profile from which 7 out of 8 samples were diagnosed as AML by the WHO2008 criteria. In cases were miRNA profiling indicated a lymphoid origin the WHO2001 criteria were more likely to diagnose a lymphoid AL than the WHO2008 criteria. Three patients (UPN 13, 18, and 22) showed expression of several lymphoid markers (either B lymphoid or T lymphoid) in combination with cMPO as single myeloid maker (*supplemental table 2*). However, with miRNA expression profiling these cases showed a clear ALL profile (*figure 3*). This might indicate that the importance of expression of cMPO as a single myeloid marker in the classification as leukemia of ambiguous lineage is overestimated. Importantly, even in the absence of informative immunophenotypic markers, miRNA expression profiling can assign a lineage to these AL cases with undefined immunophenotypic lineage. For two of the AL of ambiguous lineage patients (UPN 12 and UPN 27) the diagnosis was unclear due to absence of cCD3, cMPO and absence or low expression of CD19; miRNA profiling clustered these samples clearly within the AML group (UPN 12) and ALL group (UPN 27). Another two

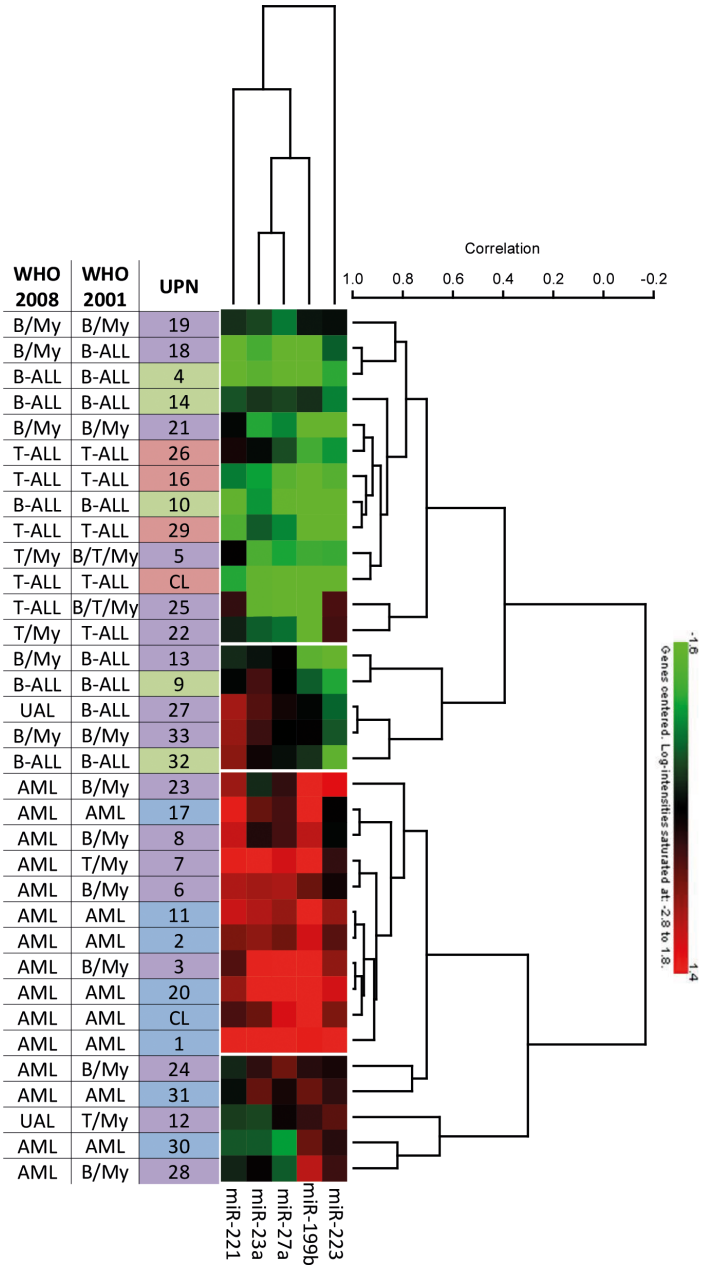


Figure 3. Unsupervised clustering of AL samples based on Q-RT-PCR expression analysis of miR-223, miR-199b, miR-221, miR-27a and miR-23a. MiRNA expression analysis using Q-RT-PCR and unsupervised clustering resulted in two separate groups, an AML and ALL group. UPN, Unique patient number; WHO2001, diagnosis when using WHO2001 criteria; WHO2008, diagnosis when using WHO2008 criteria; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoid leukemia; T-ALL, T-cell acute lymphoid leukemia; B/My, BAL/MPAL expressing B-lymphoid and myeloid markers; T/My, BAL/MPAL expressing T-lymphoid and myeloid markers; B/T/My, trilineage BAL/MPAL; UAL, unclassifiable acute leukemia; CL, cell line

leukemias of ambiguous lineage expressed markers of all three lineages (UPN 5 and UPN 25) but miRNA analysis assigned these both to the lymphoid lineage. Both these cases and UPN 22 were the only cases expressing cCD3 and all showed an underlying lymphoid lineage of origin possibly indicating the specificity of cCD3 in lymphoid lineage commitment.

Discussion

AL of ambiguous lineage differs from ALL in atypical myeloid or combined B and T lymphoid antigen expression and from AML in atypical lymphoid antigen expression. However, the immunophenotype of AL, as detected by flow cytometry, is merely a limited reflection of the genotypic state. Therefore, we hypothesized that miRNA based classification might help to distinguish between a predominant myeloid or lymphoid origin of AL of ambiguous lineage. Our results indicate that AL of ambiguous lineage is not a separate entity but can be traced back to one of the hematopoietic lineages, lymphoid or myeloid.

Reported data of gene expression profiling on thirteen pediatric MPAL cases showed that eight of the thirteen cases have a signature different from AML and B- or T-ALL. Whereas, the remaining five cases grouped with the myeloid leukemias [31]. In contrast to our data, these data suggest that at least a part of the leukemias of ambiguous lineage can be assigned as a separate identity by gene expression profiling. However, the separate group clustered very close to the B-ALL group, suggesting it could as well be a subtype within B-ALL. Since miRNA expression profiling has shown to be more successful in classification of tumors than gene expression profiling [31] we hypothesized that miRNA expression profiling might be superior in classification of leukemias without clear immunophenotypic lineage commitment. Indeed, our results show a classification of AL cases with ambiguous lineage as either AML, T-ALL or B-ALL. The difference in classification of leukemias of ambiguous lineage by gene expression profiling [18] and miRNA expression profiling is possibly due to the fact that miRNAs target a large panel of genes influencing a diverse network of signaling pathways. As a consequence, miRNA expression profiling might be superior over gene expression profiling in classification of cancers belonging to the same subtype since subtype classification is based on common impaired signaling pathways. Moreover, using miRNA expression profiling to define subclasses of leukemia might be more promising than mRNA profiling since miRNAs are more stable and can be used in smaller numbers than genes.

We identified five miRNAs that could be used to assign AL cases of ambiguous lineage to either AML or ALL. All of these five miRNAs have previously been reported to be associated with AML, such as miR-199b and miR-221 [16]. Furthermore, miR-223 has been show to function as a gene that plays a critical role in myeloid functions and differentiation [18,32]. MiR-23a and miR-27a are, together with miR-24-2, located in the same cluster which is regulated by PU-1. Each of these miRNAs are more abundantly expressed in myeloid as compared to lymphoid cells and both miR-23a and miR-27a are implicated in regulating the development of cells into the myeloid or lymphoid lineage [33]. In contrast to Wang et al [15], we could not use miR-222 to discriminate between ALL and AML, due to high expression of miR-222 in several of the B-ALL samples. All of the five selected miRNAs have enhanced expression in AML as compared to ALL which is due to their enhanced discriminative character as compared with miRNAs higher expressed in ALL. This is probably due to the fact that most miRNAs have increased expression in either B- or T-ALL and not in lymphoid

AL in general. The most significantly discriminative miRNA with enhanced expression in ALL was miR-150. Since at diagnosis, the discrimination between myeloid or lymphoid is most relevant, no miRNAs associated with only B-ALL or T-ALL were selected.

MiRNA profiling diagnosed 8 out of 17 leukemias of ambiguous lineage as AML and 9 cases as ALL. Since it might be that the karyotype and molecular aberrancies have an effect on miRNA expression and thereby potentially influence our clustering, we analyzed the distribution of the cytogenetic and molecular aberrations over the myeloid and lymphoid assigned AL of ambiguous leukemia groups. The most frequent cytogenetic abnormality was translocation t(9;22) which was positive in five leukemias of ambiguous lineage (UPN6, 8, 13, 27 and 33). Three of these cases had a miRNA profile that was similar to the ALL cases while the other two clustered with the AML cases. This indicates that despite an identical karyotype the microRNA expression profile is able to distinguish myeloid and lymphoid leukemia, suggesting that lineage specificity influences the microRNA expression profile more than the presence of cytogenetic and/or molecular aberrancies. Furthermore this suggests that translocation of BCR and ABL is not decisive in whether an AL is assigned to the myeloid or lymphoid lineage but that lineage commitment of AL with the BCR-ABL translocation is likely determined by the cell of origin receiving the mutation.

When we retrospectively classified the AL cases with ambiguous lineage containing an AML miRNA expression profile according to the WHO2001, all were diagnosed as AL with ambiguous lineage; while according to the WHO2008 seven out of eight cases were classified as AML. The other AL case with ambiguous lineage with an AML miRNA expression profile could not be classified using the WHO2008 criteria due to absence of CD19, cCD3 and cMPO. In the group AL with ambiguous lineage that had an ALL miRNA expression profiling, four out of nine cases were diagnosed as ALL by the WHO2001 criteria whereas only one out of nine cases was diagnosed as ALL by the WHO2008 criteria. Thus, classification by the criteria of the WHO2008 is less accurate in assigning ALL to the group of leukemias with a lymphoid miRNA expression profile than WHO2001, whereas it's more accurate in assigning an AL case with a AML miRNA expression profile as an AML .The immunophenotypic classification of AL cases to the myeloid lineage is done when cMPO is expressed as a single myeloid marker. This leads to assignment of AL cases containing an ALL miRNA expression profile, but with cMPO expression as a single myeloid marker, to the group of leukemias of ambiguous lineage. This might partly explain the better response to ALL treatment protocols of AL cases with ambiguous lineage [12].

Acute leukemias with ambiguous lineage are associated with a poor prognosis as compared to AML and ALL cases [7-11]. The poor prognosis of these leukemias might be due, at least partly, to their mistreatment. However, leukemias with ambiguous lineage have been shown to be associated with several poor prognostic factors such as $CD34^+$ phenotype, an unfavorable karyotype and PgP over-expression [7,9]. Our group was to small to perform survival analysis. However, generally overall survival was poor (median 20 months, range 5-110 months) for AL of ambiguous lineage.

The 5-year overall survival rate of patients with AML is considerably lower than of ALL patients and indeed, ALL patients have a better response to standard chemotherapy than AML patients in almost every age group [34]. This indicates that accurate diagnosis of all AL cases as either AML or ALL is crucial for selection of the appropriate therapy. To base treatment decisions on miRNA expression profiles it is crucial that the results of a microRNA expression test can be obtained within the timeframe of the start of appropriate treatment.

Our experience is that the turnaround time from isolation of the leukemic blasts to obtaining miRNA expression results can easily be done within 1.5 day making it rapid and within the time where a treatment decision has to be made. Whether treatment decisions based on miRNA expression profiles improve outcome of patients with AL of ambiguous lineage is difficult to determine since these leukemias are very rare and effect of treatment can only be measured in large randomized trials.
Our finding that expression analysis of five miRNAs could accurately classify AL cases with ambiguous lineage as AML or ALL might add to the already used methods to classify these AL cases of ambiguous lineage. In conclusion, our results indicate that leukemias of ambiguous lineage are not an unique entity but can be classified as either AML or ALL using miRNA expression profiling.

References

1. Brunning RD, Matutes E, Borowitz MJ et al. Acute leukaemias of ambiguous lineage. In: jaffe ES, Harris NL, Stein H, Vardiman JW, eds. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.: IARC Press, Lyon; 2001:106-107
2. Borowitz MJ, Béné M, Harris NL, Porwit A, McCann SR. Acute leukaemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL et al., eds. WHO Classification of Tumours of haematopoietic and Lymphoid Tissues, Fourth Edition: IARC Press, Lyon; 2008:150-154
3. van den Ancker W, Terwijn M, Westers TM, et al. Acute leukemias of ambiguous lineage: diagnostic consequences of the WHO2008 classification. Leukemia 2010; 7: 1392-1396
4. Buccheri V, Matutes E, Dyer MJ, Catovsky D. Lineage commitment in biphenotypic acute leukemia. Leukemia 1993; 6: 919-927
5. Bene MC, Castoldi G, Knapp W, et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995; 10: 1783-1786
6. Yan L, Ping N, Zhu M et al. Clinical, immunophenotypic, cytogenetic, and molecular genetic features in 117 adult patients with mixed-phenotype acute leukemia defined by WHO-2008 classification. Haematologica2012;97:1708-127
7. Killick S, Matutes E, Powles RL, et al. Outcome of biphenotypic acute leukemia. Haematologica 1999; 8: 699-706
8. Lee JH, Min YH, Chung CW, et al. Prognostic implications of the immunophenotype in biphenotypic acute leukemia. Leuk.Lymphoma 2008; 4: 700-709
9. Legrand O, Perrot JY, Simonin G, et al. Adult biphenotypic acute leukaemia: an entity with poor prognosis which is related to unfavourable cytogenetics and P-glycoprotein over-expression. Br.J.Haematol. 1998; 1: 147-155
10. Weir EG, li Ansari-Lari M, Batista DA, et al. Acute bilineal leukemia: a rare disease with poor outcome. Leukemia 2007; 11: 2264-2270
11. Matutes E, Morilla R, Farahat N, et al. Definition of acute biphenotypic leukemia. Haematologica 1997; 1: 64-66
12. Matutes E, Pickl WF, van 't Veer M, et al. Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. Blood 2011; 11: 3163-3171
13. Haferlach T, Kohlmann A, Bacher U, et al. Gene expression profiling for the diagnosis of acute leukaemia. Br.J.Cancer 2007; 4: 535-540

14. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999; 5439: 531-537
15. Wang Y, Li Z, He C, et al. MicroRNAs expression signatures are associated with lineage and survival in acute leukemias. Blood Cells Mol.Dis. 2010; 3: 191-197
16. Mi S, Lu J, Sun M, et al. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. Proc.Natl.Acad.Sci.U.S.A 2007; 50: 19971-19976.
17. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 2: 281-297
18. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005; 7043: 834-838
19. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. Blood 2008; 10: 5078-5085.
20. Li Z, Lu J, Sun M, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. Proc.Natl.Acad.Sci.U.S.A 2008; 40: 15535-15540
21. Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. Proc.Natl.Acad.Sci.U.S.A 2008; 10: 3945-3950
22. Garzon R, Volinia S, Liu CG, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood 2008; 6: 3183-3189
23. Marcucci G, Mrozek K, Radmacher MD, Garzon R, Bloomfield CD. The prognostic and functional role of microRNAs in acute myeloid leukemia. Blood 2011; 4: 1121-1129
24. Li Z, Huang H, Li Y, et al. Up-regulation of a HOXA-PBX3 homeobox-gene signature following down-regulation of miR-181 is associated with adverse prognosis in patients with cytogenetically abnormal AML. Blood 2012; 10: 2314-2324.
25. Schwind S, Maharry K, Radmacher MD, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J.Clin.Oncol. 2010; 36: 5257-5264
26. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 2: 249-264
27. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat.Appl.Genet.Mol.Biol. 2004; Article3
28. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 1995; 289-300
29. R Development Core Team. Language and environment for statistical computing. R Foundation for Statistical Computing. 2011
30. Smyth G. Limma: linear models for microarray data. 2012; 397-420
31. Rubnitz JE, Onciu M, Pounds S, et al. Acute mixed lineage leukemia in children: the experience of St Jude Children's Research Hospital. Blood 2009; 21: 5083-5089
32. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science 2004; 5654: 83-86
33. Kong KY, Owens KS, Rogers JH, et al. MIR-23A microRNA cluster inhibits B-cell development. Exp.Hematol. 2010; 8: 629-640
34. Provan D, Singer CRJ, Baglin T, Dokal I. Oxford Handbook of Clinical Haematology (3 ed). UK: Oxford University Press; 2009.

Supplemental material

Supplemental table 1. Literature based signature. Result of integrating the lineage discriminating miRNAs previous reported by Mi et al. [15] and Wang et al. [14]. Both reports described miRNAs that could discriminate between AML and ALL, 27 and 16 respectively. Ten miRNAs that were found by Mi et al. were also investigated by Wang et al. However, differential expression between AML and ALL could not be confirmed for two of them. The integration of all reported lineage discriminating miRNAs (excluding the two unconfirmed miRNAs) generated a literature based signature containing 32 miRNAs.

Supplemental table 1

1	hsa-let-7a	17	hsa-miR-21
2	hsa-let-7b	18	hsa-miR-210
3	hsa-let-7c	19	hsa-miR-22
4	hsa-let-7e	20	hsa-miR-221
5	hsa-miR-125a-3p	21	hsa-miR-222
6	hsa-miR-128	22	hsa-miR-223
7	hsa-miR-130a	23	hsa-miR-23a
8	hsa-miR-130b	24	hsa-miR-24
9	hsa-miR-146a	25	hsa-miR-27a
10	hsa-miR-150	26	hsa-miR-27b
11	hsa-miR-151-5p	27	hsa-miR-29a
12	hsa-miR-155	28	hsa-miR-29b
13	hsa-miR-17	29	hsa-miR-29c
14	hsa-miR-196b	30	hsa-miR-335
15	hsa-miR-199b-5p	31	hsa-miR-424
16	hsa-miR-20a	32	hsa-miR-451

Supplemental Table 2 (next page). Patient characteristics The results of immunophentyping by flow cytometry of 17 AL of ambiguous lineage patients. Markers are indicated when expressed with a fluorescence intensity of > 10% of the appropriate antibody control. Discrimination is made between weak or strong for CD19, cCD22, and CD10. Strong expression ("s") is defined as higher or as high as the expression on non-malignant cells in the same sample or, known from healthy donors. Weak expression ("w") is defined when the expression level does not reach expression of corresponding normal cells. Monoblastic appearance is defined by expression of CD14 and CD11c. UPN, Unique Patient Number; WHO2001, diagnosis when using WHO2001 criteria; WHO2008, diagnosis when using WHO2008 criteria; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoid leukemia; T-ALL, T-cell acute lymphoid leukemia; B/My, BAL/MPAL expressing B-lymphoid and myeloid markers; T/My, BAL/MPAL expressing T-lymphoid and myeloid markers; B/T/My, trilineage BAL/MPAL; UAL, unclassifiable acute leukemia; CD, cluster of differentiation; cTdT, terminal deoxynucleotidyl transferase; cMPO, cytoplasmic myeloperoxidase; cCD3, cytoplasmic CD3; cIgM, cytoplasmic immunoglobulin M; t(9;22), t(8;21), t(15;17), MLL, FLT3-ITD, Inv(16) are cytogenetic aberrancies analyzed by molecular diagnostics; Neg, negative for molecular aberrancy; Pos, positive for molecular aberrancy; ND, not done; n.a., not available; #, no CML prephase was present; Karyotype, analysis conducted by cytogenetic analysis; Complex*, 45,XX,+1,der(1;15)(p10;q10),-3,del(5)(q13q33),del(9)(q2?2),add16(p13),-17,-1,+21,add(22)(p11),+mar; Complex**, 46,XX,t(9;22)(q34:q11,2)[4] 47,XX,t(9;22)(q34;q11,2),der(22),t(9;22) [1] 46,XX [15].

Supplemental table 2.

UPN	B-cel markers	T-cel markers	Myeloid markers	Monoblastic appearance	Age	WHO 2001
B/My						
3	CD79aw, cCD22w, CD10w	CD4, CD5, CD10w	CD13, CD33, CD117, cMPO	neg	28	B/My
6	CD19w, CD79aw, cTdT	CD4, CD7, cTdT	CD13, CD33, C D117, cMPO	neg	52	B/My
8	CD19w, CD79aw, CD10s, cTdT	CD7, CD10s , cTdT	CD13, CD33, cMPO	neg	72	B/My
13	CD19s, CD79as, CD10s, CD20, cTdT	CD4, CD10s, cTdT	cMPO	neg	65	B-ALL
18	CD19s, CD10s, cTdT	CD10s, cTdT	cMPO	neg	13	B-ALL
19	CD19s, CD79as, CD10s, cTdT	CD10s, cTdT	CD13, cMPO	neg	9	B/My
21	CD19s, CD79as, CD10s, cTdT	CD4, CD10s, cTdT	CD13, cMPO	neg	14	B/My
23	CD19w, CD79aw, cTdT	cTdT	CD13, CD33, CD117, cMPO	neg	1	B/My
24	CD10, CD20, cIgM	CD7, CD10	CD13, CD33, CD117, CD15, cMPO	neg	52	B/My
27	CD19w, CD10, cTdT	CD10, cTdT	CD13, CD33	neg	62	B-ALL
28	CD19w, CD97aw, cCD22w, cTdT	CD4, cTdT	CD13, CD33, CD15, CD117, CD133, cMPO	neg	75	B/My
33	CD19s, CD79as, CD10s, cTdT	CD4, CD10s, cTdT	CD13, CD15, CD33, cMPO	neg	41	B/My
T/My						
7	none	CD2, CD4, CD5, CD7	CD13, CD33, CD15, CD65, CD117, cMPO	neg	72	T/My
12	CD10	CD2, CD4, CD5, CD7, CD10	CD13, CD33, CD117	neg	67	T/My
22	cTdT	CD2, CD5, CD7, CD8, cytCD3, cTdT	cMPO	neg	24	T-ALL
B/T/My						
5	CD79aw, CD10w, cTdT	cytCD3, CD2, CD4, CD5, CD7, CD8, CD10w, cTdT	CD13, cMPO	neg	75	B/T/My
25	CD79aw, cTdT	CD2, CD7, cytCD3, cTdT	CD13, CD15, CD117, CD65	neg	16	B/T/My

Supplemental table 2, continued

UPN	WHO 2008	Karyotype	t(9;22)	t(8;21)	t(15;17)	inv(16)	MLL	FLT3-ITD
B/My								
3	AML	Complex*	neg.	neg.	neg.	neg.	neg.	norm.
6	AML	46,XY,t(9;22)(q34;q11.2)	Pos. e14a2#	ND	ND	ND	ND	ND
8	AML	n.a.	Pos. e13a2#	neg.	neg.	neg.	neg.	norm.
13	B/My	Complex**	Pos. e1a2#	ND	ND	ND	neg.	ND
18	B/My	46,XX	neg.	neg.	neg.	neg.	neg.	norm.
19	B/My	n.a.	neg.	ND	ND	ND	neg.	ND
21	B/My	46,XY,t(9;9)(p?13;q?12)	neg.	neg.	neg.	neg.	neg.	norm.
23	AML	n.a.	neg.	neg.	neg.	neg.	neg.	norm.
24	AML	n.a.	neg.	neg.	neg.	neg.	neg.	ND
27	UAL	46,XX,t(9;22)(q34;q11.2)	Pos. e1a2#	neg.	neg.	neg.	neg.	norm.
28	AML	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
33	B/My	46,XX,t(9;22)(q34;q11.2)	Pos. e14a2#	neg.	neg.	neg.	neg.	norm.
T/My								
7	AML	46,XY,t(3;3)(q21;q26)	neg.	neg.	neg.	neg.	neg.	norm.
12	UAL	46,XX,t(2;3)(p23;q26),-7,+mar	neg.	neg.	neg.	neg.	neg.	norm.
22	T/My	46,XY	neg.	ND	ND	neg.	neg.	ND
B/T/My								
5	T/My	n.a.	neg.	neg.	neg.	neg.	neg.	norm.
25	T-ALL	n.a.	neg.	ND	ND	ND	neg.	ND

Supplemental Table 3. Significance analysis of microarray (SAM) identifies 36 significantly discriminative miRNAs between the myeloid and lymphoid clusters obtained from unsupervised clustering analysis of all samples (*figure 1A*).
The five microRNAs (miR-23a, miR-27a, miR-221, miR-223 and miR-199b) with the highest relative difference (d(i)) distinguishing AML from ALL were selected. Geometric mean intensity in AML and ALL as well as the fold change is indicated.

Supplemental Table 3

#	d(i)	Geom mean of intensities in lymphoid leukemia	Geom mean of intensities in myeloid leukemia	Fold-change	Symbol
1	-6.331	132.3	985.5	0.13	hsa-miR-27a
2	-5.997	38.8	111.9	0.35	hsa-miR-199b-5p
3	-5.617	152.2	941.8	0.16	hsa-miR-23a
4	-5.493	38.4	109.4	0.35	hsa-miR-199a-3p
5	-5.077	603.9	6323.6	0.10	hsa-miR-223
6	-5.043	78.0	238.2	0.33	hsa-miR-221
7	-4.137	148.7	530.5	0.28	hsa-miR-24
8	4.119	671.5	90.2	7.45	hsa-miR-150
9	-3.541	764.5	1980.0	0.39	hsa-miR-21
10	3.524	893.6	484.3	1.85	hsa-miR-25
11	3.417	288.3	118.8	2.43	hsa-miR-130b
12	-3.184	48.4	83.8	0.58	hsa-miR-340
13	3.109	1238.5	678.9	1.82	hsa-miR-106b
14	-2.840	47.0	68.3	0.69	hsa-miR-221*
15	2.746	73.8	44.6	1.66	hsa-miR-7
16	2.661	77.2	50.2	1.54	hsa-miR-625
17	2.563	845.4	526.6	1.61	hsa-miR-142-5p
18	2.560	340.6	172.7	1.97	hsa-miR-342-3p
19	2.505	117.4	86.4	1.36	hsa-miR-590-5p
20	2.495	180.0	81.1	2.22	hsa-miR-128
21	-2.410	34.1	41.6	0.82	hsa-miR-181c*
22	-2.408	40.9	51.4	0.80	hsa-miR-340*
23	-2.399	167.5	587.8	0.28	hsa-miR-130a
24	-2.368	80.3	186.9	0.43	hsa-miR-146b-5p
25	2.364	60.7	48.8	1.24	hsa-miR-454
26	2.324	1039.4	598.9	1.74	hsa-miR-29c
27	2.267	207.4	138.0	1.50	hsa-miR-320b
28	2.192	139.4	99.1	1.41	hsa-miR-140-3p
29	2.156	238.5	164.3	1.45	hsa-miR-320e
30	2.123	47.7	37.2	1.28	hsa-miR-210
31	2.089	76.9	57.9	1.33	hsa-miR-28-5p
32	2.072	54.0	44.0	1.23	hsa-miR-342-5p
33	2.064	100.8	74.0	1.36	hsa-miR-320a
34	2.041	326.0	220.4	1.48	hsa-miR-320d
35	2.022	199.9	147.6	1.35	hsa-miR-331-3p
36	1.975	85.5	66.9	1.28	hsa-miR-185

Supplemental Table 4. MicroRNAs are differential expressed between leukemia of ambiguous lineage, AML, B-ALL and T-ALL samples analyzed by LIMMA.
Tables show the top 25 microRNAs with the lowest FDR. (A) Top differentially expressed microRNAs between leukemia of ambiguous lineage and AML. (B) Top differentially expressed microRNAs between leukemia of ambiguous lineage and T-ALL. (C) Top differentially expressed microRNAs between leukemia of ambiguous lineage and B-ALL. (D) MicroRNAs that are differentially expressed between leukemia of ambiguous lineage and B-ALL or T-ALL are differentially expressed between B-ALL and T-ALL. Columns show t-statistic, p- and False Discovery Rate-values for the differential expressed microRNAs. 'ALAL vs. AML' represents differential expression between leukemias of ambiguous lineage and AML. 'ALAL vs. AML-T vs. AML' represents differential expression between leukemias of ambiguous lineage and T-ALL. 'ALAL vs. AML-B vs. AML' represents differential expression between leukemias of ambiguous lineage and B-ALL. 'T vs. AML-B vs. AML' represents differential expression between T-ALL and B-ALL.

Supplemental Table 4A

GeneName	t-statistic values ALAL vs AML	p-values ALAL vs AML	False Discovery Rate ALAL vs AML
hsa-miR-3607-3p	-4.267507	0.000511	0.268911
hsa-miR-150	4.194532	0.000599	0.268911
hsa-miR-181c*	-4.613656	0.000243	0.268911
hsa-miR-199b-5p	-3.593330	0.002216	0.722077
hsa-miR-181c	-3.506007	0.002680	0.722077
hsa-miR-151-5p	3.274696	0.004429	0.739704
hsa-miR-18a	3.175115	0.005491	0.739704
hsa-let-7f	-3.210894	0.005083	0.739704
hsa-miR-625	3.193295	0.005280	0.739704
hsa-miR-25*	3.184695	0.005379	0.739704
hsa-miR-611	2.989165	0.008186	0.761603
hsa-miR-20b	2.298159	0.034400	0.761603
hsa-miR-361-3p	2.153308	0.045817	0.761603
hsa-miR-27a*	2.778497	0.012802	0.761603
hsa-miR-302e	-2.248856	0.037951	0.761603
hsa-miR-151-3p	2.335972	0.031888	0.761603
hsa-miR-363	2.481153	0.023752	0.761603
hsa-miR-19a	2.120831	0.048814	0.761603
hsa-miR-502-5p	2.533943	0.021312	0.761603
hsa-miR-92a-1*	2.524618	0.021725	0.761603
hsa-miR-892b	-2.425146	0.026627	0.761603
hsa-miR-302d*	2.603964	0.018441	0.761603
hsa-miR-18b	2.735778	0.014006	0.761603
hsa-miR-1288	-2.560946	0.020158	0.761603
hsa-let-7i	-2.019358	0.059368	0.761603

Supplemental table 4B

GeneName	t-statistic values ALAL vs AML-T vs AML	p-values ALAL vs AML-T vs AML	False Discovery Rate ALAL vs AML-T vs AML
hsa-miR-513a-5p	-5.157308	0.000077	0.051812
hsa-miR-29c	-5.487066	0.000039	0.051812
hsa-miR-3923	-4.236257	0.000547	0.128627
hsa-miR-494	-4.228926	0.000556	0.128627
hsa-miR-3926	-4.214981	0.000573	0.128627
hsa-miR-1972	-4.521670	0.000296	0.128627
hsa-miR-663	-4.009495	0.000895	0.172247
kshv-miR-K12-5*	-3.795835	0.001425	0.240014
hsa-miR-29c*	-3.725784	0.001661	0.243450
hsa-miR-148a	-3.686934	0.001807	0.243450
hsa-miR-3683	3.577376	0.002295	0.257571
hsa-miR-628-5p	3.612214	0.002127	0.257571
hsa-miR-3196	-3.465031	0.002930	0.291099
hsa-miR-15b*	-3.450304	0.003026	0.291099
hsa-miR-4259	-3.394256	0.003417	0.306876
hsa-miR-146a	3.250343	0.004668	0.392993
hsa-miR-664*	-3.162368	0.005645	0.447244
hsa-miR-1288	-3.053049	0.007140	0.470665
hsa-miR-222	2.913216	0.009625	0.470665
hsa-miR-15a*	-2.922557	0.009436	0.470665
hsa-miR-345	-2.919248	0.009503	0.470665
hsa-miR-4262	3.057043	0.007079	0.470665
hsa-miR-941	3.008110	0.007861	0.470665
hsa-miR-4261	-2.959106	0.008729	0.470665
hsa-miR-7-1*	-3.036012	0.007406	0.470665

Supplemental tabel 4C

GeneName	t-statistic values ALAL vs AML-B vs AML	p-values ALAL vs AML-B vs AML	False Discovery Rate ALAL vs AML-B vs AML
hsa-miR-320a	-7.146909	0.000002	0.002099
hsa-miR-320c	-5.946545	0.000015	0.010368
hsa-miR-320b	-4.965371	0.000115	0.051651
hsa-miR-320e	-4.670534	0.000215	0.057914
hsa-miR-320d	-4.736786	0.000187	0.057914
hsa-miR-151-5p	-3.912646	0.001105	0.156747
hsa-miR-611	-3.605835	0.002157	0.156747
hsa-let-7g	-3.951326	0.001016	0.156747
hsv2-miR-H7-3p	-3.703422	0.001744	0.156747
hsa-miR-204	-3.837317	0.001302	0.156747
hsa-miR-3653	-3.887423	0.001168	0.156747
hsa-miR-3118	-3.594424	0.002211	0.156747
hsa-miR-30a	-3.769827	0.001509	0.156747
hsa-miR-1237	-3.600961	0.002180	0.156747
hsa-miR-30b	-3.712394	0.001710	0.156747
hsv1-miR-H6-3p	-3.596502	0.002201	0.156747
hsa-let-7f-1*	-3.617080	0.002105	0.156747
hsa-miR-1225-3p	-3.656371	0.001932	0.156747
hsa-let-7b*	-3.811055	0.001379	0.156747
hsa-miR-4313	-3.568319	0.002340	0.157621
hsa-miR-4305	-3.464389	0.002934	0.165389
ebv-miR-BART13	-3.368950	0.003610	0.165389
hsa-miR-29a	-3.444805	0.003062	0.165389
hsa-miR-324-3p	-3.287697	0.004306	0.165389
hsa-miR-4295	-3.417819	0.003247	0.165389

Supplemental table 4D

GeneName	t-statistic values T vs AML-B vs AML	p-values T vs AML-B vs AML	False Discovery Rate T vs AML-B vs AML
hsa-miR-513a-5p	5.173351	0.000556	0.044081
hsa-miR-320c	-5.080220	0.000632	0.047263
hsa-miR-29c	4.685986	0.001096	0.060674
hsa-miR-320e	-3.454607	0.007057	0.163881
hsa-miR-320d	-3.239787	0.009959	0.200161
hsa-miR-320a	-3.186858	0.010849	0.202963
hsa-miR-320b	-2.986355	0.015036	0.227560

Part II: Impaired antigen presentation in neoplasia: basic mechanisms and implications for acute myeloid leukemia

7

MM van Luijn
W van den Ancker
ME Chamuleau
GJ Ossenkoppele
SM van Ham
AA van de Loosdrecht

Immunotherapy. 2010
Jan;2(1):85-97

Abstract

During onset, treatment and progression of acute myeloid leukemia (AML), inadequate immune responses against certain myeloid leukemic blasts might be associated with the occurrence of minimal residual disease (MRD) and subsequent relapse. Several studies on this subject have demonstrated that, in general, solid tumor cells are able to avoid $CD8^+$ cytotoxic T cell (CTL) recognition by down-regulating HLA class I-restricted presentation of tumor-associated antigens (TAAs). In tumor cells that can express HLA class II molecules, such as myeloid leukemic blasts, abnormalities in the processing pathways of endogenous antigens could also result in impaired HLA class II-restricted TAA presentation to $CD4^+$ T helper (Th) cells. More insight into impaired TAA presentation by myeloid leukemic blasts could explain their escape from immune recognition and might be crucial for selecting appropriate strategies to improve whole-cell or dendritic cell (DC)-based tumor vaccine efficacy in the treatment of AML patients.

Introduction

One of the major obstacles in acute myeloid leukemia (AML) treatment is the recurrence of the disease after achieving complete remission. This is caused by the outgrowth of myeloid leukemic blasts that survived remission induction and consolidation therapy [1, 2]. Besides the involvement of other intrinsic factors, it could be possible that a subset of myeloid leukemic blasts, already present in the acute phase of the disease, are not recognized by the patients' own immune system. In an attempt to overcome immune escape by these leukemic blasts, current treatment protocols for AML patients include immunotherapeutic strategies such as allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusion (DLI). The application of these strategies not only contributes to immune reconstitution, but also aims to generate graft-versus-leukemia (GvL) effects via donor allogeneic NK and T cells.

The role of donor NK cells in the GvL effects was demonstrated in AML patients that received HLA haplotype-mismatched transplants. An increased donor-to-recipient NK cell alloreactivity was significantly associated with a lower relapse rate [3, 4]. For haploidentical allo-SCT, donor T cells are recognized as the main effector cells involved in GvL reactions by reacting to tumor-associated antigens (TAAs) expressed on antigen-presenting cells (APCs) [5]. It was described by several studies however, that the use of haploidentical allo-SCT followed by DLI could not prevent relapse occurrence in the majority of patients [6, 7]. In addition, donor T cells can induce severe graft-versus-host disease (GvHD), which makes it very challenging to develop approaches that improve their ability to specifically recognize myeloid leukemic blasts. These approaches include the in vitro generation of effector T cells that are specific for either leukemia-associated antigens (LAAs), such as WT-1, PRAME, RHAMM and PR-1, or hematopoiesis-restricted minor histocompatibility antigens (mHags) [8, 9].

When host and donor T cells are not able to generate long-lasting anti-leukemic immune responses in patients with AML, it may be assumed that impaired immune recognition of myeloid leukemic blasts does not result from T cell defects in the host. Instead, it is more likely that the leukemic blasts themselves are involved. Under the continuous pressure

of the host immune system, some residual leukemic blasts may escape from immune surveillance through adaptations in their phenotype, a process termed immune editing [10]. In general, tumor cells can evade eradication by T cells in different ways: 1) secreting or inducing the secretion of immunosuppressive molecules that render T cells inactive (IL-10, TGFβ, indoleamine 2,3-dioxygenase (IDO)); 2) overexpressing molecules that inhibit the apoptotic mechanisms employed by T cells to kill the tumor cell (Bcl-2, PI-9); or 3) down-modulating TAA processing and presentation pathways, which results in impaired antigen presentation and ineffective recognition by T cells. Regarding myeloid leukemic blasts, we recently reported that high expression of IDO, a metabolic enzyme that causes immune suppression via the induction of cell cycle arrest of T cells upon tryptophan degradation, is a significant predictor of poor clinical outcome in AML [11]. In addition, we demonstrated that blasts of AML patients may escape TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis by using TRAIL-R3 as a decoy receptor [12]. As myeloid leukemic blasts are capable of presenting HLA class I and II as well as co-stimulatory molecules [13], impairment in TAA presentation could be another mechanism used by these tumor cells to prevent immune recognition. Although not much is known about this type of escape in AML yet, accumulating evidence from work in solid tumor cells together with recent observations in myeloid leukemic blasts implicate that impaired TAA presentation may be an underestimated issue in myeloid neoplasia and will be discussed in the current review.

To overcome impaired TAA presentation by tumor cells, whole-cell and dendritic cell (DC)-based tumor vaccinations have been introduced in the last few years as promising immunotherapeutic strategies [14-16]. These strategies are based on the potential of tumor vaccines to present a broad range of TAAs to recipient T cells, thereby inducing a long-lasting anti-tumor response. Since TAA presentation could be disturbed in tumor cells, it is important for the efficacy of these vaccines to understand which antigen processing and presentation pathways are involved. In this review, we provide an update on the role of antigen presentation in tumor immunology and describe how this knowledge may be used to develop immunomodulatory approaches that optimize aberrant TAA presentation by whole-cell and DC-based tumor vaccines in the context of AML.

Tumor-associated antigen presentation: rationale and underlying pathways

For tumor immune surveillance, it is essential that the adaptive immune system is able to specifically recognize and eradicate tumor cells [17]. To generate effective and long-lasting anti-tumor immunity, professional APCs, e.g. DCs and macrophages, as well as B cells and tumor cells themselves, must efficiently present TAAs to $CD8^+$ cytotoxic T cells (CTLs), which allows the activation of effector CTLs that will have to lyse the tumor cells. In addition, $CD4^+$ T helper 1 (Th1) cells need to be activated to help establish an efficient TAA-specific CTL response and generate anti-tumor immune memory [18]. Upon TAA recognition and additional IFN-γ and IL-12 signalling, naive $CD4^+$ T cells differentiate into Th1 effector cells and optimize the anti-tumor effect of CTLs, either directly via co-stimulatory molecules or the production of cytokines like IL-2, or indirectly via APC activation [19]. Moreover, TAA-specific $CD4^+$ Th1 cells have been described to have potent anti-tumor effects independent of CTL action [20]. APCs use different processing pathways to present TAAs to CTLs and $CD4^+$ T cells, involving HLA class I and II molecules, respectively.

Classical HLA class I-restricted presentation: endogenous antigens

With respect to the conventional pathway of HLA class I-restricted antigen processing and presentation (*figure 1A*), endogenously synthesized proteins are first degraded into short peptides in the cytoplasm by a multicatalytic proteinase complex called the proteasome [21]. Thereafter, the proteasome-generated peptides are translocated into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) [22], another major component of the HLA class I antigen processing machinery that is localized in the ER membrane. In the ER lumen, these peptides can be further trimmed by ER aminopeptidases associated with antigen processing (ERAAPs) into the nonameric peptides necessary for optimal HLA class I loading [23]. The macromolecular peptide loading complex, which consists of TAP, tapasin, calreticulin and ERp57 [24], ensures that newly formed HLA class I heavy chain-β2-microglobulin (β2m) dimers are properly folded in the ER, allowing the allele-specific peptides to bind to the HLA class I peptide-binding groove. After peptide loading, the HLA class I/peptide complex is dissociated from the chaperones that promote ER retention and transported into the default secretory pathway, after which it eventually reaches the plasma membrane for presentation to CTLs.

HLA class I-restricted cross-presentation: exogenous antigens

The endogenous pathway of TAA loading onto HLA class I molecules explains how antigens are presented on tumor cells for recognition by activated CTLs. It does not explain however, how TAAs gain access onto HLA class I molecules in professional APCs for activation of TAA-specific CTLs in the lymph nodes. Particularly in myeloid DCs (mDCs), it has been shown that in a process termed cross-presentation, TAAs can be taken up as exogenous material (in the form of tumor cell fragments of dead tumor cells) and are subsequently presented onto HLA class I molecules. The major cell biological questions regarding cross-presentation are how exogenous TAAs in the endosomal/lysosomal pathway are trimmed to the right size for loading and in which cellular compartment these TAAs are loaded onto HLA class I molecules. To date, the pathways of cross-presentation can generally be described as being indirect or direct. The cross-presentation pathway reported by Huang and co-workers is indirect and uses the endogenous HLA class I antigen processing machinery to process exogenous antigens [25]. After entry into early endosomes, these antigens are exported into the cytoplasm, degraded by the proteasome and translocated via TAP into the ER lumen for loading onto HLA class I. Another indirect cross-presentation pathway described by Burgdorf and co-workers and Guermonprez and co-workers involves the same mechanism of antigen processing, but in these cases the proteasome-generated exogenous peptides are imported again into the early endosomes via endosomal-localized TAP and loaded onto HLA class I molecules in the endosomes [26, 27]. It remains controversial whether this peptide re-import is made possible by Toll-like receptor 4 (TLR4)- and MyD88-dependent recruitment of TAP to the early endosomes [26] or whether this involves the fusion of early endosomes with the ER [27]. In addition, recent data show that lipid bodies may also act as key organelles in the indirect pathway of cross-presentation [28]. The direct cross-presentation pathway reported by Shen and co-workers and Di Pucchio and co-workers does not rely on cytoplasmic transit of exogenous antigens nor does it involve the function of the proteasome and TAP [29, 30]. Instead, the 'classical' endosomal/lysosomal pathway serves as the processing site for these antigens, in which peptide generation is strongly dependent on cathepsin S [29]. Antigen loading occurs within the endosomal storage compartments that acquire newly formed HLA class I monomers via pathways that are currently only partially defined [30].

Classical HLA class II-restricted presentation: exogenous antigens

The endosomal/lysosomal pathway plays a central role in HLA class II antigen presentation by processing exogenously-derived antigens for loading onto HLA class II molecules [31]. Prior to peptide loading, newly synthesized HLA class II α and β heavy chains dimerize and associate with the Invariant Chain (Ii or CD74) in the ER to form a nonameric complex. This complex is then specifically targeted via two dileucine-based endocytic-sorting motifs in the cytoplasmic tail of Ii to specialized antigen-loading compartments, known as MIICs (MHC class II containing compartments) [32]. Besides its role in endosomal/lysosomal targeting, Ii also contributes to the stabilization and proper folding of HLA class II heterodimers in the ER [33]. During transport to the MIICs, Ii is degraded by specific pH-sensitive endosomal proteases (cathepsins) until a small remnant remains left in the HLA class II peptide-binding groove [34]. In the MIICs, this class II-associated invariant chain peptide (CLIP) is exchanged for an exogenous peptide, a process that is catalyzed by an HLA-like chaperone molecule, HLA-DM (DM) [35]. Especially in B cells, DM associates to yet another HLA-like chaperone, HLA-DO (DO), which down-modulates the function of DM [36]. After peptide exchange, the HLA class II molecule is transported from the MIICs to the plasma membrane, where it is presented to $CD4^+$ T cells.

HLA class II-restricted 'reverse cross-presentation': endogenous antigens

In the last few years, it has become evident that APCs, including mDCs, B cells and certain tumor cells, also have the ability to process endogenous antigens for presentation onto HLA class II molecules, which may be called 'reverse cross-presentation'. Until now, studies carried out to unravel the antigen processing pathways for this type of presentation have yielded conflicting data. On the one hand, processing of endogenous antigens for loading onto HLA class II molecules has been described to take place in the endosomal/lysosomal pathway [37]. The transport of endogenous antigens from the cytoplasm into this pathway can be accomplished through two distinct forms of the cellular housekeeping mechanism known as autophagy. During macroautophagy, large cytoplasmic particles, like endogenous antigens, are taken up into autophagosomes and delivered to the endosomal/lysosomal pathway for degradation after fusion with lysosomal compartments (*figure 1B*) [38, 39]. Additional studies demonstrated that endogenous antigen processing via this mechanism of autophagy is primarily dependent on lysosomal proteases rather than the proteasome and TAP [40, 41]. A second autophagic mechanism, termed chaperone-mediated autophagy (CMA), is more specific and involves the translocation of small cytoplasmic products, such as endogenous peptides, into the lysosomes via the chaperone protein hsc70 and its receptor LAMP-2A on the lysosomal membrane (*figure 1C*) [42]. For processing via CMA, premature degradation of endogenous antigens into peptides by the cytoplasmic proteasome is considered to be a critical step [43]. This indicates that on the other hand, the HLA class I antigen processing machinery plays an important role in 'reverse cross-presentation' of endogenous antigens. Indeed, studies by Lich and co-workers and Tewari and co-workers showed that both the proteasome and TAP were required for certain endogenous antigens to be processed and loaded onto HLA class II molecules [44, 45]. Since these molecules were capable of presenting endogenous peptides irrespectively of the endosomal/lysosomal pathway, it could be possible that proteasome- and TAP-dependent endogenous peptides are loaded onto newly synthesized HLA class II heterodimers in the ER (*figure 1D*). This type of loading however, might be disturbed by the function of Ii, as this chaperone protein is

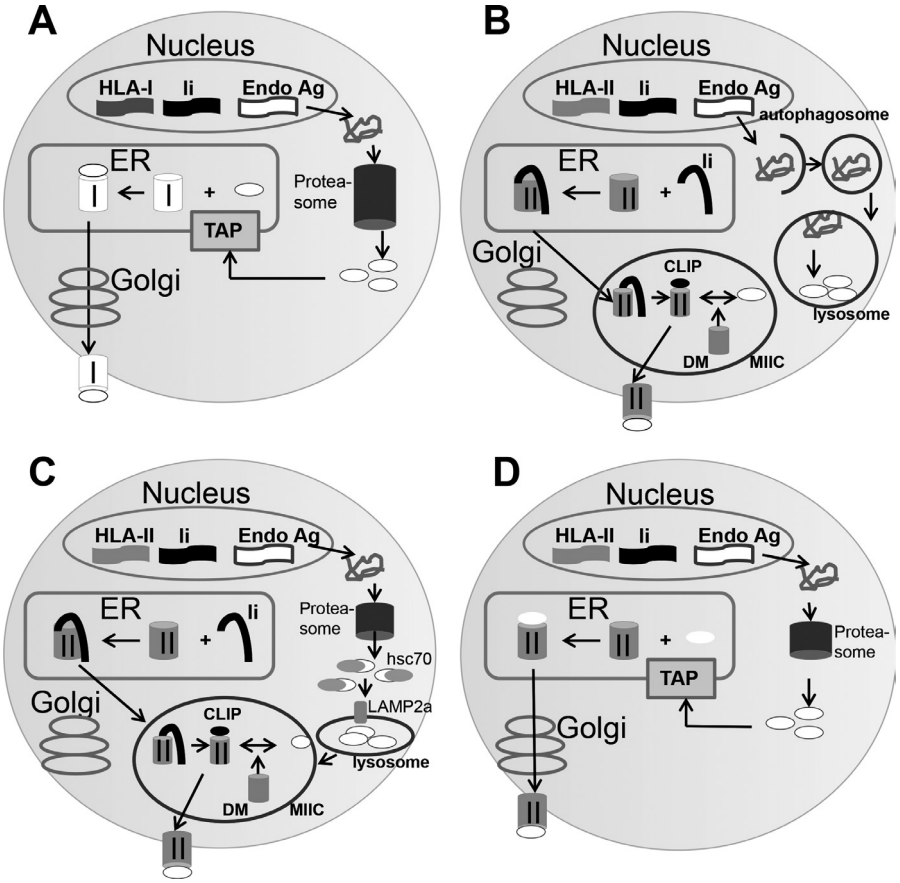


Figure 1. Potential pathways used by APCs to process and present endogenous antigens. Both HLA class I and II molecules are able to present endogenous antigens at the plasma membrane via several processing pathways. For loading onto HLA class I molecules, endogenous antigens are processed via the proteasome and transported into the ER by TAP (panel A). HLA class II molecules can be loaded with endogenous antigens in three different ways: 1) via delivery into the lysosomal compartments by autophagosomes during a process called macroautophagy (panel B); 2) via translocation into the lysosomes by the chaperone protein hsc70 and its receptor LAMP-2A on the lysosomal membrane, a process termed chaperone-mediated autophagy (CMA; panel C); or 3) via the same processing pathway used for loading onto HLA class I molecules, involving the proteasome and TAP (panel D).

able to associate with the HLA class II peptide-binding groove to prevent premature binding of endogenous peptides in the ER [46].

Defective tumor-associated antigen presentation as tumor escape mechanism

The ability of tumor cells to present their own antigens (TAAs) to the immune system makes it very challenging to develop new immunotherapeutic strategies that specifically target these TAAs. Inhibition of HLA class I- and II-restricted TAA presentation, however, is a common mechanism by which tumor cells not only evade immune surveillance, but also become less sensitive to tumor-specific immunotherapy. In order to understand and deal with this type

of tumor escape, it is important to examine tumor cells for the presence of abnormalities in the pathways normally involved in the processing and presentation of endogenous antigens, as described earlier and demonstrated in *figure 1*.

HLA class I escape mechanisms in neoplasia

In various types of solid tumors, multiple HLA class I abnormalities have been reported that can affect any phase of the processing and presentation pathway, including HLA synthesis, assembly, peptide loading and plasma membrane expression. These abnormalities are the result of genetic or epigenetic alterations involved in the expression of HLA class I molecules and components of the HLA class I antigen processing machinery. According to the definition of HLA class I deficiencies by Garrido and co-workers, HLA haplotype loss, HLA locus loss, HLA allelic loss, total HLA loss, or a combination of each represent the major altered phenotypes of tumor cells [47]. It is also possible that tumor cells are unable to upregulate HLA class I expression in response to interferons or present aberrant HLA-E after down-regulation of classical HLA-A, -B and -C molecules, but these phenotypes occur less frequently. HLA haplotype, locus and allelic losses provoke a selective absence of HLA class I molecules on tumor cells and are the consequence of loss of heterozygosity (LOH) at chromosome 6p21, transcriptional dysregulation and genetic mutations, respectively [48]. The most dramatically altered phenotype, total HLA loss, can directly result from alterations in HLA class I gene transcription. These alterations include disturbed DNA binding of nuclear transcription factors and epigenetic silencing of the HLA class I heavy chain gene, both leading to aberrant HLA class I synthesis [49, 50]. Another direct cause of total HLA loss involves β2m gene mutations, which hampers HLA class I assembly [51]. Furthermore, mutations in the genes encoding for tapasin or subunits of the proteasome (LMP-2 or -7) and TAP (TAP-1 or -2) indirectly cause total HLA class I loss by interfering with HLA class I peptide loading, release from the ER chaperones and consequently plasma membrane expression [52]. The latter deficiencies may not only result in total HLA loss, but could also stimulate cross-presentation of non-relevant exogenous antigens instead of endogenous TAAs.

HLA class II escape mechanisms in neoplasia

In contrast to HLA class I-restricted antigen presentation, the occurrence of abnormalities in the HLA class II antigen processing and presentation pathway and their functional relevance have not been studied extensively in tumor cells. The main reason is that most solid tumors do not express HLA class II molecules. Several molecular mechanisms can be responsible for deficient expression of HLA class II molecules on tumor cells. Consistent with HLA class I, repression of gene transcription contributes to a loss of HLA class II synthesis, either directly via point mutations and deletions in the HLA class II genes [53], or indirectly via hypermethylation of the activator of immune response-1 (AIR-1) locus encoding for the 'master' regulator of HLA class II gene transcription, class II transactivator (CIITA) [54]. Since CIITA additionally activates genes involved in classical HLA class II transport and peptide loading, including Ii and DM, respectively, silencing of this transcription factor also results in an inability of tumor cells to process HLA class II molecules in the endosomal/lysosomal pathway and present autophagy-dependent TAAs. Some tumor types, however, such as melanoma, lung carcinoma as well as B-cell and myeloid leukemias, do possess the capacity to express HLA class II molecules and activate tumor-specific $CD4^+$ T cells. Still, also in these

tumor cells, total and partial HLA class II loss are common altered phenotypes, which have been demonstrated to be clinically and functionally relevant [55]. For melanoma and B cells, HLA class II-restricted escape may also be the result of selectively impaired TAA processing without affecting HLA class II expression per se. Melanoma cells deficient for gamma interferon-inducible lysosomal thiol-reductase (GILT) contain low DM levels and cathepsin activity [56], suggesting that not only MIIC-restricted loading, but also degradation of endogenous TAAs within the lysosomes is impaired during macroautophagy. In a study with lymphoma B cells, it was shown that under immunosuppressive conditions glutathione depletion resulted in a down-regulation of lysosomal proteolysis, which caused impaired antigen processing [57].

HLA class I escape mechanisms in myeloid neoplasia

To explain the failure of the host immune system and currently used immunotherapeutic strategies to induce anti-leukemic immunity in AML, several studies have been performed on the role of these altered HLA class I phenotypes in the immune evasive capacity of myeloid leukemic blasts before and after treatment. It was previously described that reduced HLA class I expression on leukemic blasts is correlated with a high white blood cell (WBC) count in patients with newly diagnosed AML [58]. In other studies however, very low frequencies of total HLA class I loss in AML were detected on leukemic blasts from both diagnosis and relapse blood and bone marrow samples [59, 60], which indicates that the onset and progression of AML is not correlated with total HLA class I loss on leukemic blasts. Although no HLA haplotype loss was observed in leukemic blasts of untreated AML patients [61], recent data show that this phenotype may be important for the occurrence of relapse in patients who received DLI following haploidentical allo-SCT [62]. In 5 of 17 patients with a relapse after DLI treatment, leukemic blasts were recognized that had lost the patient-specific HLA haplotype due to acquired LOH at chromosome 6p21. As a result, donor allogeneic T cells could not eradicate these mutant variants, in contrast to the original leukemic blasts, implicating that the HLA haplotype loss rendered these blasts insensitive to GvL effects in AML patients. HLA locus loss in leukemic blasts was shown to lead to down-regulation of locus HLA-A and -B, but not -C in some patients with untreated AML [63]. Demanet and co-workers additionally found a loss of HLA-A and -B alleles in 7 of 14 patients examined, of which the majority belonged to the Bw6 group [64]. As the expression of most alleles from the Bw4 group was preserved, it was suggested that these selective HLA allelic losses serve as a compound HLA class I phenotype by which myeloid leukemic blasts circumvent both CTL and NK cell-mediated cytotoxicity during immune surveillance. Indeed, HLA allelic losses were demonstrated to be functionally relevant, but have only been reported for small numbers of AML patients [59].

HLA class II escape mechanisms in myeloid neoplasia

Since HLA class I abnormalities may not be of major importance in myeloid neoplasia, it is hypothesized that defects in the HLA class II LAA processing and presentation pathway of myeloid leukemic blasts may prevent $CD4^+$ T cell recognition and hamper the potency of an anti-leukemic immune response. In the majority of newly diagnosed AML patients, HLA class II molecules are expressed on leukemic blasts, with no changes in expression at relapse [65]. There are some cases of AML in which leukemic blasts do lack HLA class II, including acute promyelocytic leukemia (APL). Despite this fact, patients with APL have a good prognosis,

which is due to the role of other molecular mechanisms, such as the generation of the PML/RARα fusion proteins as the result of t(15;17) gene rearrangement, making these patients well treatable. Nevertheless, no differences in disease-free survival (DFS) were observed between HLA-DR-negative non-APL and HLA-DR-positive AML patients [65], indicating that the expression of HLA class II proteins on myeloid leukemic blasts may not play a role in the occurrence of relapse. We previously found indications, however, that impaired HLA class II-restricted LAA presentation is involved in AML progression, since high CLIP expression on myeloid leukemic blasts from patients significantly correlated to a shortened DFS [66]. Recently, we confirmed these findings in an expanded cohort of patients and additionally revealed that a high CLIP amount on these blasts abolishes the induction of autologous and allogeneic $CD4^+$ T cells [67].

The immune escape by $CLIP^+$ leukemic blasts likely reflects a diminished loading of LAAs onto HLA class II molecules, indicating the necessity to elucidate the molecular mechanism involved in this process. One possibility could be that the exchange of CLIP for autophagy-dependent LAAs in the lysosomal MIICs is hampered due to a lowered DM/DO ratio. This is supported by our previous observation that the DM/DO ratio inversely related to the amount of CLIP per HLA-DR molecule in leukemic blasts of patients with AML [66]. Another possible explanation for aberrant LAA loading is that the association of Ii to the HLA class II peptide-binding groove interferes with loading of proteasome- and TAP-dependent LAAs in the ER [46]. Armstrong and co-workers confirmed this function of Ii in tumor cells by showing that the presentation of an ER-retained endogenous antigen was prevented in the presence of Ii alone or both Ii and DM [68]. Less is known about proteasomal and TAP deficiencies in myeloid leukemic blasts, which may result in insufficient LAA processing and loading onto HLA class II molecules in the ER when Ii expression is down-modulated. In this regard, we recently observed in TAP^{high} myeloid leukemic blasts that HLA-DR expression at the plasma membrane occurred independently of Ii, but did depend on the function of the proteasome and TAP. HLA-DR expression by TAP^{low} myeloid leukemic blasts however, was completely dependent on Ii and did not involve the proteasome and TAP [69]. These findings suggest that HLA class II-restricted presentation of LAAs could also be affected by a deficient HLA class I antigen processing machinery in myeloid leukemic blasts silenced for Ii.

Strategies to optimize tumor-associated antigen presentation on whole-cell vaccines

To enhance the efficacy of T cell-based strategies in cancer immunotherapy, the use of TAA-presenting APCs as tumor vaccines is considered to be an attractive approach [70]. One of the ways to develop these vaccines could be loading of monocyte-derived DCs (moDCs) with TAAs. Regardless of which antigen source is the most suitable for loading, the possibility remains that immunogenic, potentially unidentified, TAAs are missing or not processed for presentation in the same manner as in tumor target cells. This might be avoided by using tumor cells as whole-cell vaccines, which are capable of presenting a broad range of TAAs at the plasma membrane. This type of vaccines may have great potential, as the presentation of endogenous TAAs to both CTLs and $CD4^+$ T cells is likely to induce an efficient anti-tumor immune response in vivo. Although almost no HLA class I defects are detected, molecular mechanisms could still exist in myeloid leukemic blasts that interfere with the onset of HLA class II-restricted LAA presentation, as mentioned previously. For the design of optimal LAA-presenting whole-cell vaccines, it is necessary to apply modulatory strategies that stimulate HLA class II-restricted LAA presentation on these blasts. The development of such strategies

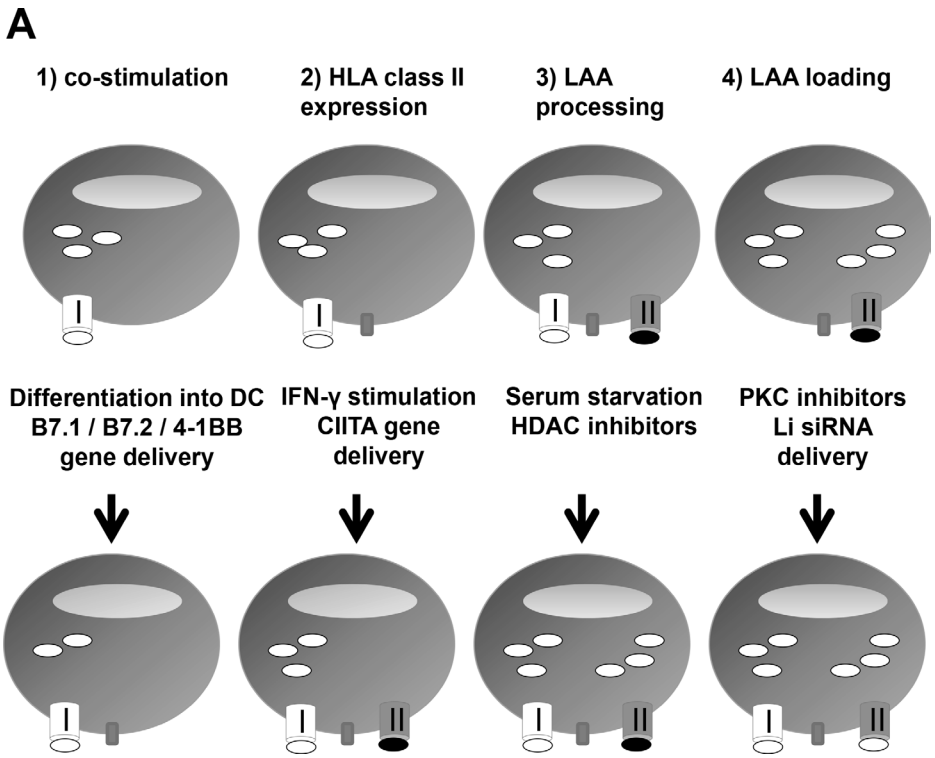
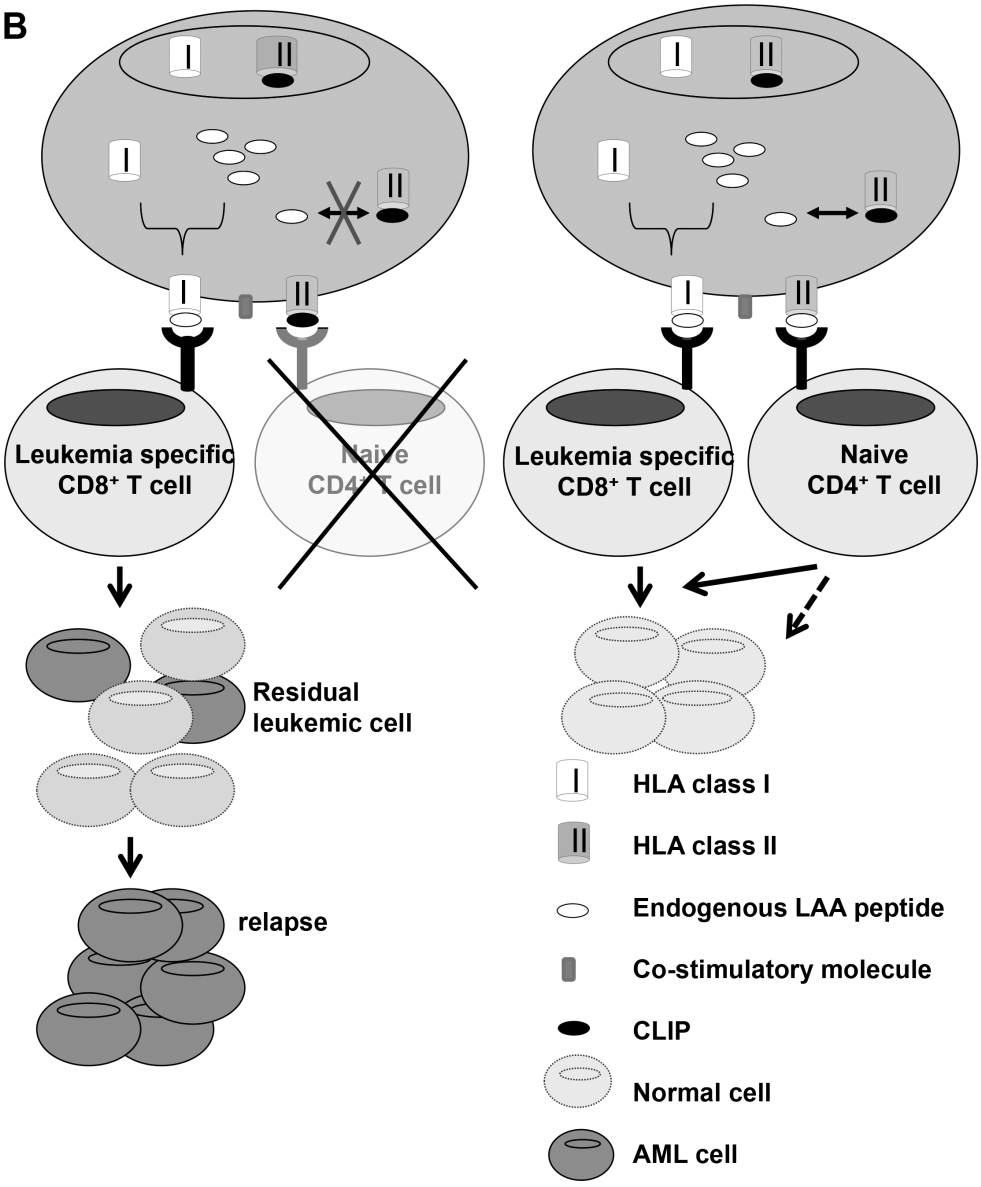


Figure 2. The application of whole-cell vaccines in the treatment of AML. (A) Schematic overview of which defects in HLA class II-restricted presentation of leukemia-associated antigens can be encountered in myeloid leukemic blasts and how these defects may be overcome by specific modulatory strategies that enhance tumor immunogenicity. To increase the expression of co-stimulatory molecules, leukemic blasts could be differentiated into AML DCs or treated with lentiviral vectors encoding for B7.1, B7.2 and/or 4-1BB. HLA class II expression might be enhanced by IFN-γ stimulation or delivery of the CIITA gene. Serum starvation in combination with HDAC inhibitors might be used to boost LAA processing and loading in the MIICs. On the other hand, the use of HDAC inhibitors together with Ii siRNAs is probably the most appropriate strategy to induce LAA processing and loading in the ER. (B) The potential clinical impact of AML whole-cell vaccines with optimal LAA presentation. In non-vaccinated patients, LAA presentation via HLA class II molecules on myeloid leukemic blasts is probably insufficient to activate $CD4^+$ T cells, leading to transient anti-leukemic $CD8^+$ CTL responses and AML recurrence. In vaccinated patients, AML whole-cell vaccines may present a broad range of LAAs via both HLA class I and II molecules, resulting in long-lasting anti-leukemic immunity and full eradication of leukemic blasts.

depends on which aspect of this type of presentation is defective, as summarized in *figure 2A*. First of all, leukemic blasts that lack co-stimulatory molecules may be differentiated into leukemic DC-like cells, resulting in increased expression of these molecules, as shown by Westers and co-workers [71]. In a recent study, another method to up-regulate co-stimulatory molecules was proposed, which involved the simultaneous delivery of B7.1, B7.2 and 4-1BB genes to blasts using lentiviral transduction [72]. Via addition of IFN-γ, HLA class II expression can be transiently restored in most blasts, as the CIITA-encoding AIR-1 locus consists of an IFN-γ-inducible promoter [73]. Some leukemic blasts, however, are



resistant to this treatment due to lack of CIITA, similarly to the majority of solid tumor cells that are negative for HLA class II. In this respect, it has been described by the group of Suzanne Ostrand-Rosenberg that CIITA gene transfection in tumor cells not only increases HLA class II expression, but also boosts processing via co-expression of DM and Ii [68]. This modality could therefore also be useful for DM-deficient leukemic blasts, since CIITA-induced DM expression might enhance loading of autophagy-dependent LAAs onto HLA class II molecules in the endosomal/lysosomal pathway.

Suppression of protein kinase C (PKC) was demonstrated to affect DO expression in activated

B cells [74], potentially leading to an increased exchange of CLIP for antigenic peptides. Thus, the use of PKC inhibitors might serve as an alternative strategy to improve MIIC-restricted LAA loading in myeloid leukemic blasts. These strategies can only be effective however, in situations where LAAs are processed and transported via autophagic mechanisms into the lysosomal compartments (*figure 1B-C*). The specific induction of autophagy using different periods of serum starvation was shown to result in the presentation of endogenous instead of exogenous antigens [38]. This approach may be extended to myeloid leukemic blasts to ensure that LAA processing occurs in the endosomal/lysosomal pathway. A second LAA processing and loading mechanism does not depend on autophagy and DM, but probably involves the proteasome and TAP (*figure 1D*). Consistent with the studies by Ostrand-Rosenberg's group using solid tumor cells [75, 76], we found that Ii silencing by specific siRNAs is also a potent strategy to enhance the immunogenicity of myeloid leukemic blasts [67]. In blasts that are able to express HLA class II molecules in the absence of Ii, it is likely that LAA loading takes place in the ER instead of the MIICs, since newly formed heterodimers require peptide binding to remain stable [33]. In this situation, suboptimal proteasome or TAP expression may prevent the sufficient supply of LAAs into the ER for loading onto HLA class II molecules. To recover the function of these components, such blasts could be treated with histone deacetylase inhibitors (HDACi), like trichostatin A, which have recently been reported to be a powerful tool to increase the expression of proteasomal and TAP subunits in tumor cells [77, 78].

Concluding remarks and future perspective

In patients with AML, the onset and progression of the disease may be explained by the potential of myeloid leukemic blasts to evade immune recognition before and after induction treatment. Although allo-SCT and DLI are potent T-cell based strategies, impairments in LAA presentation on these blasts might prevent the induction of an efficient anti-leukemic immune response in vivo. The limited studies performed demonstrated that HLA class I abnormalities are rarely found, in contrast to the situation in most solid tumors. Since AML is one of the few tumor types in which HLA class II molecules can be presented on tumor cells, defects in HLA class II-restricted LAA presentation might be involved in the capacity of myeloid leukemic blasts to escape from $CD4^+$ T cell recognition. Analyzing these defects at time of diagnosis and upon relapse of the disease could give insights in the progression of the disease and the potential failure of currently used immunotherapeutic strategies. Furthermore, knowledge about these defects is crucial for the design of an optimal AML whole-cell vaccine as immunotherapeutic strategy. This type of vaccine has the advantage that a full range of LAAs can be presented at the plasma membrane. When modulatory strategies can be applied that target specific defects in HLA class II-restricted LAA presentation, AML whole-cell vaccines might be developed that present LAAs on both HLA class I and II molecules, potentially leading to long-lasting anti-leukemic immunity in AML patients, as schematically represented in *figure 2B*. These defects could involve the expression of HLA class II and co-stimulatory molecules, but also LAA processing and loading, which all interfere with efficient HLA class II-restricted presentation of immunogenic LAAs. Immunophenotyping of blasts from patients for the expression of co-stimulatory molecules,

HLA-DR and CLIP may give indications about aberrancies in these processes. With both Ii and CLIP as possible modulatory targets for designing optimal AML whole-cell vaccines, it must be kept in mind that the pathways used for LAA processing and loading need to be characterized. We recently found in myeloid leukemic blasts that besides the endosomal/lysosomal pathway, also the cytoplasm (the proteasome) and the ER (TAP) may serve as sites for HLA class II-restricted LAA processing and loading, respectively. By transiently silencing Ii in $CLIP^+$ leukemic blasts using siRNA duplexes, insight can be gained which LAA processing pathway is active. When HLA class II expression at the plasma membrane is affected by Ii silencing, autophagy-dependent LAA or exogenous antigen processing is likely to be dominant in these blasts, as both rely on the endosomal/lysosomal pathway and the effective delivery of newly synthesized HLA-DR molecules into this pathway by means of Ii. If Ii silencing does not affect HLA-DR plasma membrane expression, the proteasome and TAP are probably involved in supplying LAAs into the ER, which are subsequently loaded onto newly synthesized HLA-DR molecules. In conclusion, a better understanding of the LAA processing pathways is needed to develop modulatory strategies that improve LAA presentation on AML whole-cell vaccines. As AML is a heterogeneous disease, future goals towards the design of optimal autologous whole-cell vaccines may include the development of an efficient monitoring system by which HLA class I and II-resticted LAA processing and presentation pathways can be characterized to choose the best-suited tailor-made strategy to improve LAA presentation in each individual patient.

References

1. San Miguel JF, Vidriales MB, Lopez-Berges C, et al. Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. Blood 2001; 6: 1746-1751.
2. Kern W, Voskova D, Schoch C, et al. Determination of relapse risk based on assessment of minimal residual disease during complete remission by multiparameter flow cytometry in unselected patients with acute myeloid leukemia. Blood 2004; 10: 3078-3085.
3. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002; 5562: 2097-2100.
4. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood 2007; 1: 433-440.
5. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood 2008; 12: 4371-4383.
6. Schmid C, Labopin M, Nagler A, et al. Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT Acute Leukemia Working Party. J.Clin.Oncol. 2007; 31: 4938-4945.
7. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995; 5: 2041-2050.
8. Falkenburg JH, Marijt WA, Heemskerk MH, Willemze R. Minor histocompatibility antigens as targets of graft-versus-leukemia reactions. Curr.Opin.Hematol. 2002; 6: 497-502.

9. Greiner J, Dohner H, Schmitt M. Cancer vaccines for patients with acute myeloid leukemia--definition of leukemia-associated antigens and current clinical protocols targeting these antigens. Haematologica 2006; 12: 1653-1661.
10. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat.Immunol. 2002; 11: 991-998.
11. Chamuleau ME, van de Loosdrecht AA, Hess CJ, et al. High INDO (indoleamine 2,3-dioxygenase) mRNA level in blasts of acute myeloid leukemic patients predicts poor clinical outcome. Haematologica 2008; 12: 1894-1898.
12. Chamuleau ME, Ossenkoppele GJ, van RA, et al. High TRAIL-R3 expression on leukemic blasts is associated with poor outcome and induces apoptosis-resistance which can be overcome by targeting TRAIL-R2. Leuk.Res. 2011.
13. Whiteway A, Corbett T, Anderson R, Macdonald I, Prentice HG. Expression of co-stimulatory molecules on acute myeloid leukaemia blasts may effect duration of first remission. Br.J.Haematol. 2003; 3: 442-451.
14. Cheuk AT, Guinn BA. Immunotherapy of acute myeloid leukaemia: development of a whole cell vaccine. Front Biosci. 2008; 2022-2029.
15. Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Leukemia-derived dendritic cells: towards clinical vaccination protocols in acute myeloid leukemia. Haematologica 2006; 3: 348-355.
16. Li L, Giannopoulos K, Reinhardt P, et al. Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts. Int.J.Oncol. 2006; 4: 855-861.
17. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. Nat.Immunol. 2001; 4: 293-299.
18. Knutson KL, Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol.Immunother. 2005; 8: 721-728.
19. Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. Immunol. Rev. 2008; 129-144.
20. Wieder T, Braumuller H, Kneilling M, Pichler B, Rocken M. T cell-mediated help against tumors. Cell Cycle 2008; 19: 2974-2977.
21. Driscoll J, Goldberg AL. The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. J.Biol.Chem. 1990; 9: 4789-4792.
22. Neefjes JJ, Momburg F, Hammerling GJ. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. Science 1993; 5122: 769-771.
23. Kanaseki T, Blanchard N, Hammer GE, Gonzalez F, Shastri N. ERAAP synergizes with MHC class I molecules to make the final cut in the antigenic peptide precursors in the endoplasmic reticulum. Immunity. 2006; 5: 795-806.
24. Cresswell P, Bangia N, Dick T, Diedrich G. The nature of the MHC class I peptide loading complex. Immunol.Rev. 1999; 21-28.
25. Huang AY, Bruce AT, Pardoll DM, Levitsky HI. In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. Immunity. 1996; 4: 349-355.
26. Burgdorf S, Scholz C, Kautz A, Tampe R, Kurts C. Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat.Immunol. 2008; 5: 558-566.

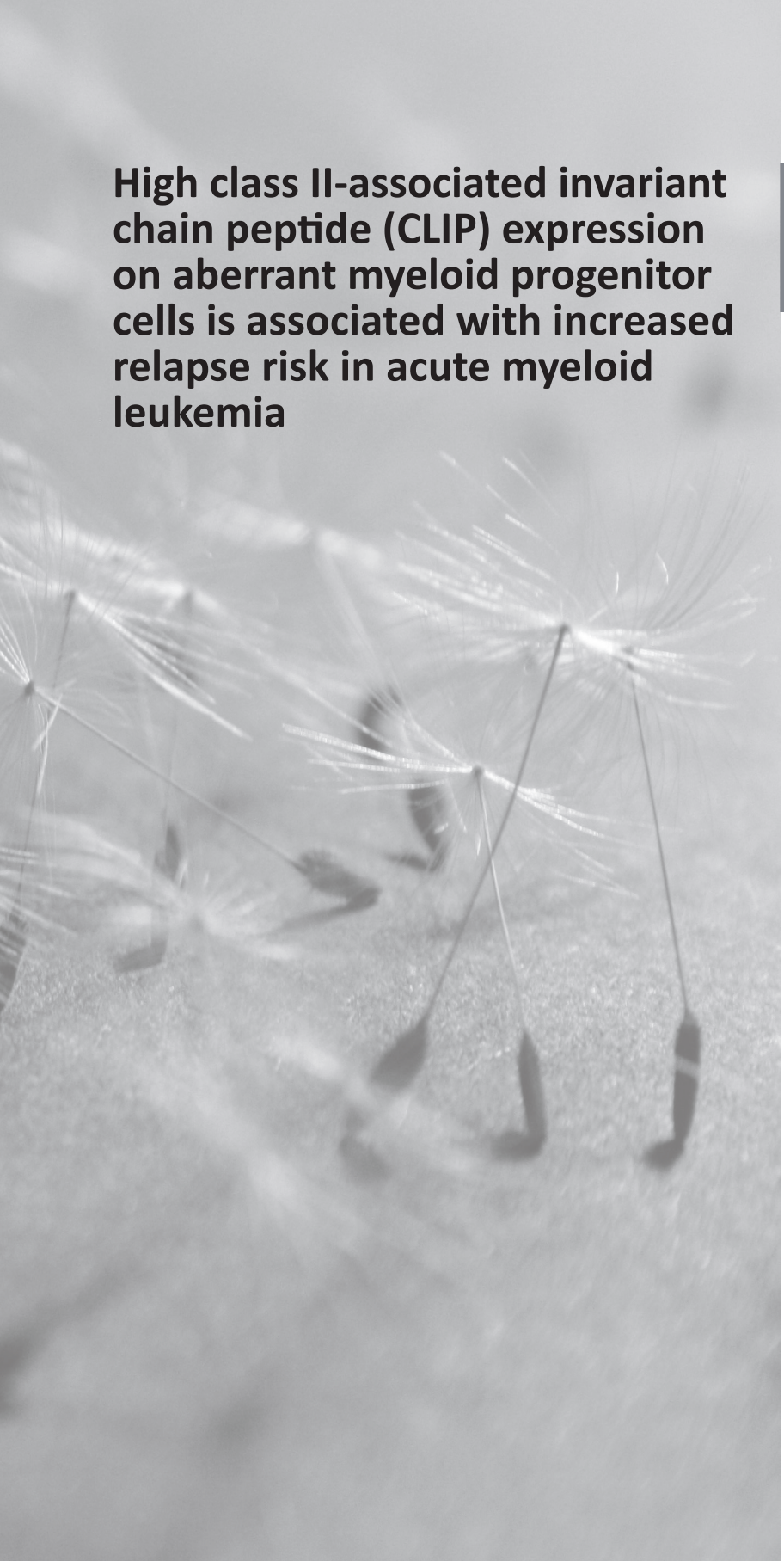
27. Guermonprez P, Saveanu L, Kleijmeer M, et al. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 2003; 6956: 397-402.
28. Bougneres L, Helft J, Tiwari S, et al. A role for lipid bodies in the cross-presentation of phagocytosed antigens by MHC class I in dendritic cells. Immunity. 2009; 2: 232-244.
29. Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity. 2004; 2: 155-165.
30. Di Pucchio T, Chatterjee B, Smed-Sorensen A, et al. Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. Nat.Immunol. 2008; 5: 551-557.
31. Wolf PR, Ploegh HL. How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. Annu.Rev.Cell Dev.Biol. 1995; 267-306.
32. Pieters J, Bakke O, Dobberstein B. The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. J.Cell Sci. 1993; 831-846.
33. Anderson MS, Miller J. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. Proc.Natl.Acad.Sci.U.S.A 1992; 6: 2282-2286.
34. Romagnoli P, Germain RN. The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. J.Exp. Med. 1994; 3: 1107-1113.
35. Sloan VS, Cameron P, Porter G, et al. Mediation by HLA-DM of dissociation of peptides from HLA-DR. Nature 1995; 6534: 802-806.
36. van Ham M, van Lith M, Lillemeier B, et al. Modulation of the major histocompatibility complex class II-associated peptide repertoire by human histocompatibility leukocyte antigen (HLA)-DO. J.Exp.Med. 2000; 7: 1127-1136.
37. Dani A, Chaudhry A, Mukherjee P, et al. The pathway for MHCII-mediated presentation of endogenous proteins involves peptide transport to the endo-lysosomal compartment. J.Cell Sci. 2004; Pt 18: 4219-4230.
38. Dengjel J, Schoor O, Fischer R, et al. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc.Natl.Acad.Sci.U.S.A 2005; 22: 7922-7927.
39. Paludan C, Schmid D, Landthaler M, et al. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 2005; 5709: 593-596.
40. Dorfel D, Appel S, Grunebach F, et al. Processing and presentation of HLA class I and II epitopes by dendritic cells after transfection with in vitro-transcribed MUC1 RNA. Blood 2005; 8: 3199-3205.
41. Nimmerjahn F, Milosevic S, Behrends U, et al. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur.J.Immunol. 2003; 5: 1250-1259.
42. Zhou D, Li P, Lin Y, et al. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity. 2005; 5: 571-581.
43. Crotzer VL, Blum JS. Cytosol to lysosome transport of intracellular antigens during immune surveillance. Traffic. 2008; 1: 10-16.
44. Lich JD, Elliott JF, Blum JS. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. J.Exp.Med. 2000; 9: 1513-1524.
45. Tewari MK, Sinnathamby G, Rajagopal D, Eisenlohr LC. A cytosolic pathway for MHC class II-restricted antigen processing that is proteasome and TAP dependent. Nat.Immunol. 2005; 3: 287-294.

46. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature 1990; 6276: 615-618.
47. Garrido F, Ruiz-Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol.Today 1997; 2: 89-95.
48. Garcia-Lora A, Algarra I, Garrido F. MHC class I antigens, immune surveillance, and tumor immune escape. J.Cell Physiol 2003; 3: 346-355.
49. Serrano A, Tanzarella S, Lionello I, et al. Rexpression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2'-deoxycytidine treatment. Int.J.Cancer 2001; 2: 243-251.
50. Blanchet O, Bourge JF, Zinszner H, et al. DNA binding of regulatory factors interacting with MHC-class-I gene enhancer correlates with MHC-class-I transcriptional level in class-I-defective cell lines. Int.J.Cancer Suppl 1991; 138-145.
51. Chang CC, Campoli M, Restifo NP, Wang X, Ferrone S. Immune selection of hot-spot beta 2-microglobulin gene mutations, HLA-A2 allospecificity loss, and antigen-processing machinery component down-regulation in melanoma cells derived from recurrent metastases following immunotherapy. J.Immunol. 2005; 3: 1462-1471.
52. Seliger B, Maeurer MJ, Ferrone S. TAP off--tumors on. Immunol.Today 1997; 6: 292-299.
53. Jordanova ES, Philippo K, Giphart MJ, Schuuring E, Kluin PM. Mutations in the HLA class II genes leading to loss of expression of HLA-DR and HLA-DQ in diffuse large B-cell lymphoma. Immunogenetics 2003; 4: 203-209.
54. van der Stoep N, Biesta P, Quinten E, van den Elsen PJ. Lack of IFN-gamma-mediated induction of the class II transactivator (CIITA) through promoter methylation is predominantly found in developmental tumor cell lines. Int.J.Cancer 2002; 4: 501-507.
55. Campoli M, Ferrone S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. Oncogene 2008; 45: 5869-5885.
56. Goldstein OG, Hajiaghamohseni LM, Amria S, et al. Gamma-IFN-inducible-lysosomal thiol reductase modulates acidic proteases and HLA class II antigen processing in melanoma. Cancer Immunol.Immunother. 2008; 10: 1461-1470.
57. Lemaire G, Guittet O, Vesin MF, Lepoivre M, Cottet MH. Glutathione depletion reveals impairment of antigen processing and inhibition of cathepsin activity by nitric oxide in antigen-presenting cells. Mol.Immunol. 2009; 6: 1100-1108.
58. Savoia P, D'Alfonso S, Peruccio D, et al. Loss of surface HLA class I molecules in leukemic myeloblasts is correlated with an increased leukocyte concentration at onset. Haematologica 1992; 2: 127-129.
59. Brouwer RE, van der HP, Schreuder GM, et al. Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but functionally relevant, and can be restored by interferon. Hum.Immunol. 2002; 3: 200-210.
60. Wetzler M, Baer MR, Stewart SJ, et al. HLA class I antigen cell surface expression is preserved on acute myeloid leukemia blasts at diagnosis and at relapse. Leukemia 2001; 1: 128-133.
61. Masuda K, Hiraki A, Fujii N, et al. Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts. Cancer Sci. 2007; 1: 102-108.
62. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. N.Engl.J.Med. 2009; 5: 478-488.
63. Majumder D, Bandyopadhyay D, Chandra S, et al. Analysis of HLA class Ia transcripts in human leukaemias. Immunogenetics 2005; 8: 579-589.

64. Demanet C, Mulder A, Deneys V, et al. Down-regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic cells: an escape mechanism from CTL and NK attack? Blood 2004; 8: 3122-3130.
65. Wetzler M, McElwain BK, Stewart CC, et al. HLA-DR antigen-negative acute myeloid leukemia. Leukemia 2003; 4: 707-715.
66. Chamuleau ME, Souwer Y, van Ham SM, et al. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res. 2004; 16: 5546-5550.
67. van Luijn MM, Chamuleau ME, Thompson JA, et al. Class II-associated invariant chain peptide down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased CD4+ T-cell responses. Haematologica 2010; 3: 485-493.
68. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc.Natl.Acad.Sci.U.S.A 1997; 13: 6886-6891.
69. van Luijn MM, Chamuleau ME, Ressing ME, et al. Alternative Ii-independent antigen-processing pathway in leukemic blasts involves TAP-dependent peptide loading of HLA class II complexes. Cancer Immunol.Immunother. 2010; 12: 1825-1838.
70. de Gruijl TD, van den Eertwegh AJ, Pinedo HM, Scheper RJ. Whole-cell cancer vaccination: from autologous to allogeneic tumor- and dendritic cell-based vaccines. Cancer Immunol. Immunother. 2008; 10: 1569-1577.
71. Westers TM, Stam AG, Scheper RJ, et al. Rapid generation of antigen-presenting cells from leukaemic blasts in acute myeloid leukaemia. Cancer Immunol.Immunother. 2003; 1: 17-27.
72. Cheuk AT, Chan L, Czepulkowski B, et al. Development of a whole cell vaccine for acute myeloid leukaemia. Cancer Immunol.Immunother. 2006; 1: 68-75.
73. Muhlethaler-Mottet A, Otten LA, Steimle V, Mach B. Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. EMBO J. 1997; 10: 2851-2860.
74. Roucard C, Thomas C, Pasquier MA, et al. In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. J.Immunol. 2001; 12: 6849-6858.
75. Thompson JA, Dissanayake SK, Ksander BR, et al. Tumor cells transduced with the MHC class II Transactivator and CD80 activate tumor-specific CD4+ T cells whether or not they are silenced for invariant chain. Cancer Res. 2006; 2: 1147-1154.
76. Thompson JA, Srivastava MK, Bosch JJ, et al. The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4(+) T lymphocytes. Cancer Immunol.Immunother. 2008; 3: 389-398.
77. Setiadi AF, Omilusik K, David MD, et al. Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. Cancer Res. 2008; 23: 9601-9607.
78. Khan AN, Gregorie CJ, Tomasi TB. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. Cancer Immunol. Immunother. 2008; 5: 647-654.

High class II-associated invariant chain peptide (CLIP) expression on aberrant myeloid progenitor cells is associated with increased relapse risk in acute myeloid leukemia

8



W van den Ancker
MM van Luijn
MED Chamuleau
A Kelder
N Feller
M Terwijn
A Zevenbergen
GJ Schuurhuis
SM van Ham
TM Westers
GJ Ossenkoppele
AA van de Loosdrecht

submitted

Abstract

The presence of minimal residual disease (MRD) determined by flow cytometry is highly predictive for relapse of acute myeloid leukemia (AML). Impaired immunogenicity of low frequency MRD cells might play an important role in relapse of leukemia. Class II-associated invariant chain peptide (CLIP) plays a role in the immune escape of leukemic cells. Here, we evaluated CLIP and HLA-DR expression on leukemia-associated phenotype (LAP)-positive cells during follow-up and before relapse by flow cytometry. We found that CLIP expression on residual LAP^+ cells significantly correlated with a shortened relapsefree survival, even in those cases with a percentage of LAP^+ cells below 0.14 which is generally considered as MRD^{low}. Consequently, analysis of CLIP expression on LAP^+ cells during follow-up could be of additional value in the evaluation of MRD to predict relapse of AML.

Introduction

In acute myeloid leukemia (AML) the arrest in differentiation due to acquired mutations in hematopoietic (stem) cells results in excessive proliferation and accumulation of immature myeloid cells. Although high-dose chemotherapy effectively reduces the tumor burden in most AML cases, some leukemic cells survive treatment and cause a relapse. Presence of aberrant immunophenotypic markers on malignant cells, the so-called leukemia-associated phenotype (LAP), provides a powerful tool to monitor minimal residual disease (MRD) [1]. By flow cytometric identification of LAP^+ cells, information is gained about MRD frequency in the bone marrow (BM), which serves as a highly reliable predictor of relapse free survival (RFS) and overall survival (OS) after chemotherapy. One possible mechanism for outgrowth of residual leukemic cells is their escape from immune surveillance due to a non-immunogenic phenotype [2]. During immune surveillance, presence of HLA class II on antigen-presenting cells (APC) is critical for optimal anti-tumor immunity by inducing a T helper cell response [3,4]. To enable loading and presentation of antigenic peptides, HLA class II molecules should release class II-associated invariant chain peptide (CLIP) from their antigen-binding groove [5,6]. Besides professional APC, also tumor cells can serve as potent APC by expressing costimulatory and HLA molecules [7,8]. Previously, we showed that CLIP expression on primary $HLA\text{-}DR^+$ leukemic cells interferes with effector T helper cell activation, indicating that it may serve as an immune escape mechanism in AML [9]. Additionally, high CLIP expression (>35%) on leukemic cells at diagnosis is associated with a shortened disease-free survival [7,10]. Here, we investigate the clinical impact of CLIP expression on LAP^+ cells during follow-up in AML.

Materials and methods

Patient samples

For our study, we selected blood and BM samples from 50/412 AML patients who were admitted to our hospital between 2001 and 2010. From the total of 412 patients, ~60%

were eligible for both induction and consolidation therapy and reached complete remission. Since we were interested in the role of CLIP on leukemic cells in the context of HLA class II, we excluded acute promyelocytic leukemia (APL) cases, which are HLA-DR^{low} or HLA-DR-negative. Also AML without a leukemia-associated phenotype (LAP) had to be excluded because follow-up analysis using flow cytometry is not possible. In approximately one third of the remaining patients, CLIP could be analyzed and evaluated during follow-up, resulting in the final inclusion of 50 AML cases. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Patients received chemotherapy according to the Dutch-Belgian cooperative trial group for Hematology Oncology (HOVON)-protocols (www.hovon.nl).

Immunophenotypical analysis

The presence of a LAP was determined as previously described [1]. In short, a panel of different monoclonal antibodies (moAb) was used to define a LAP on leukemic cells at diagnosis (summarized in *Supplementary table 1*.). BM was obtained after each treatment cycle or stem cell transplantation (SCT) and thereafter on a regular base. The last available follow-up sample before relapse was used for MRD and CLIP analysis. During follow-up, at least 1x10^6 BM-derived WBC were labeled after red blood cell lysis with the moAb that defined the LAP, together with a primitive marker (i.e. CD34 and/or CD117), CD45 and anti-CLIP (cerCLIP.1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-HLA-DR moAb [1, 7]. First, the total WBC compartment was characterized by CD45 expression and FSC. Next, aberrancies were analyzed at diagnosis on primitive marker-positive cells with a low to intermediate SSC. In general, a single LAP marker was sufficient to analyze CLIP and HLA-DR expression on LAP$^+$ cells; all samples were measured using a FACS Calibur flow cytometer (four-color based; Becton Dickenson). If an extra LAP-defining marker was necessary for CLIP and HLA-DR analysis on LAP$^+$ cells, data files from different immunofluorescent stainings were merged based on the presence of common markers using Infinicyt software (Cytognos, Salamanca, Spain). In this case at least two markers (e.g. CD45 and CD34) and FCS/SSC were used as common markers. For detection of LAP$^+$ cells a cut-off of 0.01% was used (i.e. 1x10^2 positive cells out of a total of 1x10^6 leukocytes); presence of 0.14% or more LAP$^+$ cells (defined as LAP$^+$ cells but corrected for percentage LAP$^+$ cells at diagnosis)[1] was considered as MRD^{high}, while presence of 0.01-0.14% LAP$^+$ cells was regarded as MRD^{low}. The frequencies of CLIP-expressing cells within the LAP$^+$ population were determined as indicated in Supplementary *figure 1*. A 35% cut-off was used to discriminate between patients with CLIP^{high} and CLIP^{low} expression, as previously described [7]. Relative CLIP expression was determined by calculating the ratio between CLIP and HLA-DR expression based on both the percentage and mean fluorescence intensity [9].

Statistical analysis

Relapse free survival (RFS) was defined as the interval between the date of complete remission (CR) and relapse (respectively <5% and ≥5% leukemic cells in BM, as determined by cytomorphology). For survival data analysis, Kaplan Meier curves were compared by means of log-rank test; Cox univariate linear regression analysis was performed to evaluate the predictive value of LAP and CLIP percentages, $p<0.05$ was considered significant.

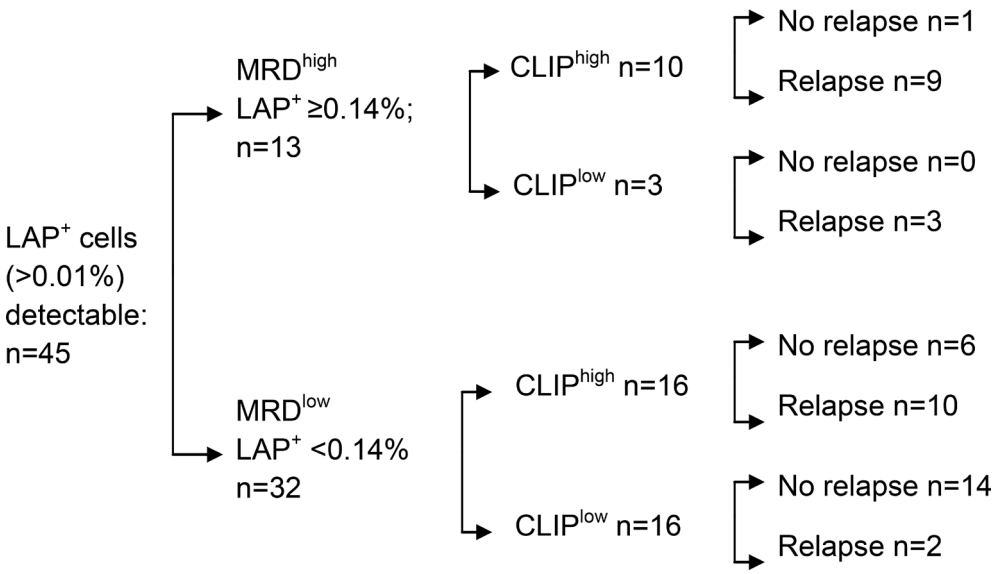


Figure 1. Schematic overview of the analyzed AML patient group with respect to leukemia associated phenotype (LAP), minimal residual disease (MRD), class II associated invariant chain peptide (CLIP) expression and occurrence of relapse.

Results and discussion

Previously, we reported that high MRD frequency is associated with poor prognosis and early relapse in AML [1]. This could be confirmed in our current cohort of 50 AML patients. Presence of MRD^{high} (>0.14% LAP^+ cells) was largely discriminative for a shortened RFS; all but one of the 13 MRD^{high} patients experienced a relapse compared to 14/37 patients considered MRD^{low} or MRD-negative (<0.14% LAP^+ cells; p<0.001; data not shown) [1]. The median frequency of LAP^+ cells in the 26 patients who showed a relapse was 0.05% (range 0.00-4.53%) as compared to 0.03% (range 0.00-0.09%) in the 24 patients who remained in continuous remission (p=0.03, n=50, data not shown). There was no difference in distribution of the LAP in the MRD^{low} and MRD^{high} group.

Subsequently, we assessed the role of CLIP expression on LAP^+ cells regarding relapse occurrence during follow-up. In 5 patients, LAP^+ cells as defined at diagnosis were not detected during follow-up. Consequently, we were able to evaluate CLIP on LAP^+ cells in 45 patients; 24 experienced a relapse, while 21 remained in continuous CR (summarized in *figure 1*). Both the percentage of LAP^+ cells and the percentage of $CLIP^+$ cells within the LAP^+ population significantly predicted the occurrence of a relapse (p=0.025 and p=0.038, respectively; Cox univariate analysis, data not shown). Notably, high CLIP expression on LAP^+ cells was negatively associated with RFS (p=0.001 [log rank]; *figure 2A*). No significant differences were found in leukocyte counts, percentages of blasts in the bone marrow, cytogenetic risk scores at diagnosis and inclusion of an allogeneic stem cell transplantation in the treatment regimens between $CLIP^{high}$ and $CLIP^{low}$ patients. Recently, we showed that the presence of CLIP on leukemic cells from $HLA\text{-}DR^+$ AML patients inhibits activation

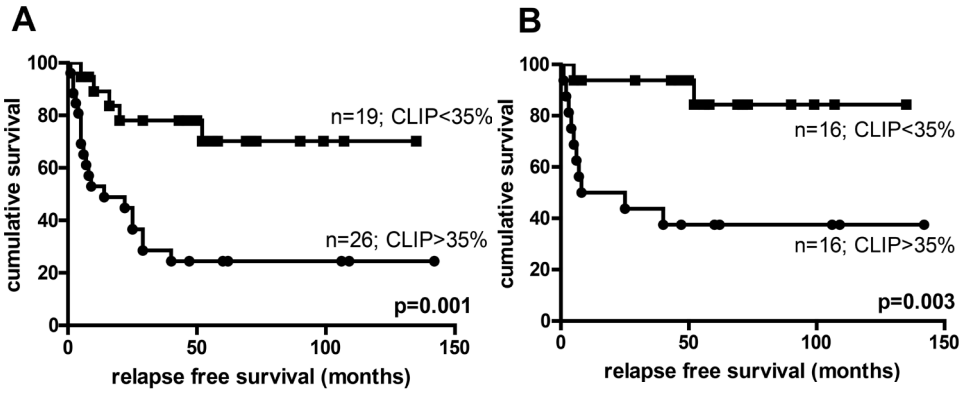


Figure 2. The association of class II-associated invariant chain peptide (CLIP) on leukemia-associated phenotype (LAP)-positive cells with relapse-free survival (RFS). CLIP expression on LAP$^+$ cells was analyzed using a previously defined cut-off of 35%.7 Its impact on RFS was evaluated in all patients with LAP$^+$ cells, i.e. minimal residual disease-high (MRD^{high}) and MRD^{low} (A) and in patients with 0.01-0.14% LAP$^+$ cells and thus considered MRD^{low} (B).

of autologous leukemia-reactive CD4$^+$ T cells [9], indicating that HLA-DR-mediated CLIP presentation might be an immune escape mechanism of leukemic cells. Moreover, in concordance with CLIP as a sole marker, the CLIP/DR ratio on leukemic cells at diagnosis was shown to be of importance in predicting RFS in AML [7]. For all cases in the present study, more than 90% of LAP$^+$ cells were HLA-DR positive at diagnosis, however, the mean fluorescence did vary (MFI range 35-470). In 16 patients we analyzed both CLIP and HLA-DR expression on LAP$^+$ cells during follow-up. A shortened RFS was found for patients with a high CLIP/DR ratio (CLIP/DR ratio cut off: 0.05; p=0.03 [log rank], data not shown).

In the group regarded as MRD^{low} (0.01%-0.14% LAP$^+$ cells), a minority of patients experienced a relapse (12 out of 32, *figure 1*). Interestingly, the presence of CLIP on LAP$^+$ cells predicted a significantly shortened RFS in these MRD^{low} cases (p=0.003 [log rank]; *figure 2B*). Previously, it has been reported that flow cytometric detection of MRD is important for predicting a relapse in AML [1]. However, for a few patients, the presence of MRD is not predictive for a relapse although a relapse is experienced. In these cases, analysis of CLIP on LAP$^+$ cells during follow-up may help to better define MRD and to identify patients who will likely develop a relapse. Consequently, current treatment modalities may be utilized earlier in these high risk cases. In addition, CLIP-positive cases might benefit from developments in immunomodulatory treatment that can enhance the immunogenicity of MRD cells. For instance, modulation of CLIP or other components of the HLA class II antigen presentation pathway could offer new possibilities for immunotherapy of AML patients.

Remarkably, no correlation was found between CLIP expression on LAP$^+$ cells during follow-up and CLIP expression in AML at diagnosis; from 11 patients with CLIP^{high}LAP$^+$ cells during follow-up, 7 patients showed low CLIP expression (<35%) at diagnosis, while 4 patients had high CLIP expression (>35%; data not shown). In cases with CLIP^{low} expression at diagnosis, but CLIP^{high} expression at relapse, it remains to be investigated whether the small group of CLIP^{high} leukemic cells preferentially survived chemotherapy and escaped immune surveillance or residual leukemic cells acquired CLIP expression during follow-up. In either case, the high CLIP expression could reflect a state of leukemic cells in which endogenous antigen

presentation is hampered due to a deficiency in antigen processing machinery. For example, the function of the transporter associated with antigen processing (TAP) could be impaired, which is a common immune escape mechanism of solid tumors [11,12] and might also be involved in CLIP$^+$ leukemic cells [13]. As a result, TAP-mediated translocation of potentially leukemia-associated endogenous antigens into the ER or endosomal compartments may be downregulated, resulting in less antigen loading of HLA molecules. This may not only impair HLA class I-, but also HLA class II-mediated presentation of endogenous antigens, as previously reported for solid tumor cells [14,15] Moreover, there are indications that CLIP is also involved in HLA class I antigen presentation, since it can bind to various HLA class I allotypes (e.g. HLA-A2) [16]. The role of CLIP presented by HLA class I molecules in tumor immune escape has to be further elucidated.

In conclusion, the data presented here demonstrate the clinical impact of CLIP on LAP$^+$ cells during follow-up in AML. Expression of CLIP on LAP$^+$ cells resulted in poor survival, not only in MRD-positive AML patients, but also in patients with low MRD (<0.14% LAP$^+$ cells). This may point to CLIP expression as a mechanism for MRD cells to escape immune surveillance and consequently a promising target for future research aiming at prevention of relapse.

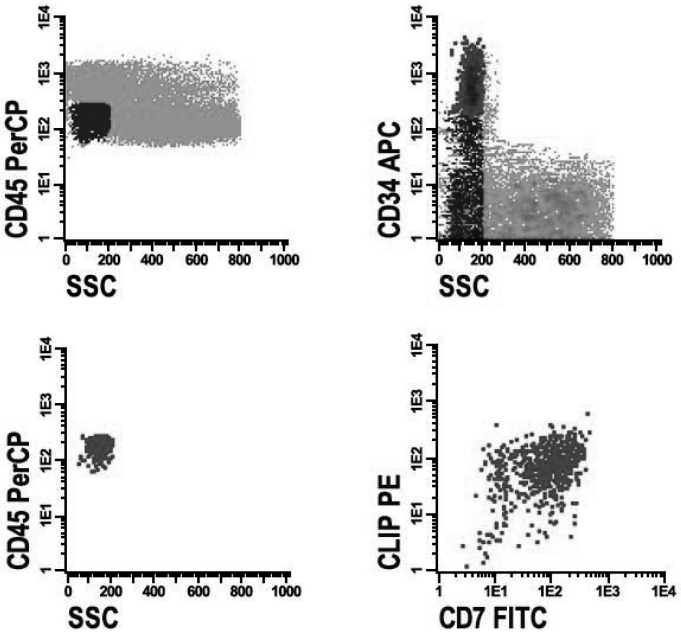
Reference List

1. Feller N, van der Pol MA, van SA, Weijers GW, Westra AH, Evertse BW, Ossenkoppele GJ, Schuurhuis GJ (2004) MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. Leukemia 18(8):1380-1390
2. Zitvogel L, Tesniere A, Kroemer G (2006) Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 6(10):715-727
3. Ostrand-Rosenberg S (2005) CD4$^+$ T lymphocytes: a critical component of antitumor immunity. Cancer Invest 23(5):413-419
4. Kremer AN, van der Meijden ED, Honders MW, Goeman JJ, Wiertz EJ, Falkenburg JH, Griffioen M (2012) Endogenous HLA-class-II epitopes that are immunogenic in vivo show distinct behavior towards HLA-DM and its natural inhibitor HLA-DO. Blood 120(16):3246-55
5. Rocha N, Neefjes J (2008) MHC class II molecules on the move for successful antigen presentation. EMBO J 27(1):1-5
6. Romagnoli P, Germain RN (1994) The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. J Exp Med 180(3):1107-1113
7. Chamuleau ME, Souwer Y, Van Ham SM, Zevenbergen A, Westers TM, Berkhof J, Meijer CJ, van de Loosdrecht AA, Ossenkoppele GJ (2004) Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res 64(16):5546-5550
8. Brouwer RE, Zwinderman KH, Kluin-Nelemans HC, van Luxemburg-Heijs SA, Willemze R, Falkenburg JH (2000) Expression and induction of costimulatory and adhesion molecules on acute myeloid leukemic cells: implications for adoptive immunotherapy. Exp Hematol 28(2):161-168
9. van Luijn MM, van den Ancker W, Chamuleau ME, Zevenbergen A, Westers TM, Ossenkoppele G, Van Ham SM, van de Loosdrecht AA (2011) Absence of class II-associated invariant chain peptide on leukemic blasts of patients promotes activation of autologous leukemia-reactive CD4$^+$ T cells. Cancer Res 71(7):2507-17

10. van Luijn MM, Chamuleau ME, Thompson JA, Ostrand-Rosenberg S, Westers TM, Souwer Y, Ossenkoppele GJ, Van Ham SM, van de Loosdrecht AA (2010) Class II-associated invariant chain peptide down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased $CD4^+$ T-cell responses. Haematologica 95(3):485-493
11. Seliger B, Maeurer MJ, Ferrone S (1997) TAP off--tumors on. Immunol Today 18(6):292-299
12. Johnsen AK, Templeton DJ, Sy M, Harding CV (1999) Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis. J Immunol 163(8):4224-4231
13. van Luijn MM, van den Ancker W, Chamuleau ME, Ossenkoppele GJ, Van Ham SM, van de Loosdrecht AA (2010) Impaired antigen presentation in neoplasia: basic mechanisms and implications for acute myeloid leukemia. Immunotherapy 2(1):85-97
14. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S (1997) Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc Natl Acad Sci U S A 94(13):6886-6891
15. Thompson JA, Srivastava MK, Bosch JJ, Clements VK, Ksander BR, Ostrand-Rosenberg S (2008) The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4(+) T lymphocytes. Cancer Immunol Immunother 57(3):389-398
16. van Luijn MM, van de Loosdrecht AA, Lampen MH, van Veelen PA, Zevenbergen A, Kester MG, de Ru AH, Ossenkoppele GJ, van HT, van Ham SM (2012) Promiscuous binding of invariant chain-derived CLIP peptide to distinct HLA-I molecules revealed in leukemic cells. PLoS One 7(4):e34649

Supplementary Figure legends

Supplementary Figure 1: Gating strategy for flow cytometric analysis of CLIP expression on LAP$^+$ cells
First CD45dim cells (i.e. blasts) were gated (A) and selected for expression of CD34 as primitive marker in this example (B); when CD34 expression was absent, CD117 was used as primitive marker. Subsequently, cells were backgated from plot A and B to ensure that precursors cells formed a distinct population in CD45 expression, SSC (see panel C) and in FSC (not shown). In dotplot D, CLIP expression is depicted against expression of the aberrant marker as defined at diagnosis. In this example, 88% of LAP$^+$ cells (aberrant CD7 expression) were CLIP-positive.



Supplementary Table 1. Patient characteristics

UPN	LAP marker	WHO classification
1	CD7$^+$	acute erythroid leukemia
2	CD7$^+$	acute erythroid leukemia
3	CD7$^+$	acute erythroid leukemia
4	TdT$^+$	acute monoblastic and monocytic leukemia
5	CD56$^+$	acute monoblastic and monocytic leukemia
6	CD34$^-$	acute monoblastic and monocytic leukemia
7	CD7$^+$	acute monoblastic and monocytic leukemia
8	CD7$^+$	acute monoblastic and monocytic leukemia
9	CD7$^+$	acute myeloid leukemia without differentiation
10	CD7$^+$	acute myeloid leukemia without differentiation
11	CD7$^+$	acute myeloid leukemia with minimal differentiation
12	CD56$^+$	acute myeloid leukemia with minimal differentiation

UPN	LAP marker	WHO classification
13	$CD19^{+}$	acute myeloid leukemia with minimal differentiation
14	$CD11c^{+}$	acute myeloid leukemia with minimal differentiation
15	$CD11b^{+}$	acute myeloid leukemia not otherwise specified
16	$CD11b^{+}$	acute myeloid leukemia not otherwise specified
17	$CD19^{+}$	acute myeloid leukemia with maturation
18	$CD56^{+}$	acute myeloid leukemia with maturation
19	$CD7^{+}$	acute myeloid leukemia with maturation
20	$CD7^{+}$	acute myeloid leukemia with maturation
21	$CD7^{+}$	acute myeloid leukemia with maturation
22	$CD15^{+}$	acute myeloid leukemia with maturation
23	$CD7^{+}$	acute myeloid leukemia with maturation
24	$CD117^{+++}$	acute myeloid leukemia with maturation
25	$CD7^{+}$	acute myeloid leukemia with t(8;21)
26	$CD56^{+}$	acute myeloid leukemia with t(8;21)
27	$CD11c^{+}$	acute myelomonocytic leukemia
28	$CD56^{+}$	acute myelomonocytic leukemia
29	$CD7^{+}$	acute myelomonocytic leukemia
30	$CD7^{+}$	acute myelomonocytic leukemia
31	$CD7^{+}$	acute myelomonocytic leukemia
32	$CD34^{-}$	acute myelomonocytic leukemia
33	$CD7^{+}$	acute myelomonocytic leukemia
34	$CD15^{+}$	acute myeloid leukemia with inv(16)
35	$CD13^{-}$	acute myeloid leukemia with inv(16)
36	$CD15^{+}$	acute myeloid leukemia with inv(16)
37	TdT^{+}	acute myeloid leukemia with inv(16)
38	$CD2^{+}$	acute myeloid leukemia with inv(16)
39	$CD15^{+}$	acute myeloid leukemia with inv(16)
40	$CD15^{+}$	acute myeloid leukemia with inv(16)
41	$CD7^{+}$	AML with multilineage dysplasia
42	$CD13^{-}$	AML with multilineage dysplasia
43	$CD7^{+}$	AML with multilineage dysplasia
44	$CD33^{-}$	AML with multilineage dysplasia
45	$CD13^{-}$	AML with multilineage dysplasia
46	$CD13^{-}$	AML with multilineage dysplasia
47	$CD7^{+}$	AML with multilineage dysplasia
48	$CD13^{-}$	AML with multilineage dysplasia
49	$CD5^{+}$	AML with multilineage dysplasia
50	$CD56^{+}$	AML with multilineage dysplasia

The various leukemia-associated phenotypes (LAP) used for determination of CLIP on LAP positive cells are shown. Phenotypes considered as aberrant were: presence of CD7, CD11c or CD56; over expression of CD13; absence of CD13 or CD33 combined with presence of another myeloid marker; absence of CD34 but presence of primitive marker CD117 or diminished expression of CD45. UPN: Unique Patient Number.

Absence of class II-associated invariant chain peptide on leukemic blasts of patients promotes activation of autologous leukemia-reactive CD4$^+$ T cells

9

MM van Luijn
W van den Ancker
MED Chamuleau
A Zevenbergen
TM Westers
GJ Ossenkoppele
SM van Ham
AA van de Loosdrecht

Cancer Res. 2011 Apr 1;71(7):2507-17

Abstract

The capacity of tumor cells to escape from immune surveillance has become a substantial obstacle to successful cancer immunotherapy. Multiple tumor-related mechanisms underlie defects in HLA class I antigen presentation, but less is known about the role of impaired HLA class II antigen presentation. Our previous findings showed that presence of the class II-associated invariant chain peptide (CLIP) on HLA class II$^+$ leukemic blasts is correlated to a poor survival in acute myeloid leukemia (AML). In this study, we thoroughly examined the functional significance of CLIP expression on leukemic blasts of AML patients. CD4$^+$ T cells of patients were co-cultured with autologous CLIP$^-$ and CLIP$^+$ primary leukemic blasts and analyzed for several functional parameters by flow cytometry. Increased HLA-DR and IFN-γ expression was observed for CD4$^+$ T cells stimulated with CLIP$^-$ leukemic blasts, in contrast to CLIP$^+$ leukemic blasts, indicating activation and polarization toward T helper 1 cells. Also, CLIP$^-$ leukemic blasts induced more outgrowth of effector memory CD4$^+$ T cells with HLA-DR-restricted TCR Vβ repertoires as well as leukemia-specific reactivity compared to CLIP$^+$ leukemic blasts. These data provide a rationale for using immunotherapeutic strategies that can down-modulate CLIP on leukemic blasts to generate effective leukemia-specific T cell immunity in patients with AML.

Introduction

Many approaches instrumental for developing additional immunotherapeutic strategies in the treatment of cancer patients are currently investigated. These include the ex vivo generation of autologous T cells that specifically recognize and eradicate tumor cells [1], and loading of professional antigen-presenting cells (APCs), such as dendritic cells (DCs), with apoptotic tumor cells to present tumor-associated antigens (TAAs) and activate tumor-specific T cells in vivo [2]. It is of utmost importance to better understand how target tumor cells interact with the immune system of patients ('immune editing') and which tumor-associated mechanisms are present that affect immune recognition ('immune escape'; reviewed in ref. [3], potentially leading to the onset and progression of the disease).

Especially in solid tumors, but also in acute myeloid leukemia (AML), one of the main mechanisms of tumor cells to circumvent immune surveillance is T cell suppression. Certain tumor cells inhibit the effector function of T cells by interacting with negative costimulatory receptors or expression and secretion of immunosuppressive cytokines [4]. To escape from specific recognition by T cells, many solid tumors not only aberrantly express HLA class I molecules and components of antigen processing machinery (reviewed in ref. [5]), but also lack HLA class II expression. The absence of TAA presentation via HLA class II molecules prevents tumor cells from being recognized by CD4$^+$ T cells, which impairs T helper 1 (Th1) cell activation that is crucial for effective anti-tumor immunity [6;7].

In patients with HLA class II-negative tumors, tumor-specific CD4$^+$ T cells can only be activated by professional APCs that engulf, process and present exogenous TAAs derived from tumor cells. Studies to augment HLA class II expression and processing in such tumors demonstrated that tumor cells are also able to present endogenous TAAs by HLA class II molecules and mount a tumor-specific CD4$^+$ T cell response [8;9]. Moreover, the ability of these tumor cells to activate CD4$^+$ Th1 cells was strongly enhanced in the absence of the Invariant Chain (Ii; ref. [10], a chaperone protein important for HLA class II function [11].

Therefore, it is of interest to examine the impact of HLA class II presentation machinery on the immune escape potential of tumor cells that do express HLA class II molecules, such as leukemic blasts.

According to the classical HLA class II antigen presentation pathway, newly formed HLA class II molecules associate with Ii and are transported to specialized lysosomal antigen-loading compartments (MIICs; ref. [12;13]). During transport, Ii is cleaved leaving a small remnant bound to the HLA class II peptide-binding groove, the class II-associated invariant chain peptide (CLIP; ref. [14;15]). This self-peptide is then exchanged for an antigenic peptide in the MIIC compartments by the peptide editor HLA-DM [16], and the formed HLA class II/peptide complex is transferred to the plasma membrane for presentation. In the absence of HLA-DM, peptide exchange in the MIICs does not occur and CLIP is abundantly expressed at the plasma membrane [17;18].

Previously, we described that high CLIP expression on HLA class II$^+$ leukemic blasts negatively correlated to the clinical outcome of patients with AML [19;20]. In addition, down-modulation of CLIP on myeloid leukemia cell lines resulted in an increased ability to induce allogeneic CD4$^+$ T cells [20], suggesting that CLIP on leukemic blasts influences CD4$^+$ T cell recognition in AML patients. Here, we investigate the functional impact of CLIP on leukemic blasts directly derived from patients by performing co-cultures with autologous CD4$^+$ T cells. We show differences in CD4$^+$ T cell activation, Th1 skewing, effector memory differentiation, TCR Vβ repertoire and leukemia-specific reactivity, which points to a critical role for CLIP in T cell recognition of leukemic blasts in AML patients.

Materials and Methods

Patient selection

Diagnosis of AML was based on the French-American-British (FAB) criteria [21]. Cytogenetic risk groups were classified as previously reported [22]. AML patients received induction and consolidation treatment in accordance with HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) protocols (www.hovon.nl). For co-cultures with autologous CD4$^+$ T cells, we needed to select patients who were in complete remission for at least 6 months and did not receive an allogeneic stem cell transplantation. Additionally, a large number of leukemic blasts from the same patients at diagnosis had to be able to stimulate CD4$^+$ T cells, which were screened for high expression of CD40 and HLA-DR using flow cytometry. The clinical features and immunophenotypical characteristics of leukemic blasts of 8 selected patients are summarized in *table 1*.

Patient samples

We collected bone marrow samples at the time of diagnosis and peripheral blood samples during remission of the same patients after obtaining informed consent and according to the declaration of Helsinki. Mononuclear cell fractions were freshly isolated by the use of density-gradient centrifugation (Ficoll-PaquePLUS, Amersham Biosciences, Freiburg, Germany), frozen at -80°C and stored in liquid nitrogen using RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 20% heat-inactivated FBS (Greiner, Alphen a/d Rijn, The Netherlands) and 10% dimethylsulphoxide (Merck, Darmstadt, Germany). For their use in co-cultures, cells were rapidly thawed, washed twice in RPMI supplemented with 40% FBS and resuspended in the required medium for cell sorting, as described below.

Antibodies

The mouse monoclonal antibodies (mAbs) used for flow cytometry included: PE-conjugated CD25 (Dako, Glostrup, Denmark), CD27 (Beckton Dickinson (BD), San Jose, CA), CD40 (Beckman Coulter, San Diego, CA), anti-CLIP (clone cerCLIP.1; Santa Cruz Biotechnology, Santa Cruz, CA), anti-IFN-γ and anti-IL-4 (both BD); FITC-conjugated CD28 (Dako), CD45RA (Sanquin, Amsterdam, The Netherlands), CD45RO (Dako), CD4, CD14, CD86, anti-HLA-DR (clone L243) and anti-IFN-γ; APC-conjugated CD3; PerCP-conjugated CD45; and 7-amino-actinomycin D (7AAD; Via-Probe, all BD). Anti-HLA-DR blocking mAb L243 was prepared from supernatants of the HB-55 hybridoma (ATCC, Teddington, UK).

Immunofluorescence staining

Before antibody labeling, 1 x 10^5 cells were incubated with 10% human gamma-globulin (60 mg/ml; Sanquin) for 10 min. Mouse mAbs were added during 15 min at room temperature (RT). T cell receptor (TCR) Vβ repertoires were analyzed using the IOTest Beta Mark TCR Vβ Repertoire kit (Beckman Coulter), consisting of PE-, FITC- or both PE- and FITC-conjugated mAbs directed against 24 different TCR Vβ families. Before Vβ-specific staining, cells were stained with APC-conjugated CD3 and PerCP-conjugated CD4 mAbs and washed with PBS-0.1% HSA-0.05% sodium azide.

For cytokine expression, leukemic blast-activated $CD4^+$ T cells were restimulated at day 12 and 20 after activation with PMA (25 ng/ml) and ionomycin (1 μg/ml) for 6 h in culture medium [RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% human AB serum (ICN Biochemicals, Aurora, OH, USA), 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO) and 50 μM 2-ME (Gibco) at 37°C in a humidified atmosphere containing 5% CO2. To address $CD4^+$ T cell specificity, we used different autologous cells (1:1 ratio) as stimulators instead of PMA/ionomycin. After 2 h of stimulation, cells were incubated with brefeldin A (10 μg/ml), fixed with PBS-1% paraformaldehyde, permeabilized with PBS-0.1% saponin (Sigma-Aldrich) and stained with anti-cytokine mAbs (30 min at RT).

Stained cells were washed with PBS-0.1% HSA-0.05% sodium azide and evaluated by a FACSCalibur flow cytometer (BD). Flow cytometric analysis was performed with CellQuest software (BD). Myeloid blasts were defined as side scatter (SSC) low, CD45 intermediate [23]; the percentage of positive cells was determined as compared to PBS controls. Mean fluorescence index (MFI) was defined by the mean value of positive cells devided by that of the negative control. The following formula was applied to calculate relative expression: (% of positively stained cells / 100) * mean fluorescence intensity of positively stained cells.

Cell sorting of primary leukemic blasts and $CD4^+$ T cells

To select $CLIP^-$ and $CLIP^+$ primary myeloid leukemic blasts, thawed mononuclear cells from patients with *de novo* AML were resuspended in PBS-0.1% HSA, pre-incubated with 10% human gamma-globulin for 10 min and then stained with sterile-filtered PerCP-conjugated CD45 (BD) and PE-conjugated anti-CLIP (Santa Cruz Biotechnology) mAbs for 15 min on ice. Selected $HLA\text{-}DR^+$ patients revealed differential CLIP expression, in line with previous observations [19]. After washing with PBS, stained cells were analyzed and sorted for $CLIP^-$ and, if present, $CLIP^+$ cells within the CD45dim/SSClow population by using a FACSAria flow cytometer (BD). The viability of the sorted cell fractions was confirmed by using trypan blue dye exclusion (>90%). Sorted cells were washed twice in PBS and used immediately for

Table 1. Patient characteristics.

AML patient number	1	2	3	4	5	6	7	8
Gender (M/F)	F	M	F	M	F	F	M	M
Age at diagnosis (years)	37	55	56	61	72	70	40	61
WBC at diagnosis (109/l)	110	84	21	20	18	79	56	68
FAB classification	M2	M5	M4	M4	M2	M5	M1	M4
Cytogenetic risk group*	favor-able	stan-dard	ad-verse	favo-rable	stan-dard	stan-dard	stan-dard	stan-dard
Immunophenotype of blasts								
CD45dim/SSClow (%)	89.0	85.7	78.5	18.5	50.1	26.5	51.4	46.8
CD40 (%)	47.8	77.7	21.0	52.7	84.8	70.8	48.1	76.1
CD86 (%)	1.6	11.9	5.4	34.3	35.3	96.5	21.7	83.3
HLA-DR (%)	94.2	59.1	83.4	99.5	96.1	99.7	89.7	100
CLIP (%)	1.0	17.6	53.4	13.3	42.7	49.0	76.1	50.9

Screening of selected AML patients that were in remission for at least 6 months and did not receive allogeneic stem cell transplantation. CD40, CD86, HLA-DR and CLIP expression is shown as the percentage of positive CD45dim/SSClow-gated primary leukemic blasts at diagnosis. Abbreviations: WBC, white blood cells; FAB, French-American-British; int, intermediate. * Groups were classified as previously described.[22]

culture experiments. The remaining cell fractions were frozen in several aliquots for later use.

For the isolation of $CD4^+$ T cells, we thawed mononuclear cells obtained from peripheral blood samples of patients in remission, as described above, and selected $CD14^-CD4^+$ cells with anti-CD14 and anti-CD4 microbeads using magnetic cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. The purity of cells that were negatively selected for CD14 and positively selected for CD4 exceeded 95%, as determined by flow cytometric analyses (data not shown). Depleted $CD14^+$ cell fractions were frozen and stored in liquid nitrogen.

Autologous co-cultures

Flow cytometrically sorted leukemic blasts were irradiated (30 Gy) and added to 2.5-5 x 10^5 remission $CD4^+$ T cells of the same patient at a stimulator-to-responder ratio of 1:1 in culture medium supplemented with IL-7 (10 ng/ml; Miltenyi Biotec). $CD4^+$ T cells were cultured with irradiated autologous blasts for 3 weeks at 37°C in a humidified atmosphere containing 5% CO_2. To study HLA-DR restriction of the $CD4^+$ T cell responses, we used $CLIP^-$-sorted leukemic blasts pre-treated with the anti-HLA-DR blocking antibody L243 [24] (9.2 µg/ml) at the start of culture. Following one week of stimulation, $CD4^+$ T cells were re-stimulated with the same irradiated and sorted blasts (1:1 ratio) and cultured again in fresh medium. IL-2 (50 U/ml; Miltenyi Biotec) was added 2 days after each round of stimulation. Samples were taken from co-cultures at the same day to investigate functional parameters indicative for T cell function by using flow cytometry.

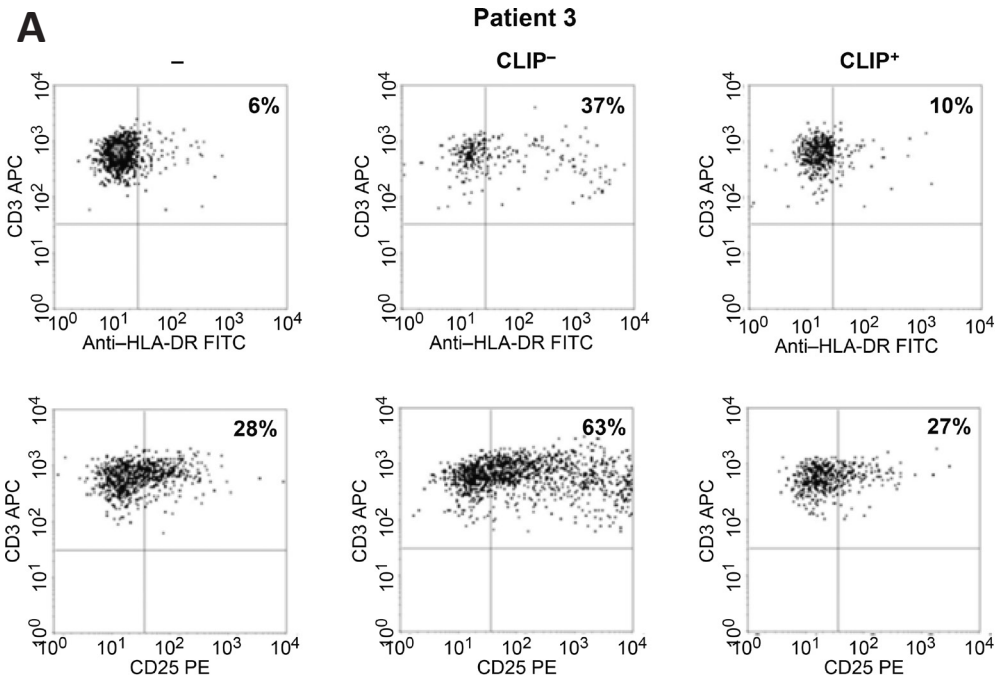
Functional analyses

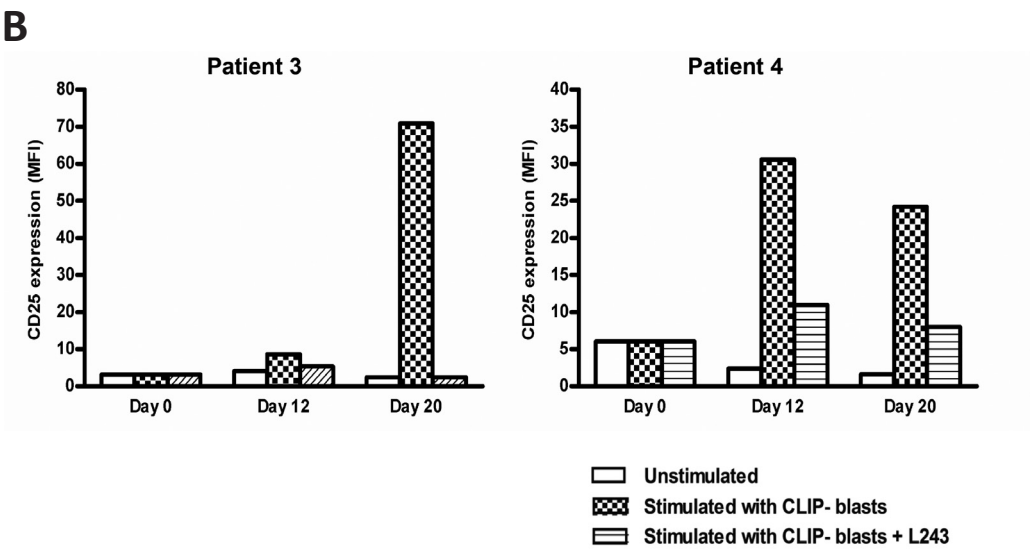
Paired t tests were used to compare IFN-γ and IL-4 expression in $CD4^+$ T cells between co-cultures of patients. Only $CD4^+$ T cells that received two or more rounds of stimulation with leukemic blasts were used for statistical analysis. For assessment of TCR Vβ repertoires, a given TCR Vβ family was interpreted as positive when Vβ expression was enhanced at least 20% after stimulation with leukemic blasts, as compared to unstimulated $CD4^+$ T cells, and greater than two times the standard deviation over the average for healthy controls.

Results

Establishment of $CLIP^-$ and $CLIP^+$ co-cultures from the same AML patients

We analyzed the functional impact of CLIP on primary leukemic blasts by performing co-cultures of $CD4^+$ T cells with autologous $CLIP^-$ and $CLIP^+$ leukemic blasts from eight AML patients, who were selected as described in Materials and Methods and shown in *table 1*. In all cases, we could flow cytometrically sort $CLIP^-$ primary leukemic blasts for at least two rounds of stimulation of autologous $CD4^+$ T cells. For patient 1, too few viable $CD4^+$ T cells were left after co-culture with $CLIP^-$ leukemic blasts to do functional analyses. For patient 1 and 2, it was technically impossible to acquire sufficient amounts of $CLIP^+$ leukemic blasts to stimulate $CD4^+$ T cells, as the majority of leukemic blasts were negative for CLIP (*table 1*). For





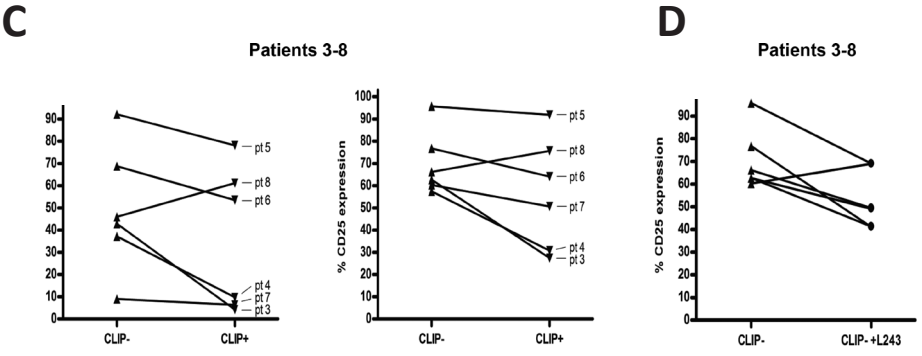


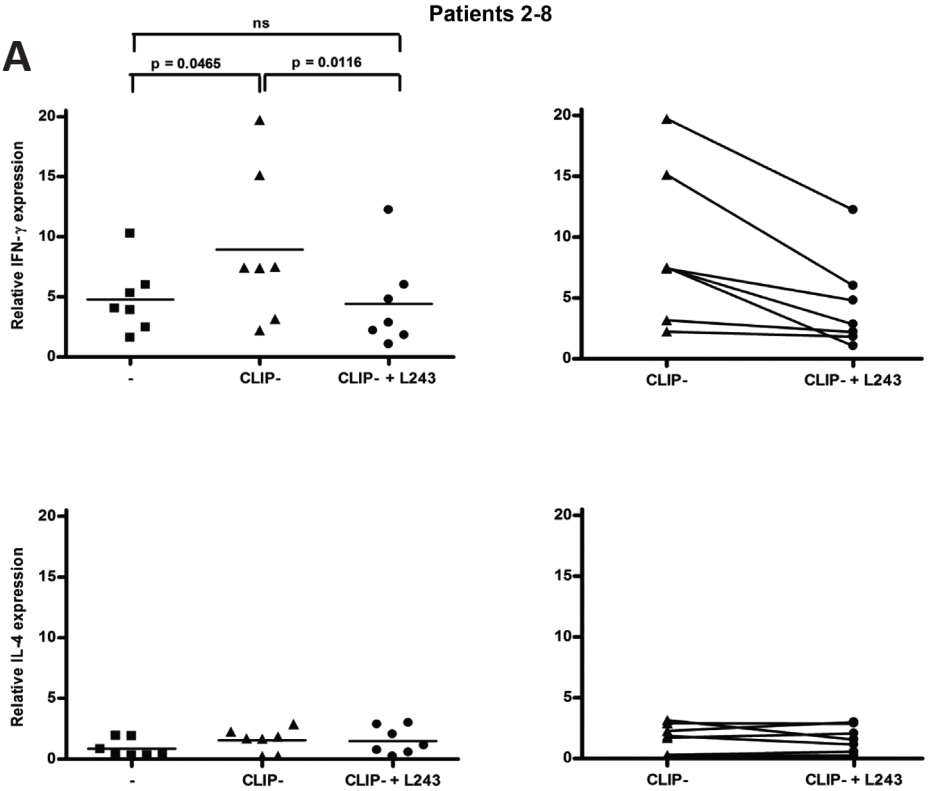
Figure 1. CD4⁺ T cell activation following stimulation with autologous CLIP⁻ and CLIP⁺ primary leukemic blasts. (A) Flow cytometric analysis of HLA-DR and CD25 expression on unstimulated CD4⁺ T cells ('-') and CD4⁺ T cells stimulated with three rounds of autologous CLIP⁻CD45dim or CLIP⁺CD45dim leukemic blasts. (B) MFI of CD25 expression on CD4⁺ T cells following two (day 12) and three (day 20) rounds of stimulation with untreated and L243-treated autologous CLIP⁻ leukemic blasts. (C, D) Patients analyzed for HLA-DR and CD25 expression (%) on CD4⁺ T cells stimulated with equal rounds of CLIP⁻, L243-treated CLIP⁻ or CLIP⁺ leukemic blasts. HLA-DR (%): 'CLIP⁻ vs CLIP⁺', p=0.13; CD25 (%): 'CLIP⁻ vs CLIP⁺', p=0.10; 'CLIP⁻' vs 'CLIP⁻ +L243', p=0.035 (paired t tests). As compared to CLIP⁻ co-cultures, only patient 8 showed an increase in the percentage of positive cells.

patients 3-8, it was possible to study the influence of both CLIP$^-$ and CLIP$^+$ leukemic blasts on CD4$^+$ T cells after equal rounds of stimulation.

CLIP$^-$ leukemic blasts activate CD4$^+$ T cells more efficiently than CLIP$^+$ leukemic blasts from the same patient

To examine the capability of CLIP$^-$ and CLIP$^+$ primary leukemic blasts to activate autologous CD4$^+$ T cells, we assessed CD4$^+$ T cells from different co-cultures for expression of HLA-DR and CD25, two common activation markers.

As indicated for patient 3, HLA-DR and CD25 expression levels were strongly increased on CD4$^+$ T cells after three rounds of stimulation with CLIP$^-$ leukemic blasts, as compared to unstimulated CD4$^+$ T cells (day 20; *figure 1A* and *figure 1B*). For patient 4, CD4$^+$ T cells from CLIP$^-$ co-cultures revealed a similar increase in expression, which was already observed after two rounds of stimulation with CLIP$^-$ leukemic blasts (day 12; *figure 1B*). In CLIP$^+$ co-cultures, the expression of HLA-DR and CD25 on CD4$^+$ T cells were reduced compared to CLIP$^-$ co-cultures (*figure 1A* and *figure 1C*), demonstrating that CLIP on leukemic blasts affects CD4$^+$ T cell activation. To investigate if CD4$^+$ T cell activation by CLIP$^-$ leukemic blasts was HLA-DR restricted, we blocked HLA-DR expression on these blasts with the L243 mAb. Stimulation with L243-treated CLIP$^-$ leukemic blasts resulted in decreased CD25 expression on CD4$^+$ T cells compared to CLIP$^-$ co-cultures without co-incubation with L243 (*figure 1B*



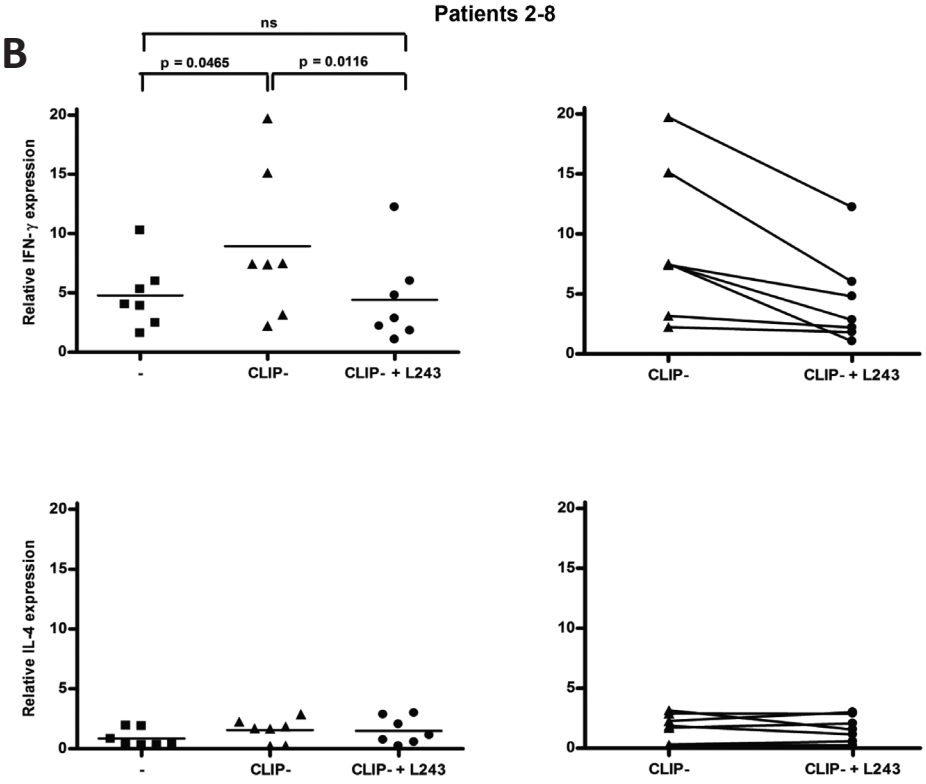


Figure 2. The role of CLIP on primary leukemic blasts in CD4⁺ T helper 1/T helper 2 skewing. Th1 and Th2 polarization of CD4⁺ T cells in CLIP⁻ and CLIP⁺ co-cultures of the same AML patients. After PMA/ionomycin restimulation, CD3⁺CD4⁺ cells were analyzed at the same day of culture for intracellular IFN-γ and IL-4 expression by flow cytometry. The relative expression was defined as described in Materials and Methods. (A) IFN-γ and IL-4 expression in CD4⁺ T cells cultured alone or with autologous CLIP- or L243-treated leukemic blasts. (B) IFN-γ and IL-4 expression in CD4⁺ T cells cultured with autologous CLIP⁻ or CLIP⁺ leukemic blasts.

and *figure 1D*). For patient 6 and 7, we found additional increases in HLA-DR-restricted CD4⁺ T cell proliferation during CLIP⁻ co-cultures, in contrast to CLIP⁺ co-cultures and controls, as recently reported [20]. One patient (patient 8) showed excessive spontaneous proliferation of CD4⁺ T cells in culture (data not shown), which possibly explains the aberrant activation differences between CLIP⁻ and CLIP⁺ co-cultures for this patient only (*figure 1C*).
To conclude, for 5 out of 6 AML patients, CD4⁺ T cells were generally more activated in cultures with CLIP⁻ leukemic blasts than in cultures with CLIP⁺ leukemic blasts, indicating that the presence of CLIP on primary leukemic blasts negatively influences autologous CD4⁺ T cell priming.

CLIP⁻ primary leukemic blasts prime CD4⁺ T cells toward a T helper 1 phenotype in contrast to CLIP⁺ primary leukemic blasts

For effective anti-tumor T cell immunity, it is necessary that CD4⁺ T cells are activated and differentiate into Th1 cells, which augment tumor-specific CTL responses by triggering APCs

or secreting IFN-γ [25]. Therefore, we next investigated whether CLIP on primary leukemic blasts had an effect on Th1 and Th2 skewing of autologous $CD4^+$ cells.
$CLIP^-$ leukemic blasts induced significant IFN-γ production in $CD4^+$ T cells as compared to unstimulated $CD4^+$ T cells (p=0.0465; *figure 2A*). This induction was HLA-DR-dependent, since L243 treatment of these blasts prior to stimulation abrogated the effect. No significant increases in IL-4 were encountered. $CD4^+$ T cells that received equal rounds of stimulation with $CLIP^+$ leukemic blasts hardly produced IFN-γ, in accordance with unstimulated $CD4^+$ T cells (*figure 2B*). IFN-γ expression between $CD4^+$ T cells of $CLIP^-$ and $CLIP^+$ co-cultures was significantly different (p=0.0477), in contrast to IL-4 (*figure 2B*). Interestingly, in addition to a decrease in IFN-γ^+ T cell number, an increased amount of IL-4^+ $CD4^+$ T cells was found in $CLIP^+$ compared to $CLIP^-$ co-cultures of patient 6 and 8 (*Supplementary figure S1*).
Overall, for 7 out of 8 AML patients, $CLIP^-$ leukemic blasts induced IFN-γ expression in

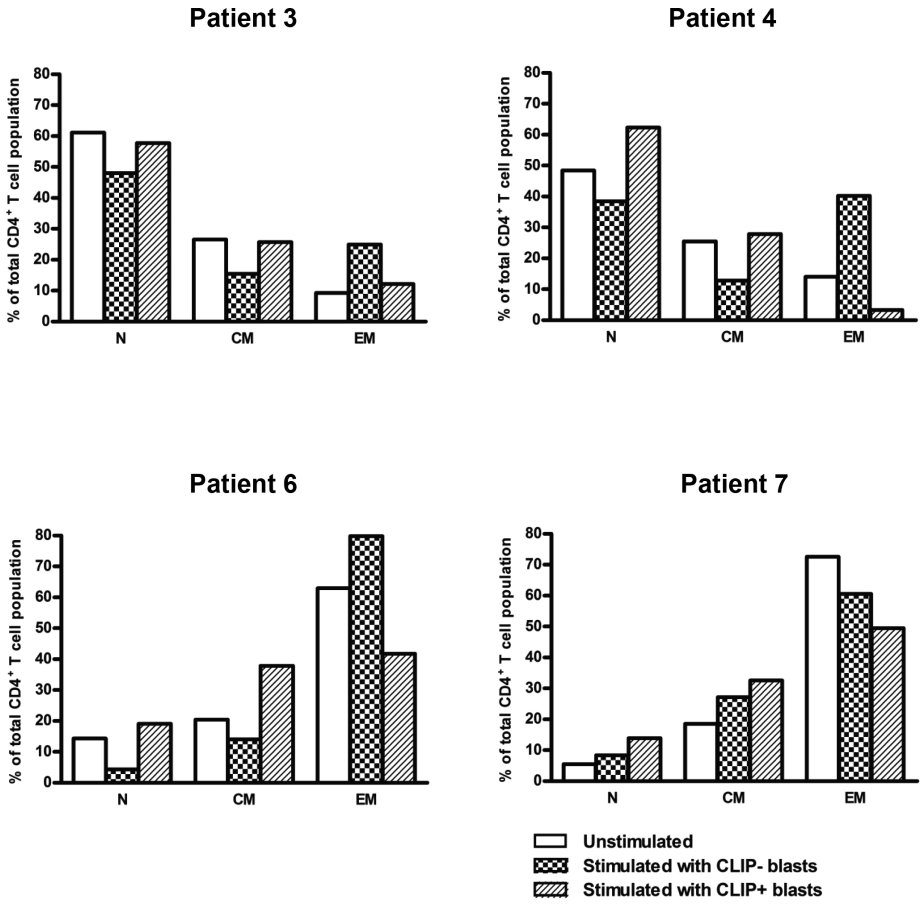


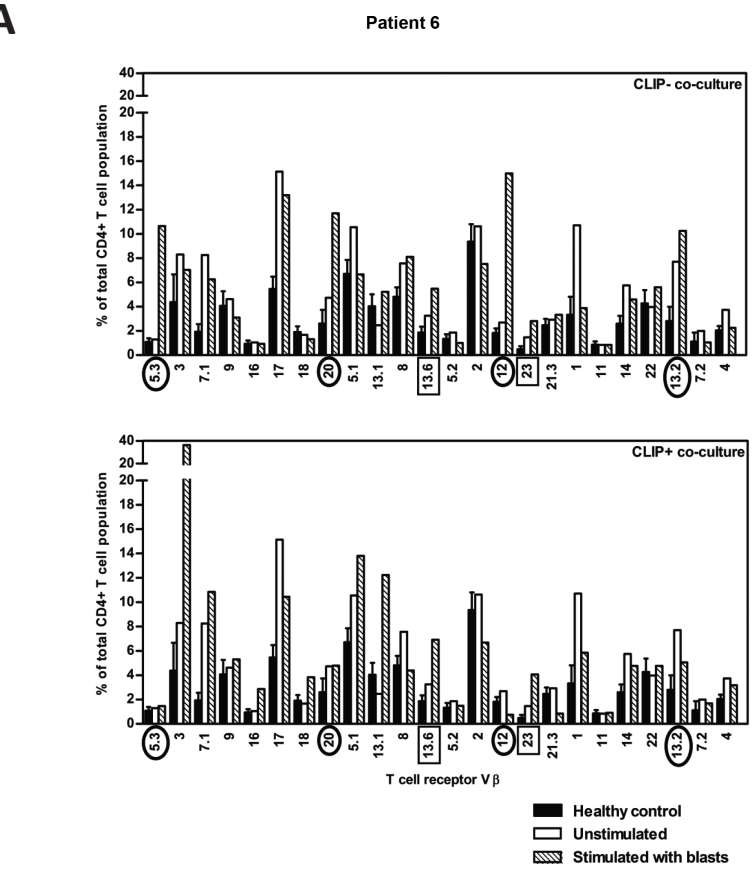
Figure 3. Increases in effector memory phenotype of highly activated $CD4^+$ T cells in $CLIP^-$ compared to $CLIP^+$ co-cultures. Percentages of naive (N; $CD45RA^+CD27^+$), central memory (CM; $CD45RA^-CD27^+$) and effector memory (EM; $CD45RA^-CD27^-$) $CD4^+$ T cells in different co-cultures of AML patients, as determined by flow cytometry. Patients showing the largest increases in $CD4^+$ T cell activation between $CLIP^-$ and $CLIP^+$ were assessed. $CD4^+$ T cells received equal rounds of stimulation.

autologous $CD4^+$ T cells, demonstrating polarization toward a Th1 phenotype. Moreover, for all AML patients tested, $CLIP^+$ leukemic blasts were not able to significantly enhance IFN-γ levels, which indicates that absent CLIP expression on primary leukemic blasts boosts Th1 differentiation.

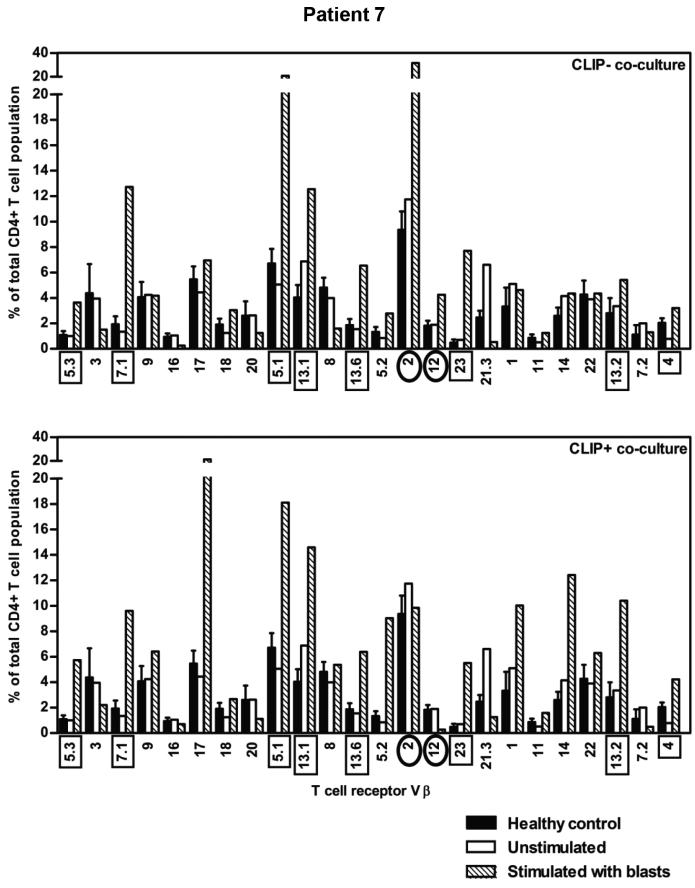
Highly activated $CD4^+$ T cells in $CLIP^-$ co-cultures have an increased effector memory phenotype as compared to $CD4^+$ T cells in $CLIP^+$ co-cultures

As $CD4^+$ T effector memory cells are critical for long-term immune protection against tumor growth [26], we studied if the presence of CLIP on primary leukemic blasts was associated with changes in $CD4^+$ T cell memory phenotype. This was analyzed for patients whose $CD4^+$ T cells revealed a large difference in activation following stimulation with $CLIP^-$ and $CLIP^+$ leukemic blasts (n=4; *figure 1C*).

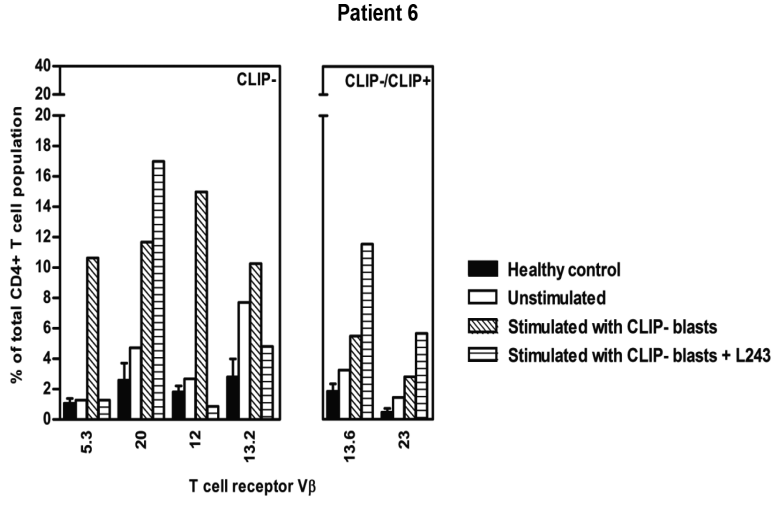
For patient 3 and 4, an increased amount of $CD4^+$ T cells obtained an effector memory ($CD27^-CD45RA^-$) phenotype in $CLIP^-$ co-cultures, as compared to control cultures (25% and 40% versus 9% and 14%, respectively; *figure 3*). This increase was HLA-DR restricted, as it was abolished by stimulation with L243-treated $CLIP^-$ leukemic blasts (data not shown), and accompanied by a decreased percentage of naive ($CD27^+/CD45RA^+$) and central memory ($CD27^+/CD45RA^-$) cells (*figure 3*). In $CLIP^+$ co-cultures, we observed a reduced percentage of effector memory cells (12% and 3%, respectively), whereas the percentage of naive and



B


Patient 7
CLIP- co-culture
CLIP+ co-culture
% of total CD4+ T cell population
5.3
3
7.1
9
16
17
18
20
5.1
13.1
8
13.6
5.2
2
12
23
21.3
1
11
14
22
13.2
7.2
4
T cell receptor Vβ
Healthy control
Unstimulated
Stimulated with blasts

C


Patient 6
CLIP-
CLIP-/CLIP+
% of total CD4+ T cell population
5.3
20
12
13.2
13.6
23
T cell receptor Vβ
Healthy control
Unstimulated
Stimulated with CLIP- blasts
Stimulated with CLIP- blasts + L243

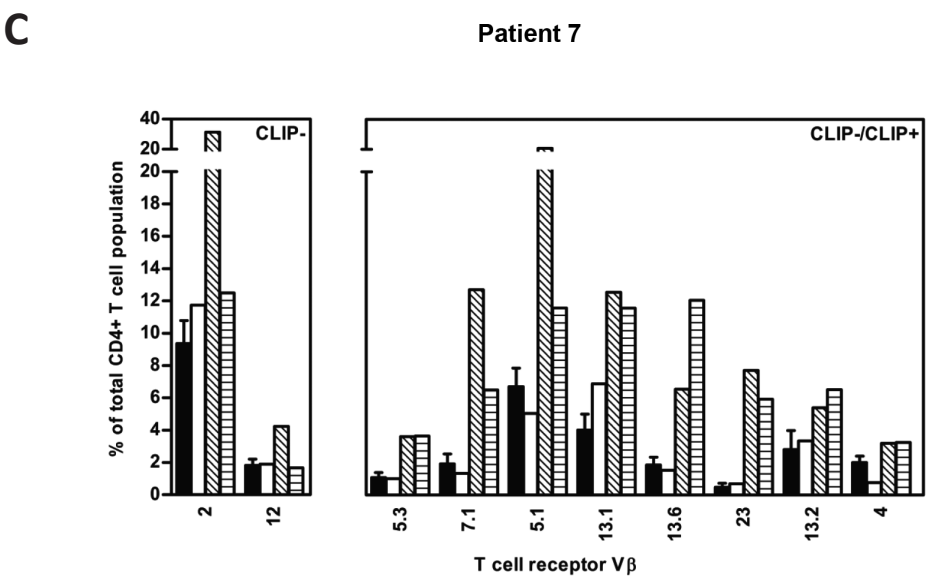


Figure 4. TCR Vβ CD4⁺ T cell expansion in long-term cultures with CLIP⁻ and CLIP⁺ primary leukemic blasts. TCR Vβ analyses were performed for well-expanding CD4⁺ T cells in culture with autologous CLIP⁻ and CLIP⁺ primary leukemic blasts (panel A and B). The positive TCR Vβ families of CLIP⁻ co-cultures that were present (rectangles) or absent (circles) in corresponding CLIP⁺ co-cultures are highlighted. To assess HLA-DR restriction, we analyzed the presence of positive families in co-cultures with L243-treated CLIP⁻ leukemic blasts (panel C).

central memory cells was enhanced. For patient 6 and 7, despite the high effector memory and low naive and central memory cell numbers in control cultures, similar differences in memory phenotype were seen between CLIP⁻ and CLIP⁺ co-cultures (*figure 3*). These data were confirmed by CD27 and CD45RO expression analyses (data not shown).
Thus, for all AML patients tested, the activation of autologous CD4⁺ T cells in CLIP⁻ co-cultures caused enhanced differentiation of naive and/or central memory cells into effector memory cells, as compared to CLIP⁺ co-cultures. This implicates that the ability of primary leukemic blasts to generate effector CD4⁺ T cells involves the expression of CLIP.

CLIP⁻ primary leukemic blasts activate distinct subsets of HLA-DR-restricted CD4⁺ T cells in comparison with CLIP⁺ primary leukemic blasts

To provide evidence that CLIP⁻ leukemic blasts can present leukemia-associated antigens (LAAs) and thus activate distinct antigen-specific CD4⁺ T cell subsets, we studied the relation of CLIP on primary leukemic blasts and TCR Vβ repertoires of outgrowing CD4⁺ T cells. The well-expanding CD4⁺ T cell populations of patient 6, 7 and 8 were compared for the presence of 24 different TCR Vβ families after three rounds of stimulation with CLIP⁻ or CLIP⁺ leukemic blasts using flow cytometry.
We detected six TCR Vβ families for patient 6 and ten TCR Vβ families for patient 8 that were strongly expanded in cultures of CD4⁺ T cells stimulated with CLIP⁻ leukemic blasts as compared to unstimulated CD4⁺ T cells and healthy controls. (*figure 4A* and *figure 4B*). Four and two of these families, respectively, were exclusively expanded by CLIP⁻ and not by CLIP⁺

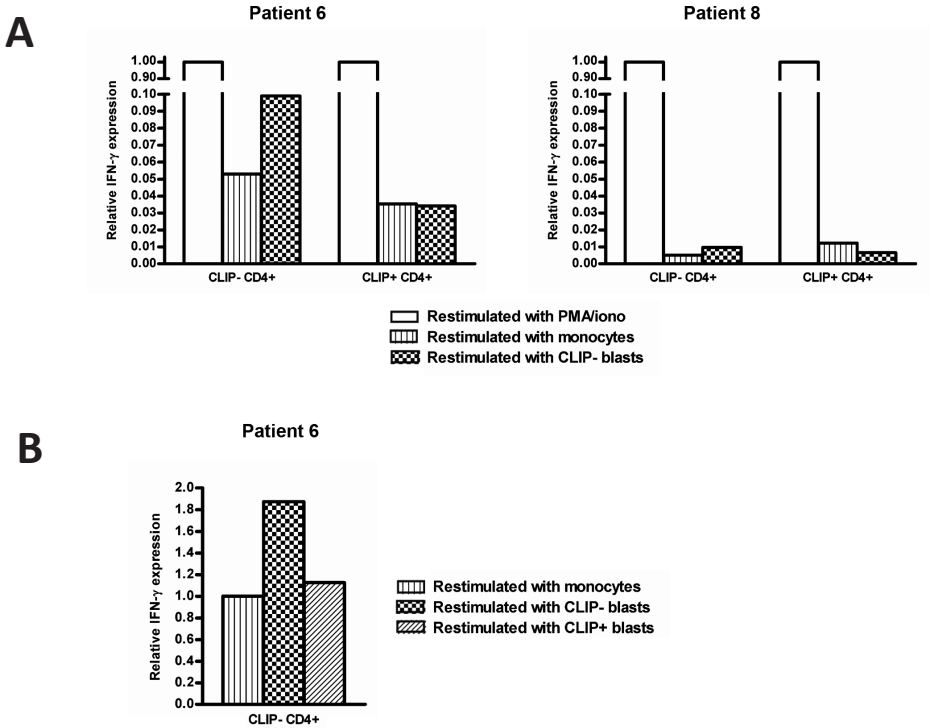


Figure 5. Leukemia-specific reactivity of highly activated CD4⁺ T cells primed by CLIP⁻ primary leukemic blasts. CD4⁺ T cells from long-term CLIP⁻ and CLIP⁺ co-cultures were restimulated with autologous CLIP⁻ primary leukemic blasts and analyzed for intracellular IFN-γ expression by flow cytometry. Results were compared to restimulation with autologous non-malignant monocytes (A) or CLIP⁺ primary leukemic blasts (B). The relative expression was defined as described in Materials and Methods and, in panel A, normalized to that of CD4⁺ T cells restimulated with PMA/ionomycin in the same co-culture.

leukemic blasts. The majority of CLIP⁻-specific TCR Vβ families did not expand in L243 CLIP⁻ co-cultures (*figure 4C)*, demonstrating that activation was primarily restricted to HLA-DR. The activation of all TCR Vβ families equally present in CLIP⁻ and CLIP⁺ co-cultures was not HLA-DR restricted. For patient 8, no differences in TCR Vβ expression were found between CD4⁺ T cells from CLIP⁻ and CLIP⁺ co-cultures (data not shown), consistent with the earlier described strong spontaneous CD4⁺ T cell proliferation.
In conclusion, for 2 out of 3 AML patients, we reveal that CLIP⁻ leukemic blasts activate specific subsets of HLA-DR-restricted CD4⁺ T cells that are not activated by CLIP⁺ leukemic blasts. These results show that CLIP on primary leukemic blasts interferes with expansion of antigen-specific CD4⁺ T cells, and thus may prevent activation of leukemia-reactive CD4⁺ T cells.

CD4⁺ T cells stimulated with CLIP⁻ primary leukemic blasts reveal leukemia-specific reactivity in contrast to those stimulated with CLIP⁺ primary leukemic blasts
To further examine leukemia specificity, we evaluated if the well-expanding 'CLIP⁻-primed' CD4⁺ T cells from patient 6 could detect antigens presented by other autologous APCs.

$CD4^+$ T cells from $CLIP^-$ and $CLIP^+$ co-cultures were studied for IFN-γ expression after restimulation with autologous non-malignant monocytes or $CLIP^-$ primary leukemic blasts. An almost 2-fold reduction in IFN-γ expression was observed in '$CLIP^-$-primed' $CD4^+$ T cells restimulated with non-malignant monocytes compared to those restimulated with $CLIP^-$ leukemic blasts (*figure 5A*). This difference was not found for '$CLIP^+$-primed' $CD4^+$ T cells, confirming that recognition of $CLIP^-$ leukemic blasts by these $CD4^+$ T cells is determined by specific priming. As another control, similar experiments for the spontaneously outgrowing $CD4^+$ T cells from patient 8 showed hardly any recognition of $CLIP^-$ leukemic blasts or non-malignant monocytes (*figure 5A*).

To evaluate whether the recognition of primary leukemic blasts by '$CLIP^-$-primed' $CD4^+$ T cells of patient 6 was dependent on CLIP expression, we compared the ability of these $CD4^+$ T cells to recognize autologous $CLIP^-$ or $CLIP^+$ leukemic blasts. Restimulation with $CLIP^-$ leukemic blasts clearly induced IFN-γ expression in '$CLIP^-$-primed' $CD4^+$ T cells, in contrast to restimulation with $CLIP^+$ leukemic blasts (*figure 5B*). This shows that the expansion of '$CLIP^-$-primed' $CD4^+$ T cells is antigen-specific and relies on a $CLIP^-$ background.

Taken together, these data indicate that $CD4^+$ T cell priming by $CLIP^-$ leukemic blasts can result in increased leukemia-specific reactivity, demonstrating the potential relevance of CLIP expression on primary leukemic blasts in the induction of an effective leukemia-specific $CD4^+$ T cell response.

Discussion

Impaired T cell recognition of tumor cells is an essential issue to address in translational cancer research, since it could not only explain disease progression, but also provide new immunotherapeutic targets to induce effective anti-tumor immunity. In AML patients, this may be a pathologic event as T cells constantly encounter leukemic blasts in the peripheral blood without the ability to control or eradicate the disease. Recent studies have shown that the number of peripheral blood T cells is increased at diagnosis [27], but that, despite the excess of leukemic blasts as APCs, these cells are not fully activated [28]. This suggests that AML patients induce an immune response, but lack leukemia-specific T cells due to mechanisms used by leukemic blasts that prevent activation. In this study, we demonstrate that one of these mechanisms could be the expression of CLIP, a self-peptide known to be presented by HLA class II molecules instead of antigenic peptides to $CD4^+$ T cells.

By using cells from the same AML patients, co-culture experiments were performed in a setting that might be representative for the in vivo situation. In this way, we also minimized the influence of inter-patient variability, a feature commonly observed in AML. To acquire autologous $CD4^+$ T cells, we had to select AML patients that were still in complete remission. This restricted the number of cases that could be assessed, as CLIP expression in patients with prolonged remission is generally low at diagnosis in contrast to early-relapsing patients [19]. For the patients whose $CD4^+$ T cells could be primed with $CLIP^-$ and $CLIP^+$ leukemic blasts, strong differences in T cell function were found, caused by either a direct or indirect effect of CLIP.

For most patients, $CLIP^+$ leukemic blasts were less able to activate $CD4^+$ T cells during co-cultures (*figure 1C*). This points to a direct role for CLIP in the induction of aberrant T cell activation pathways, which were recently found in AML T cells that lacked the ability to form

immunologic synapses after conjugation with leukemic blasts [27]. Interestingly, for patient 6 and 7, CD25 expression on $CD4^+$ T cells remained relatively high after stimulation with $CLIP^+$ leukemic blasts, which might represent $CD25^{high}$ regulatory T cells (Tregs). In agreement with this, $CLIP^+$-restricted $CD4^+$ T cell priming was seen for both patients, as TCR Vβ families 3 and 17 were markedly expanded in $CLIP^+$, but not in $CLIP^-$ co-cultures, respectively (*figure 4*). Self-peptides can indeed promote Treg expansion [29] and a role for CLIP in this context has been proposed [30]. Also Tregs are highly prevalent in patients with untreated AML [31], but further investigation is needed to confirm this association. Although expression levels of IL-4 were relatively low compared to IFN-γ, for some patients, $CLIP^+$ leukemic blasts seemed to stimulate IL-4^+ Th2 cells more efficiently than $CLIP^-$ leukemic blasts (*Supplementary figure S1*). This suggests that high expression of CLIP together with CD86 (patient 6 and 8; *table 1*) contributes to a tumor-protective Th2 immune response, in line with the findings of Rohn and co-workers for maturing DCs [32]. In AML, CLIP may therefore be involved in the correlation of CD86 with hyperleukocytosis and the capacity of CD86-positive leukemic blasts to boost Treg- or Th2-related cytokine production [33].

Another possibility is that CLIP on leukemic blasts indirectly affects $CD4^+$ T cell function. In the absence of CLIP, we observed increases in T cell activation, Th1 skewing and effector memory differentiation (*figures 1-3*). HLA-DR-specific blocking on leukemic blasts abrogated these effects, which points to the involvement of a contact-dependent mechanism in which peptides other than CLIP are recognized and cause an effective $CD4^+$ T cell response. Since not only the amount of naïve cells, but also of central memory cells was lowered in $CLIP^-$ co-cultures (*figure 3*), $CD4^+$ T cells from patients in remission probably have already encountered these immunogenic peptides at disease onset. Earlier studies reported that the precursor of CLIP, Ii, blocks loading of endogenous peptides onto HLA class II molecules in the ER [34-36], thereby preventing peptide loading until the MIICs are reached for exchange of CLIP with exogenous peptides. Thus, Ii has an important role in discriminating HLA class I and II-mediated presentation of endogenous and exogenous peptides, respectively [37]. Based on these findings, it is likely that in $CLIP^-$ leukemic blasts, HLA class II complexes are loaded with endogenous peptides for presentation to $CD4^+$ T cells. A $CLIP^-$ myeloid leukemic cell line, KG-1, was already shown to present HLA-DR molecules occupied with endogenous rather than exogenous peptides [38]. Recently, we extended this by showing that contrary to $CLIP^+$ leukemic blasts, $CLIP^-$ KG-1 blasts present such peptides via a proteasome- and TAP-dependent pathway [39]. The current study indicates that this type of processing in $CLIP^-$ primary leukemic blasts may lead to activation of specific subsets of HLA-DR-restricted $CD4^+$ T cells (*figure 4*) with leukemia-specific reactivity (*figure 5*). Collectively, these data support our hypothesis that the absence of CLIP on leukemic blasts is critical for endogenous LAA presentation and the induction of a leukemia-specific T cell response, resulting in prolonged disease-free survival of $CLIP^-$ AML patients [19;20].

Until now, little was known about the functional consequences of CLIP on HLA class II-bearing tumor cells. Here, we reveal that the presence of CLIP on leukemic blasts of AML patients interferes with optimal activation of leukemia-reactive $CD4^+$ T cells. This deserves more attention in the development of immunotherapy for patients with such tumors. Current immunotherapeutic strategies are based on the delivery of optimal DCs or T cells to boost the immune system of patients against tumor cells. In AML, we now demonstrate that CLIP can also be detrimental for the recognition of primary leukemic blasts, indicating that

priming or delivery of leukemia-specific T cells by such strategies, respectively, might not be enough for the generation of anti-leukemic immunity in vivo. CLIP$^+$ leukemic blasts also have to be recognized well by leukemia-specific T cells via presentation of endogenous LAAs instead of CLIP, which otherwise leads to tumor immune escape and outgrowth. Recent studies report that gene expression of the two main processors of endogenous antigens, the proteasome and TAP, can be increased in tumor cells using histone deacetylase inhibitors (HDACi; ref. [40]. Furthermore, preliminary experiments in our laboratory showed strongly reduced CLIP levels on leukemic blasts of AML patients after treatment with the clinically applicable HDACi suberoylanilide hydroxamic acid (SAHA). Therefore, we propose that CLIP down-modulation by pharmacological agents may serve as a promising strategy to enhance endogenous LAA presentation on primary leukemic blasts *in vivo*, leading to improved immunotherapy in AML.

Reference List

1. Morris E, Hart D, Gao L et al. Generation of tumor-specific T-cell therapies. Blood Rev. 2006;20:61-69.
2. Reichardt VL, Brossart P, Kanz L. Dendritic cells in vaccination therapies of human malignant disease. Blood Rev. 2004;18:235-243.
3. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat.Immunol. 2002;3:991-998.
4. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res. 2006;66:5527-5536.
5. van Luijn MM, van den Ancker W, Chamuleau ME et al. Impaired antigen presentation in neoplasia: basic mechanisms and implications for acute myeloid leukemia. Immunotherapy 2010;2:85-97.
6. Chakraverty R, Eom HS, Sachs J et al. Host MHC class II+ antigen-presenting cells and CD4 cells are required for CD8-mediated graft-versus-leukemia responses following delayed donor leukocyte infusions. Blood 2006;108:2106-2113.
7. Knutson KL, Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol.Immunother. 2005;54:721-728.
8. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. Proc.Natl.Acad.Sci.U.S.A 1997;94:6886-6891.
9. Humphreys RE, Hillman GG, von HE, Xu M. Forcing tumor cells to present their own tumor antigens to the immune system: a necessary design for an efficient tumor immunotherapy. Cell Mol.Immunol. 2004;1:180-185.
10. Thompson JA, Srivastava MK, Bosch JJ et al. The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4(+) T lymphocytes. Cancer Immunol.Immunother. 2008;57:389-398.
11. Anderson MS, Miller J. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. Proc.Natl.Acad.Sci.U.S.A 1992;89:2282-2286.
12. Bakke O, Dobberstein B. MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. Cell 1990;63:707-716.

13. Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. Nature 1991;354:392-394.
14. Lotteau V, Teyton L, Peleraux A et al. Intracellular transport of class II MHC molecules directed by invariant chain. Nature 1990;348:600-605.
15. Riese RJ, Chapman HA. Cathepsins and compartmentalization in antigen presentation. Curr. Opin.Immunol. 2000;12:107-113.
16. Sherman MA, Weber DA, Jensen PE. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. Immunity. 1995;3:197-205.
17. Martin WD, Hicks GG, Mendiratta SK et al. H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. Cell 1996;84:543-550.
18. Denzin LK, Cresswell P. HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. Cell 1995;82:155-165.
19. Chamuleau ME, Souwer Y, van Ham SM et al. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res. 2004;64:5546-5550.
20. van Luijn MM, Chamuleau ME, Thompson JA et al. CLIP down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased $CD4^+$ T cell responses. Haematologica 2010;95:485-93.
21. Bennett JM, Catovsky D, Daniel MT et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann. Intern.Med. 1985;103:620-625.
22. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N.Engl.J.Med. 1999;341:1051-1062.
23. Lacombe F, Durrieu F, Briais A et al. Flow cytometry CD45 gating for immunophenotyping of acute myeloid leukemia. Leukemia 1997;11:1878-1886.
24. Souwer Y, Griekspoor A, Jorritsma T et al. B cell receptor-mediated internalization of salmonella: a novel pathway for autonomous B cell activation and antibody production. J.Immunol. 2009;182:7473-7481.
25. Wang RF. The role of MHC class II-restricted tumor antigens and $CD4^+$ T cells in antitumor immunity. Trends Immunol. 2001;22:269-276.
26. Kennedy R, Celis E. Multiple roles for $CD4^+$ T cells in anti-tumor immune responses. Immunol. Rev. 2008;222:129-144.
27. Le DR, Taussig DC, Ramsay AG et al. Peripheral blood T cells in acute myeloid leukemia (AML) patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts. Blood 2009;114:3909-3916.
28. Ersvaer E, Hampson P, Wendelbo O et al. Circulating T cells in patients with untreated acute myelogenous leukemia are heterogeneous and can be activated through the CD3/TCR complex. Hematology. 2007;12:199-207.
29. Cozzo C, Larkin J, III, Caton AJ. Cutting edge: self-peptides drive the peripheral expansion of $CD4^+CD25^+$ regulatory T cells. J.Immunol. 2003;171:5678-5682.
30. Hess AD. Modulation of graft-versus-host disease: role of regulatory T lymphocytes. Biol. Blood Marrow Transplant. 2006;12:13-21.
31. Szczepanski MJ, Szajnik M, Czystowska M et al. Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia. Clin.Cancer Res. 2009;15:3325-3332.

32. Rohn TA, Boes M, Wolters D et al. Upregulation of the CLIP self peptide on mature dendritic cells antagonizes T helper type 1 polarization. Nat.Immunol. 2004;5:909-918.
33. Tamura H, Dan K, Tamada K et al. Expression of functional B7-H2 and B7.2 costimulatory molecules and their prognostic implications in de novo acute myeloid leukemia. Clin.Cancer Res. 2005;11:5708-5717.
34. Bodmer H, Viville S, Benoist C, Mathis D. Diversity of endogenous epitopes bound to MHC class II molecules limited by invariant chain. Science 1994;263:1284-1286.
35. Long EO, LaVaute T, Pinet V, Jaraquemada D. Invariant chain prevents the HLA-DR-restricted presentation of a cytosolic peptide. J.Immunol. 1994;153:1487-1494.
36. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature 1990;345:615-618.
37. Teyton L, O'Sullivan D, Dickson PW et al. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. Nature 1990;348:39-44.
38. Harris PE, Maffei A, Colovai AI et al. Predominant HLA-class II bound self-peptides of a hematopoietic progenitor cell line are derived from intracellular proteins. Blood 1996;87:5104-5112.
39. van Luijn MM, Chamuleau ME, Ressing ME et al. Alternative Ii-independent antigen processing pathway in leukemic blasts involves TAP-dependent peptide loading of HLA class II complexes. Cancer Immunol.Immunother. 2010;59:1825-38.
40. Setiadi AF, Omilusik K, David MD et al. Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. Cancer Res. 2008;68:9601-9607.

Part III: The role of immunotherapy in leukemia

10

Adapted from: Recent advances in antigen-loaded dendritic cell-based strategies for treatment of minimal residual disease in acute myeloid leukemia and

W van den Ancker
MM van Luijn
TM Westers
HJ Bontkes
JM Ruben
TD de Gruijl
GJ Ossenkoppele
AA van de Loosdrecht

Immunotherapy. 2010 Jan;2(1):69-83

Adapted from: Dendritic cell-based immunotherapy in myeloid leukaemia: translating fundamental mechanisms into clinical applications

AA van de Loosdrecht
W van den Ancker
I Houtenbos
GJ Ossenkoppele
TM Westers

Handb Exp Pharmacol. 2009;(188):319-48

Summary

Therapeutic vaccination with dendritic cells (DC) is recognized as an important experimental therapy for the treatment of minimal residual disease (MRD) in acute myeloid leukemia (AML). Many sources of leukemia associated antigens (LAA) and different methods for antigen (Ag) loading of DC have been used in an attempt to optimize anti-tumor responses. For instance, monocyte-derived DC (moDC) have been loaded with apoptotic whole-cell suspensions, necrotic cell lysates, tumor-associated peptides, eluted peptides and cellular DNA or RNA. Furthermore, MoDC can be chemically or electrically fused with leukemic blasts and DC have been cultured out of leukemic blasts. However, it remains unclear and a challenge in cancer immunotherapy to identify which of these methods is the most optimal for Ag loading and activation of DC. This review discusses recent advances in DC research and the application of this knowledge towards new strategies for Ag loading of DC in the treatment of MRD in AML.

Introduction

Acute myeloid leukemia (AML) is characterized by proliferation of clonal neoplastic myeloid hematopoietic precursor cells and impaired hematopoiesis. About 30% of AML cases can be classified as AML with myelodysplasia related changes, often preceded by a myelodysplastic syndrome (MDS). Chemotherapeutic induction regimens achieve complete remission (CR) in 70-80% of adult AML patients younger than 60 years; when older than 60, CR is achieved only in 50-60% of patients [1]. Despite intensive consolidation chemotherapy, relapses occur in approximately 50-60% of patients, probably caused by the presence of minimal residual disease (MRD). In the last decade, new treatment modalities have revealed the important role of the immune system in the prevention and control of leukemia, e.g. the graft versus leukemia (GvL) effect induced by allogeneic stem cell transplantation (SCT) and the reinduction of CR after donor lymphocyte infusion (DLI) after allogeneic SCT for patients with relapsed (chronic myeloid) leukemia. These effects are considered to be mediated by anti-tumor T cells. The induction, regulation and maintenance of such responses in AML are thought to be coordinated by dendritic cells (DC).

In 1995, the first cancer patients were vaccinated with DC and since then DC vaccines have been administered to patients with a variety of solid tumors, most frequently melanoma followed by prostate cancer and renal cell carcinoma, as well as to multiple myeloma and leukemia patients [2;3]. So far, more than 180 trials have been conducted; an overview of the DC-based clinical studies for cancer can be found at www.mmri.mater.org.au [4]. In the majority of trials immune responses against tumor associated antigens (TAA) could be detected. However, the same data emphasized the need for improving vaccination strategies, as in only a minority of patients clinical responses were achieved [5;6]. These poor clinical results could be partly explained by the use of immature DC and the late disease stage in which patients were treated. Overall, it was concluded that therapeutic cancer vaccines are feasible, easy to administer in an outpatient setting and do not cause many side effects. The number of randomized trials remains limited and evaluation of the efficacy of DC vaccination regimens is difficult [5;7]. To our knowledge, only one placebo controlled vaccination trial in prostate cancer patients has been performed with DC-like cells; encouragingly, results suggest a survival advantage for the vaccinated group [8]. Questions remain about which DC

precursor should be used for DC preparation, how immature DC should be activated and which source of TAA should be used. Moreover, there is no consensus yet on the timing and route of vaccine administration and methods of immunomonitoring. To date, most research has focused on solid tumors. This review discusses recent advances in DC research and the application of this knowledge towards new strategies for Leukemia Associated Antigen (LAA) loading of DC in the treatment of AML.

Principles of immunotherapy

Immune surveillance

Both unlimited growth and a differentiation block are needed for a cell to evolve into a malignant clone able to cause all clinical symptoms of solid or leukemic tumors. A model has been proposed for solid tumors by which the immune system plays an important role in the elimination of tumor cells, called immune surveillance [9]. The immune surveillance model describes the role of the immune system in the evolution of solid malignancies in three different phases. Hypothetically, the same model can be applied to AML and is summarized in *figure 1*. The first phase encompasses a state in which the immune system recognizes and eliminates (pre-)leukemic cells and the immune response is not yet inhibited by immune suppressive effects of (pre-) leukemic cells and/or their microenvironment, i.e. the elimination phase. This phase is followed by an equilibrium phase in which there is still a low tumor burden and immune cells are able to remove part of the malignant cells and prevent full-blown leukemia (equilibrium phase). In this phase, the mutation of malignant cells into escape-variants is promoted. In AML, the MDS prophase might be considered as the equilibrium state in some cases. In about 30% of the MDS cases, leukemia will develop which might at least be in part due to escape of immune surveillance [10]. Additionally, the immune system might control MRD cells in patients in first CR; in 50% of patients who reach CR relapse of AML occurs, even years after the last cycle of chemotherapy. The equilibrium phase is followed by the escape phase: malignant variants are no longer recognized by the immune system. This will result in uncontrolled expansion of leukemic blasts and development of manifest disease [9;11].

The role of DC in immune surveillance

DC are professional antigen (Ag)-presenting cells orchestrating the immune response. Immature DC sense Ag with their pattern recognition receptors (PRR). Antigenic peptides are thought to be chaperoned by peptide-binding heat shock proteins (HSP) such as glycoprotein 96 (gp96), HSP90, HSP70 and calreticulin. These chaperone-peptide complexes bind phagocytic PRR (pPRR) receptors, resulting in internalization and cross-priming [12]. As tumor cells constitute an exogenous source of Ag for the DC, TAA need to be processed and presented by MHC class I via a process called cross-presentation [13]. As well as binding of pPRR, instructive PRR (iPRR) are involved in providing DC with signals that regulate their capacity to elicit T cell responses, for instance Toll Like Receptors (TLR) [14;15]. Activation of TLR results in enhanced Ag presentation, production of inflammatory cytokines, upregulation of co-stimulatory molecules and overall activation of adaptive immune responses [16;17]. When the T cell receptor (TCR) of a naive T cell recognizes the TAA-derived epitopes presented by the DC, the T cells will become activated and a TAA-specific T

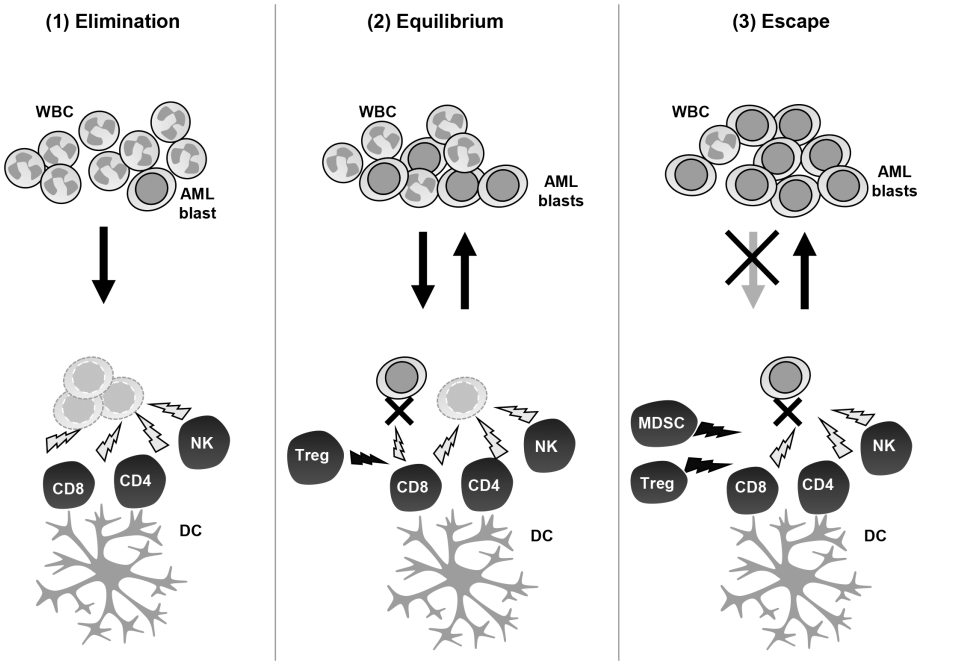


Figure 1. The three phases recognized in immune surveillance applied to AML. (1) The first phase, the elimination state, shows an immune system capable of eliminating (pre-)malignant AML blasts by NK and T cells activated by dendritic cells (DC). (2) This phase is followed by the equilibrium phase, wherein the immune cells remove part of the AML blasts, but some of the blasts cells escape with help of, for instance, T regulatory cells (Treg) or myeloid-derived suppressor cells (MDSC). (3) When these malignant cells are not recognized anymore by the immune system, this will result in an uncontrolled expansion of AML blasts and impaired numbers and function of granulocytes and other white blood cells (WBC). Adapted from Dunn and co-workers [8].

cell clone will expand. In AML, relatively few LAA have been identified, such as: WT1 (Wilms' tumor-gene product), PRAME (preferentially expressed Ag of melanoma), RHAMM (receptor for hyaluronic acid mediated motility), PR1 (a proteinase 3 derived peptide), hTERT (human telomerase reverse transcription Ag) and Survivin [11;18].

Immune escape

Various immunosuppressive effects, directly or indirectly induced by leukemic blasts, are known. Leukemic blasts can escape T cell-mediated cytoxicity directly by upregulating T cell inhibitory molecules, such as Programmed Death receptor ligand 1 (PD-L1) [19]. Furthermore, presentation of LAA by MHC class II, and thereby T helper (Th) cell activation, is reduced when the class II-associated invariant chain peptide (CLIP) is not replaced by an (immunogenic) peptide [20;21]. Without T cell help cytoxic T lymphocyte (CTL) responses cannot be maintained and tolerance will be induced. In addition, in AML indoleamine-2,3-dioxygenase expression is associated with poor clinical outcome, possibly by skewing the immune response towards a tolerogenic state [22;23]. Furthermore, increased frequency of and suppression by regulatory T cells (Treg) in patients with acute myelogenous leukemia

is observed in AML [24]. Factors such as vascular endothelial growth factor (VEGF), IL6, GM-CSF, IL-10 and TGFβ are associated with inhibition of DC function and maturation and induction of suppressive cells [22;23].

Immunotherapy

All the escape pathways discussed above are potential targets for immunotherapy. For instance, passive immunotherapy with adoptive transfer of specific T cells can restore rates of functional CTL [25]. Unfortunately, no memory response will be elicited and therefore after the lifespan of the CTL, the effect will disappear allowing dormant MRD cells to grow out. In contrast, active immunotherapy, for instance with DC vaccination, induces CTL and memory responses *in vivo*. In myeloid leukemia various sources of DC can be thought of: leukemia-derived-DC, AML-derived DC lines with DC differentiation capacity, $CD34^+$ normal precursor cell-derived DC or monocyte-derived DC (MoDC). Most *in vitro* generated immature DC can be primed and matured with cytokines and/or with tumor Ag in different ways [26]. There are numerous ways to interfere with and to modulate the escape pathways of blasts, which all may be of help to enhance the efficacy of DC vaccine regimens [27]. A selection is discussed in this paper focussing on the current state of the art.

Dendritic cells for immunotherapy; leukemic cell derived DC

Leukemic blasts, being malignantly transformed precursor cells, often have the ability to differentiate into DC-like cells. These AML-derived DC could provide a distinct opportunity to generate Ag presenting cells (APC) that harbor the full range of identified and unidentified LAA (*figure 2D*). AML-derived DC have the capacity to induce leukemia-specific T cell responses *in vitro* as well as *in vivo*, thus offering an immunotherapeutic modality for AML patients with MRD [28;29]. Unfortunately, leukemic DC might still harbor features of the leukemic blast, such as reduced expression of MHC molecules and molecules related to adhesion and induction of the co-stimulatory pathway as well as inhibitory features such as IDO expression [22]. Furthermore, vaccination with AML-DC is limited to 60-70% of cases where AML blasts can be cultured into leukemic DC [29;30]. Therefore, a more reliable and continuous source for differentiation into DC may be an AML-derived cell line, such as MUTZ-3 [*figure 2D*]. This cell line behaves as the immortalized equivalent of $CD34^+$ DC malignant precursor cells. Upon stimulation with cytokine cocktails, MUTZ-3 progenitors acquire a phenotype consistent with either interstitial DC or Langerhans cells (LC), superior to other AML cell lines such as THP-1, KG1 and HL60 [31-33]. As MUTZ-3 has an $HLA\text{-}A2^+$/ $HLA\text{-}A3^+$ phenotype, it can be used as a vaccine in patients with matching alleles, i.e. 70% of the Caucasian population. However, similar to leukemic DC, MUTZ-3 DC might exhibit features which potentially influence the efficacy of MUTZ-3 DC vaccines [34]. Therefore, other sources of DC, for instance MoDC cultured at the time of complete remission, loaded with tumor-specific peptides or patients' tumor material may serve as an alternative. A few papers have compared MoDC with AML-DC and indicate MoDC as a valid alternative for clinical application, especially for those patients where no leukemic DC can be obtained [35-39].

Dendritic cells for immunotherapy; monocyte-derived DC

Source of MoDC

Immature MoDC can be cultured with GM-CSF and IL-4 from monocytes of AML patients at the time of complete remission after induction therapy or from monocytes of matched healthy donors. Subsequently, maturation can be induced by various stimuli, such as cytokine cocktails (IL1β, TNF-α, IL6, and PGE_2 being the most widely used clinically) or Toll

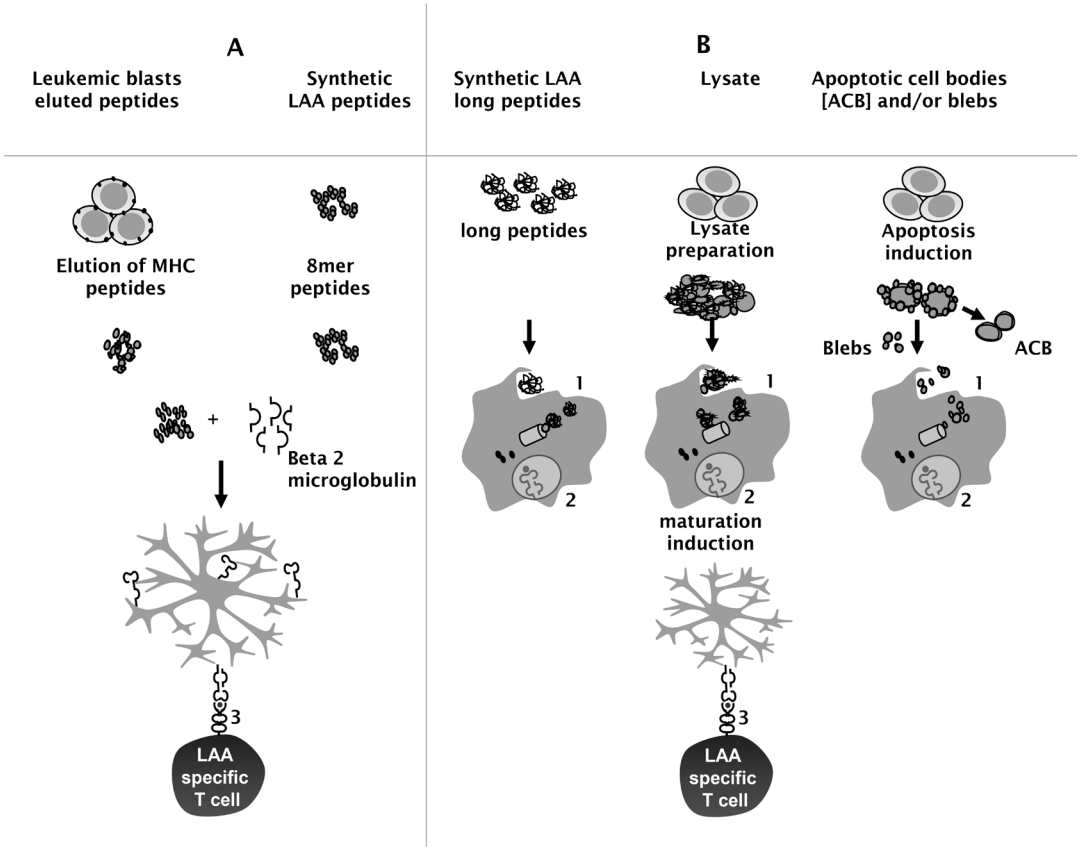
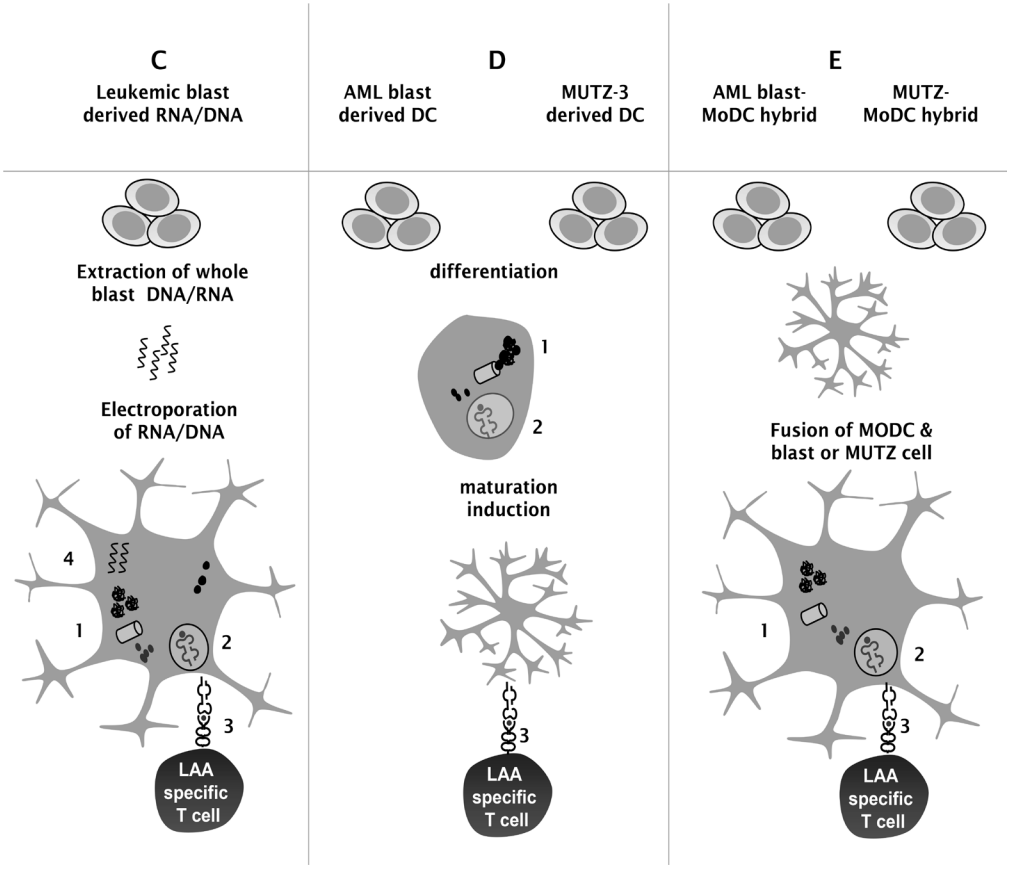


Figure 2. Dendritic cell (DC) vaccine preparation for clinical purposes. A. Mature DC can be loaded with peptides eluted from leukemic blasts or with synthetic 8-mer peptides. B. Immature DC can be loaded with synthetic long peptides, leukemic blast-derived lysate or apoptotic cells. Apoptotic cells can be subdivided in blebs or apoptotic cell bodies (ACB). Upon phagocytosis of tumor derived cell material, antigens are cleaved by the proteasome (1), cross-presented onto MHC class I molecules (2) and presented to LAA specific naive Tcells (3). Subsequently maturation can be induced with

like receptor ligands TLR-L (such as LPS) [7]. During active disease and shortly after complete remission the yields of functional MoDC are low, probably due to suppressive effects of leukemic blasts and intensive chemotherapy. Approximately three weeks after the last cycle of chemotherapy negative effects wane, suggesting that MoDC can best be obtained at a later timepoint after first CR [40;41]. For patients with an HLA-matched sibling, vaccination with MoDC derived from the donor might be an option, thereby circumventing leukemia- and treatment-related problems in the culture of patient-derived MoDC. Although the use of matched allogeneic MoDC is limited to those patients who have an eligible donor; emerging donor-banks for matched unrelated donors might overcome this problem. Furthermore, the



a cytokine cocktail. C. Mature DC can be eletroporated with tumor-derived DNA or RNA. This is transcribed intracellularly and processed via the proteasome onto MHC class I molecules. D. AML blasts or MUTZ-3 cells can be cultured with a cytokine cocktail into AML-DC or MUTZ-3-DC. These cells will present endogenous tumor-assaociated peptides by MHC class I molecules. E. Mature DC can be fused with an AML blast or AML cell line, such as the MUTZ-3.

use of non-matched allogeneic DC has been explored. An estimated 1-10% of the circulating T cell repertoire is directed against allo-Ag; alloDC are expected to trigger a broad T cell response thereby activating tumor-reactive T cells through cross-reactivity. On the other hand, high frequencies of allo-reactive T cells could overwhelm the proliferation of rare LAA-specific precursors [42]. Overall, clinical trials using allogeneic non-matched MoDC loaded with autologous lysates or apoptotic cells in solid malignancies were feasible and in general well tolerated [42-45].
Whereas, leukemic DC harbor a full range of endogenous LAA, MoDC need to be pulsed with exogenous LAA. Various sources of LAA have been used in an attempt to optimize anti-tumor responses. These include known LAA-derived peptides, apoptotic whole-cell suspensions, necrotic cell lysates, eluted peptides, cellular DNA or RNA, as well as chemical or electrical fusion of whole tumor cells with DC, and will be discussed in the next paragraps (summarized in *figure 2*.) [3;46].

Peptides derived from LAA

As mentioned above, WT1, PRAME, RHAMM, PR1, hTERT and survivin may all be considered as relevant peptide sources for DC loading. Unfortunately, some of these LAA are also expressed in normal hematopoietic progenitor cells and their overexpression is common but not uniform in leukemia [47]. Other obstacles to the use of MHC-I binding peptides for DC loading are low affinity of peptide binding to MHC class I molecules, the short time peptides reside on the surface of DC and a lack of Th cell activation [48]. Moreover, targeting a single Ag may result in immunologic pressure against the parent protein resulting in selection of Ag-loss leukemia variants [49]. Promotion of Ag-loss variants might be prevented by combining epitopes from different LAA. Unfortunately, HLA restriction of peptides limits the application to patients with certain HLA profiles. Many of these problems might be overcome by the use of longer peptides where the DC needs to process the long peptide into smaller parts before presentation onto MHC class I and II molecules [50]. Additionally, electroporation of RNA encoding for LAA epitopes might be preferable over the use of LAA peptides [51].

Eluted peptides

Naturally processed and presented peptides can be eluted from the surface of AML blasts. These peptides comprise all known and unknown presented LAA and therefore are hypothetically advantegous over known LAA, where only a few, mostly HLA-A2 associated epitopes, can be used. Eluted peptides from patients' blasts are unique for every patient and not hampered by HLA-restriction. In contrast to known LAA, eluted peptides can bind both HLA class I and II molecules and thereby activate both $CD8^+$ and $CD4^+$ T cells. Although further research is required concerning the low affinity of peptides binding to MHC molecules and the short time peptides reside on the surface of DC [48]. Elution of peptides from leukemic blasts can be done by using either citrate-phosphate (CP) or trifluoroacetic acid (TFA) buffer; for AML the TFA elution method to extract immunogenic AML-associated peptides was found to be favourable [52;53]. Application of this method might even be more efficacious when combined with depletion of regulatory T cells [54]. A direct comparison of eluted peptides and other LAA sources for loading strategies has not yet been made.

Apoptotic cells and blebs

Apoptosis, i.e. physiological programmed cell death, is associated with diverse cellular

changes to ensure that cells are cleared by the immune system. These changes include changes in the distribution of membrane lipids, membrane blebbing, condensation of both cytoplasm and chromatin, and fragmentation of the nucleus and cellular constituents into membrane-enclosed apoptotic bodies. Changes of molecules in the membrane make cells palatable for phagocytes. For example, translocation of soluble molecules such as phosphatidylserine and calreticulin to the plasma membrane or adsorption of soluble proteins from outside the cell such as C1q and thrombospondin are known to enhance uptake of apoptotic cells by DC and to lead to their activation [55-57]. One of the first studies showed that $CD34^+$-derived DC loaded with irradiated blasts were able to prime $CD8^+$ T cells into anti-leukemic CTL [58]. Furthermore, apoptotic cell loaded MoDC were able to generate anti-leukemic Th cells [59]. For loading of DC with apoptotic cells, induction of apoptosis should be as immunogenic as possible. For instance, upregulation of calreticulin by irradiation with UV light or by treatment with chemotherapeutic drugs, is associated with immunogenic apoptosis of colon carcinoma cells [55]. In myeloma, the proteosome inhibitor bortezomib induces upregulation of HSP90 and thereby enhances DC-mediated induction of immunity [60]. Furthermore, activation of cells before apoptosis induction, for instance with PHA, might trigger DC responses [61]. The importance of identification of immunogenic forms of cell death is further emphasized by the observation that apoptotic cell bodies can inhibit NF-κB signalling and thereby induce tolerogenic DC [62] This is in agreement with data showing that apoptotic cells cannot induce maturation of DC to the same level as lysates [63;64]. Cell death associated with upregulation of for instance TLR-L, might change a potentially tolerogenic source to an immunogenic source of Ag for DC loading [57;65]. Artificial introduction of TLR-L into leukemic blasts might be another way to make otherwise tolerogenic blasts more immunogenic prior to DC loading [66].

It is believed that in the process of dying, apoptotic cells shed immunogenic particles in the form of so-called blebs. After blebbing a remnant remains which is termed the apoptotic cell body (ACB). It was shown that these small blebs are taken up more efficiently by DC than (whole-cell) apoptotic bodies [67;68]. Blebs are vesicles with a size of 1-3µm, and can thereby be differentiated from other smaller extracellular microvesicles such as exosomes (size 40-100nm). This is of importance in tumor immunology as exosomes derived from tumor cells are thought to exert immunosuppressive effects [69]. Due to their size and weight, blebs can easily be separated from ACB and exosomes by centrifugation and thereby constitute a source of tumor Ag easily applicable for loading strategies of DC. Furthermore, in mouse studies blebs were shown to skew the immune response to a Th1 type [68]

Whole-cell lysate or necrotic material

It has long been assumed that apoptotic cell death is poorly immunogenic or even tolerogenic, whereas necrotic cell death is considered immunogenic [64;70-72]. However, the association of necrosis with local immune suppression in solid tumors has been shown as well [73]. Freeze/thaw cycles, used to generate whole-cell lysates, represent a form of brisk tumor cell death, most closely related to necrosis. These lysates harbor both known and unknown LAA. This advantage is a limitation as well, as lysates contain many non-malignant peptides which might increase the risk of auto-immunity [74]. Moreover, cell lysates may also contain intracellular proteases with inhibitory activity and immunosuppressive cytokines, such as TGFβ which might impair the immune response [75;76]. In order to combine the positive effect of upregulation of apoptosis related molecules with the immunogenicity of

lysates, a combination of heating before induction of necrosis by freeze-thaw cycles has been found favourable in a colon cancer model [77]. On the other hand, in *in vitro* assays lysate-loaded DC have been effective in priming $CD8^+$ cells, even more so than leukemic DC [35]. Furthermore, CTL primed *in vitro* with lysate-loaded DC demonstrated cytotoxic activity against leukemic targets [78;79]. Lysate-loaded DC vaccines administered to two AML patients were well tolerated without apparent side effects. In these two patients, an increase in autologous T cell stimulatory capacity of the DC and immunological responses by positive delayed-type hypersensitivity (DTH) skin reactivity could be shown [80].

Whole cell RNA/DNA

Whole cell RNA or cDNA encode for all cellular peptides and, hence, cover all known and unknown LAA and can be easily extracted or generated from whole-cell lysates and transfected into MoDC.[81-83]. Furthermore, amplification of RNA enables the use of small populations, such as the stem cell compartment, presumably containing the leukemia-initiating cell. Mature DC can be efficiently transfected through square wave electroporation with RNA (*figure 2D*). Peptides encoded by the transfected RNA are produced intracellularly and, therefore, directly processed and loaded onto MHC class I molecules, bypassing the need for cross presentation. On the other hand, $CD4^+$ T cells are of crucial importance to induce effective and long lasting immune responses [84]. This problem may be overcome by introducing both intracellular and extracellular Ag. Loading of MoDC with RNA and lysates from AML blasts was shown to be supplementary, leading to an enhancement of Ag loading onto both MHC class I and II molecules [85]. Other strategies with for instance LAMP-1 gene fusion for routing peptides to the lysosomes and class II presentation pathways have been explored for solid malignancies and might also be an option for AML [86].

Hybrids

Autologous AML blasts can be fused with mature MoDC, thereby generating hybrids that express LAA derived from the blast fusion partner as well as co-stimulatory and adhesion molecules from the MoDC (*figure 2E*). Although specific features of AML blasts such as low expression of co-stimulatory or adhesion molecules might hamper the Ag presenting and immunologic capacity of the DC. Banat and co-workers showed that DC hybridisation with AML blasts containing the chromosomal aberration inversion 16 (inv(16)) generated memory T cells able to react to AML blasts upon re-stimulation. This was not found for other aberrations and implies that different forms of AML could affect the outcome of this approach [87]. When using non-matched allogeneic MoDC, a fusion cell is generated expressing MHC molecules from the patients' leukemic blasts and co-stimulatory molecules from the donors' MoDC. These hybrids are able to induce anti-leukemic CTL *in vitro* [38]. Therefore, allogeneic MoDC [38;88] or MUTZ-3-DC fusions with AML blasts from patients might be an attractive option. AML is thought to originate from a malignant stem cell, which might be less sensitive for chemotherapeutics. These AML initiating cells with a $CD34^+CD38^-$ phenotype might be the cause of relapse. Ideally, these malignant stem cells should be targeted in immunotherapeutic protocols. By selecting $CD34^+CD38^-$ AML stem cells for the preparation of DC fusion cells, induced CTL may potentially eradicate AML by directly or indirectly targeting the leukemia initiating cells [89]. However, this approach is restricted by low numbers of stem cells and persistent controversy about the definition of malignant and normal hematopoietic stem cells.

Comparison of loading strategies

Weigel and co-workers compared AML-derived lysate-loaded MoDC with hybrids in mice but could not classify one as favourable over the other [90]. One in vitro study however, showed better CTL responses with DC-AML hybrids as compared to apoptotic cell-loaded DC or lysate-loaded DC [91]. *In vitro* anti-leukemic T cell responses could be found after co-culture of autologous remission lymphocytes with irradiated AML blast–loaded DC and fusion hybrids of AML blasts with DC. Interestingly, responses were observed against MoDC loaded with irradiated blasts in 2 out of 6 patients and against hybrids in 3 other patients out of the same 6 patients tested [88]. These results might imply that various AML patients need different vaccine preparations in order to achieve improved outcome and might explain the different results in small studies. Furthermore, different DC preparation protocols were used, complicating comparison. Translating results of DC loading of other tumors to AML is complicated due to different protocols in preparation of MoDC, the low number of patients tested, and varying results. [92;93]. Therefore, no definite conclusion can be drawn so far about which source of LAA and loading methods are preferred.

Vaccine adjuvants

The term adjuvant describes all compounds with intrinsic immunostimulatory and/or immunomodulatory properties. Adjuvants are used to manipulate or modulate different steps of the immune response. For instance, binding of pPRR enhances uptake of Ag and ligation of iPRR enhances processing and triggering of specific peptide presentation by MHC molecules combined with maturation of immunogenic DC. Different adjuvants may enhance the induction of different co-stimulatory signals in order to improve T cell responses or may inhibit negative co-stimulatory signals. The challenge is to define an optimal and safe combination of these different adjuvants which will result in an effective anti-tumor immune response when combined with MoDC vaccination [94].

Using adjuvants *in vitro* **to improve DC loading protocols**

Uptake of immunogeneic material by DC can be mediated by various receptors. The most well-known receptor family in this regard is the Fc receptor family. Coating of apoptotic or necrotic tumor cells with an Fc-domain containing antibody has been explored. Phagocytosis via Fc receptors is known to facilitate efficient cross-priming of exogenous Ag via MHC class I molecules. Tumor cells coated with an IgG antibody are internalized via Fcγ receptors on DC. In a murine B-cell lymphoma model this resulted in improved tumor protection [95;96]. Other groups of receptors related to phagocytosis are the scavenger receptors and TLR [16]. Upregulation of for instance the TLR-L calreticulin can be induced by irradiation with UV light or treatment with antracyclins [55;56]. Furthermore, various ways of inducing apoptosis or necrosis, such as heat shock, are associated with enhanced heat-shock protein (HSP) expression. HSP-peptide complexes are more easily taken up via scavenger receptors and TLR and induce cross-priming and skew the immune response towards a Th1 response [97;98]. Various other TLR-L have been used for *in vitro* vaccine preparation and administered as adjuvant together with the vaccine. Of note, careful dosing of these TLR-agonists *in vivo* is important, as high concentrations can be immunosuppressive [99;100].

Roughly, TLR-L can be divided into two groups: those with extracellular receptors (TLR-1, -2, -4, -5, and -11), and those with intracellular receptors (TLR-3, -7, -8 and -9). Since binding of TLR-1, -2 and -6 mostly results in a Th2 response and TLR-7 and -9 are not expressed on human MoDC, most research focused on other TLR-L. The most well known TLR-L is the TLR-4-agonist LPS, which is frequently used for maturation of DC *in vitro*. Since LPS itself is not clinically applicable, substrates from LPS have to be used, such as monophosphoryl lipid A (mLPA) [101]. In addition, components of the frequently applied adjuvant Bacillus Calmette Guérin (BCG) act as TLR-2/4 agonists stimulating cross-priming of Ag. Intracellular binding of TLR-3 and -8 (by their natural virus-derived ligands single- and double stranded RNA respectively) acts as a danger signal and results in enhanced cross-priming of exogenous Ag [102]. R848 (a TLR-7/8 agonist) and imiquimod (a TLR-7 agonist) have been shown to increase IFNα production in plasmacytoid DC and even induce perforin and granzyme B expression in myeloid DC [103] . The latter indicates the induction of direct cytotoxic immune responses and tumor destruction by myeloid DC. Next to activation of DC, TLR-L can facilitate the effector arm of the immune response: TLR-9 ligand cytosine-phophate-guanosine (CpG) that contains motifs from bacterial DNA, is able to skew the host immune response towards a Th1 response [104]. Nevertheless, CpG should be used with caution in future clinical trials as CpG has been shown to also recruit Treg in mice [105]. Smits and co-workers showed that electroporation of the TLR3-L poly-IC into AML blasts resulted in upregulated expression of MHC and co-stimulatory molecules and enhanced production of interferons by the AML blast itself. Furthermore, upon phagocytosis of these electroporated blasts by MoDC and subsequent co-incubation with T cells, their production of pro-inflammatory cytokines and Th1-polarizing capacity was enhanced [66].

In vivo comparison of loading strategies in AML

In AML only a few clinical phase-I vaccination trials with AML-DC have been performed and even less clinical trials using MoDC loaded with whole-cell derived LAA. The available data are summarized below.

Clinical studies on leukemic DC-based vaccination

The first AML-DC vaccine phase 1 trial in 5 patients was published in 2006 and showed promising results. T cells specificly recognizing a PRAME-derived peptide could be detected upon vaccination [106]. Another phase I/II study with AML-DC was performed by Roddie and co-workers (2006) [29]. Five patients, four *de novo* and one relapse, achieved CR and were vaccinated with autologous leukemic blasts that underwent DC differentiation *ex vivo*. Increased anti-leukemic T cell responses in four patients were observed. MRD was monitored by RT-PCR for WT1 gene expression. Two patients relapsed shortly after start of vaccination; the remaining patients had relatively constant WT1 expression levels following vaccination. Two patients remained in remission for more than 12 months post-vaccination [29].

Clinical applicability of these vaccines is limited by many factors, i.e. insufficient numbers of leukemic blasts for culture of AML-DC, failure of leukemic blasts to undergo DC differentiation and failure of patients to achieve CR due to progressive disease or death before vaccination. Overall, it could be concluded that leukemia-derived DC-based vaccination is only feasible in a subgroup of patients [30;107].

Clinical studies on MoDC-based vaccination

Clinical experiences with immunotherapy mostly focus on vaccination with synthetic peptides. For instance, vaccination with a RHAMM peptide resulted in clinical and immunological responses in MDS, AML and Multiple Myeloma patients and comparable results were found with WT1 and PR1 peptide vaccines [108-111]. Beside vaccination with AML-DC, there is little clinical experience with other DC vaccination strategies in the treatment of AML. In 2007, the first AML patient (who relapsed after allogeneic stem cell transplantation) was vaccinated with WT1 and KLH peptide-loaded MoDC derived from a healthy donor. Vaccination induced immune responses to KLH, whereas immune responses to WT1 could not be detected and the leukemia gradually progressed [112]. Another Phase I study using MoDC electroporated with RNA encoding for the WT1 antigen showed that this approach is safe in a clinical setting [51].

Even less studies have been performed with DC loaded with whole-blast cell derived LAA. DC loaded with blast lysates were administered at a 2- to 3-week interval to two patients with relapse after autologous peripheral blood SCT (PBSCT) in four infusions. Mixed leukocyte reaction and DTH tests with leukemic lysates showed no response before vaccination but did so afterwards, though no hematological responses were observed [80]. More DC-based clinical vaccination trials in AML have been started and are expected to be published in the near future. These include, amongst others, loading of MoDC with whole-cell RNA and mRNA encoding for WT1 or hTERT, fusion of MoDC with leukemic cells, and can be found at http://www.clinicaltrials.gov or http://clinicaltrialsfeeds.org.

Timing of vaccination in relation to standard AML therapy

DC vaccination is thought to be more effective when the immune system is intact and not affected by cancer [7;113]. Although frequencies are low in healthy donors, LAA-specific T cells can easily be expanded upon stimulation with Ag loaded autologous MoDC. In CML, it has been demonstrated that significantly higher percentages of BCR-ABL specific CTL can be detected in patients by tetramer (Tm) screening, but these Tm-specific CTL could not be expanded [114]. Chemotherapy and stem cell transplantation (SCT) treatment are likely to influence the effect of immunotherapy by affecting composition and functionality of T cell compartments. *In vitro* priming studies with AML-DC at various time points during remission resulted in different immune responses. During early remission anti-AML immune responses seem to be largely MHC-restricted, but to shift towards non-MHC restricted immune response later in CR [115]. Furthermore, leukemic blasts induce T cell suppression by production of suppressive cytokines and activation of suppressive cells such as MDSC and Treg. When considering these suppressive effects of both leukemic blasts and chemotherapy, DC vaccines should ideally be administered during CR. With sensitive detection methods, for instance by flow cytometry or with molecular tools, minimal residual disease (MRD) cells can be followed and a relapse can be detected in an early phase [116;117]. Therefore, the time frame in which a vaccine can be administered can be accurately determined by intensive follow up for MRD. After SCT it takes about 6 months before $CD8^+$ cells are fully reconstituted; for $CD4^+$ T cells this can take more than one year. As a consequence, the immune system is not fully capable of controlling MRD or responding to a DC vaccine. Donor

lymphocyte infusion (DLI) is used clinically for controlling MRD and preventing relapse of AML [117]. DLI might be of interest to combine with DC vaccination protocols. Furthermore, *in vivo* depletion of $CD25^+$ Treg cells, has been shown to increase the efficacy of an eluted peptide-loaded DC vaccine in an AML mouse model [54]. In order to administer the vaccine early after treatment, other options such as reducing the immunosuppressive effects of chemotherapy should be explored. Various chemotherapeutic immunomodulatory drugs have been described, such as antracyclins, able to stimulate anti-cancer immune responses [55]. Other new immunomodulaory drugs are of interest, such as lenalidomide, histone deacetylase inhibitors (HDACi) and 5-Azacitidine. 5-Azacitidine is known to enable cell proliferation and differentiation, which may allow normal hematopoietic progenitor cells to grow and differentiate and HDAC inhibitors have been shown to induce MHC class I and II expression on tumor cell lines and enhance differentiation of AML blasts into DC. Furthermore, lenalidomide has been demonstrated to enhance in vitro expansion and Th1 skewing of cells [118-121]. The immunomodulatory effects of these drugs might thus increase the window in which a vaccine could be administered.

The choice of vaccination site

Next to timing, the site of vaccination is a matter of debate. Intradermal, subcuteanous, intravenous and intranodal vaccination differentially affects the primary immune response and the distribution of memory cells and thereby the ability to control the outgrowth of tumors [122]. When a vaccine is administered intradermally or subcutaneously, this may improve T cell responses as compared to intravenous administration [123;124]. Intradermal or subcutaneous vaccination requires the DC to migrate to the lymph node. In this respect, addition of PGE_2 in DC preparation protocols is necessary [125;126]. However, PGE_2 diminishes IL-12 secretion and adversely affects CTL stimulatory ability of DC [127;128]. In addition, it was recently demonstrated that PGE_2 matured DC can recruit Treg [129]. Addition of TLR-L might (partially) overcome these inhibitory effects. Therefore, vaccine preparation protocols might determine the preferred site of vaccination [130]. Intranodal administration may circumvent this problem but requires technical expertise and includes the risk of damaging the architecture of the lymph node [131]. Available evidence points to the dermis as a preferred site of vaccine administration [132].

Conclusion and future perspectives

DC vaccination in AML holds promise as a treatment modality. LAA-loaded MoDC-based vaccines can be produced on a large scale, are easy to administer in an outpatient setting and are generally well-tolerated. The use of leukemia-derived DC is feasible in approximately 60% of AML patients. As an alternative, MoDC have been loaded with apoptotic whole-cell suspensions, necrotic cell lysates, tumor-associated peptides, eluted peptides and cellular DNA or RNA, but none of these loading strategies has yet been definitively proven favourable over another. In addition, MoDC have been chemically or electrically fused with leukemic blasts and DC have been cultured out of leukemic blasts. *In vitro* research on monocyte-derived vaccines in AML is limited by the low number of patients tested, differences

in vaccine preparation protocols and the heterogeneity of AML. The identification of an optimal approach for inducing tumor cell death or obtaining peptides that would lead to effective endocytosis and activation of MoDC remains controversial. Ongoing research is needed in order to achieve consensus about which Ag loading strategy for MoDC combined with administration of optimal adjuvants is most suitable. In addition, major progress can be made through the development of a read out system in which the TAA loading efficiency of various MoDC preparations can be tested.

References

1. Provan D, Singer CRJ, Baglin T, Lilleyman J. Acute myeloblastic leukaemia (AML). In: Provan D, Singer CRJ, Baglin T, Lilleyman J, eds. Oxford handbook of clinical haematology. Oxford: Oxford University Press; 2004:149-157.
2. Ridgway D. The first 1000 dendritic cell vaccinees. Cancer Invest 2003;21:873-886.
3. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat.Rev. Immunol. 2005;5:296-306.
4. Ballestrero A, Boy D, Moran E et al. Immunotherapy with dendritic cells for cancer. Adv.Drug Deliv.Rev. 2008;60:173-183.
5. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat.Med. 2004;10:909-915.
6. Itoh K, Yamada A, Mine T, Noguchi M. Recent advances in cancer vaccines: an overview. Jpn.J.Clin.Oncol. 2009;39:73-80.
7. Figdor CG, de V, I, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat.Med. 2004;10:475-480.
8. Small EJ, Schellhammer PF, Higano CS et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. J.Clin.Oncol. 2006;24:3089-3094.
9. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat.Immunol. 2002;3:991-998.
10. Chamuleau ME, Westers TM, van DL et al. Immune mediated autologous cytotoxicity against hematopoietic precursor cells in patients with myelodysplastic syndrome. Haematologica 2009;94:496-506.
11. Koebel CM, Vermi W, Swann JB et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature 2007;450:903-7
12. Binder RJ, Srivastava PK. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of $CD8^+$ T cells. Nat.Immunol. 2005;6:593-599.
13. Burgdorf S, Kautz A, Bohnert V, Knolle PA, Kurts C. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. Science 2007;316:612-616.
14. Pardoll DM. Spinning molecular immunology into successful immunotherapy. Nat.Rev. Immunol. 2002;2:227-238.
15. Padovan E, Landmann RM, De LG. How pattern recognition receptor triggering influences T cell responses: a new look into the system. Trends Immunol. 2007;28:308-314.
16. Schnare M, Barton GM, Holt AC et al. Toll-like receptors control activation of adaptive immune responses. Nat.Immunol. 2001;2:947-950.

17. West MA, Wallin RP, Matthews SP et al. Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. Science 2004;305:1153-1157.
18. Greiner J, Schmitt M, Li L et al. Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. Blood 2006;108:4109-4117.
19. Saudemont A, Quesnel B. In a model of tumor dormancy, long-term persistent leukemic cells have increased B7-H1 and B7.1 expression and resist CTL-mediated lysis. Blood 2004;104:2124-2133.
20. Van luijn M, Chamuleau ME, Thompson JA et al. CLIP down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased $CD4^+$ T cell responses. Haematologica 2010;95:485-93
21. Chamuleau, M. E., Feller, N., Kelder, A., van de Loosdrecht, A. A., and Ossenkoppele, G. J. High CLIP (Class II Associated Invariant Chain Peptide) Expression on Minimal Residual Disease Cells Is Associated with High Relapse Rate in AML Patients. Blood 2006;108:Abstract #2300
22. Chamuleau ME, van de Loosdrecht AA, Hess CJ et al. High INDO (indoleamine 2,3-dioxygenase) mRNA level in blasts of acute myeloid leukemic patients predicts poor clinical outcome. Haematologica 2008;93:1894-1898.
23. Bronte V, Mocellin S. Suppressive influences in the immune response to cancer. J.Immunother. 2009;32:1-11.
24. Szczepanski MJ, Szajnik M, Czystowska M et al. Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia. Clin.Cancer Res. 2009;15:3325-3332.
25. Jedema I, Meij P, Steeneveld E et al. Early detection and rapid isolation of leukemia-reactive donor T cells for adoptive transfer using the IFN-gamma secretion assay. Clin.Cancer Res. 2007;13:636-643.
26. O'Neill DW, Adams S, Bhardwaj N. Manipulating dendritic cell biology for the active immunotherapy of cancer. Blood 2004;104:2235-2246.
27. Andersen MH, Sorensen RB, Schrama D et al. Cancer treatment: the combination of vaccination with other therapies. Cancer Immunol.Immunother. 2008;57:1735-1743.
28. Li L, Reinhardt P, Schmitt A et al. Dendritic cells generated from acute myeloid leukemia (AML) blasts maintain the expression of immunogenic leukemia associated antigens. Cancer Immunol.Immunother. 2005;54:685-693.
29. Roddie H, Klammer M, Thomas C et al. Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia. Br.J.Haematol. 2006;133:152-157.
30. Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Feasibility of clinical dendritic cell vaccination in acute myeloid leukemia. Immunobiology 2006;211:677-685.
31. Santegoets SJ, Masterson AJ, van der Sluis PC et al. A $CD34(^+)$ human cell line model of myeloid dendritic cell differentiation: evidence for a CD14(+)CD11b(+) Langerhans cell precursor. J.Leukoc.Biol. 2006;80:1337-1344.
32. Masterson AJ, Sombroek CC, de Gruijl TD et al. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from $CD34^+$ precursors. Blood 2002;100:701-703.

33. Santegoets SJ, van den Eertwegh AJ, van de Loosdrecht AA, Scheper RJ, de Gruijl TD. Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. J.Leukoc. Biol. 2008;84:1364-1373.
34. Rasaiyaah J, Noursadeghi M, Kellam P, Chain B. Transcriptional and functional defects of dendritic cells derived from the MUTZ-3 leukaemia line. Immunology 2009;127:429-441.
35. Lee JJ, Choi BH, Kang HK et al. Monocyte-derived dendritic cells from HLA-matched allogeneic donors showed a greater ability to induce leukemic cell-specific T cells in comparison to leukemic cell-derived dendritic cells or monocyte-derived dendritic cells from AML patients. Leuk.Res. 2008;32:1653-1660.
36. Lee JJ, Nam CE, Nam JH et al. Generation of cytotoxic donor $CD8^+$ T cells against relapsing leukemic cells following allogeneic transplantation by stimulation with leukemic cell- or leukemic lysate pulsed donor cell-derived dendritic cells. Leuk.Res. 2004;28:517-524.
37. van de Loosdrecht AA. The dendritic cell: the piano player in orchestrating the immune response in leukemia. Leuk.Lymphoma 2007;48:217-218.
38. Gong J, Koido S, Kato Y et al. Induction of anti-leukemic cytotoxic T lymphocytes by fusion of patient-derived dendritic cells with autologous myeloblasts. Leuk.Res. 2004;28:1303-1312.
39. Spisek R, Chevallier P, Morineau N et al. Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. Cancer Res. 2002;62:2861-2868.
40. Hasebe H, Nagayama H, Sato K et al. Dysfunctional regulation of the development of monocyte-derived dendritic cells in cancer patients. Biomed.Pharmacother. 2000;54:291-298.
41. Royer PJ, Bougras G, Ebstein F et al. Efficient monocyte-derived dendritic cell generation in patients with acute myeloid leukemia after chemotherapy treatment: Application to active immunotherapy. Exp.Hematol. 2008;36:329-339.
42. Merrick A, Diaz RM, O'Donnell D et al. Autologous versus allogeneic peptide-pulsed dendritic cells for anti-tumour vaccination: expression of allogeneic MHC supports activation of antigen specific T cells, but impairs early naive cytotoxic priming and anti-tumour therapy. Cancer Immunol.Immunother. 2008;57:897-906.
43. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. Nat.Rev.Immunol. 2007;7:942-953.
44. Hus I, Rolinski J, Tabarkiewicz J et al. Allogeneic dendritic cells pulsed with tumor lysates or apoptotic bodies as immunotherapy for patients with early-stage B-cell chronic lymphocytic leukemia. Leukemia 2005;19:1621-1627.
45. Holtl L, Ramoner R, Zelle-Rieser C et al. Allogeneic dendritic cell vaccination against metastatic renal cell carcinoma with or without cyclophosphamide. Cancer Immunol. Immunother. 2005;54:663-670.
46. van der Bruggen P., Van den Eynde BJ. Processing and presentation of tumor antigens and vaccination strategies. Curr.Opin.Immunol. 2006;18:98-104.
47. Greiner J, Ringhoffer M, Taniguchi M et al. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. Int.J.Cancer 2004;108:704-711.
48. Amoscato AA, Prenovitz DA, Lotze MT. Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. J.Immunol. 1998;161:4023-4032.

49. Toes RE, Offringa R, Blom RJ, Melief CJ, Kast WM. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. Proc.Natl.Acad.Sci.U.S.A 1996;93:7855-7860.
50. Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat.Rev.Cancer 2008;8:351-360.
51. Van Driessche A, Van de Velde AL, Nijs G et al. Clinical-grade manufacturing of autologous mature mRNA-electroporated dendritic cells and safety testing in acute myeloid leukemia patients in a phase I dose-escalation clinical trial. Cytotherapy. 2009:11:653-6
52. Delluc S, Tourneur L, Fradelizi D et al. DC-based vaccine loaded with acid-eluted peptides in acute myeloid leukemia: the importance of choosing the best elution method. Cancer Immunol.Immunother. 2006;56:1-12.
53. Delluc S, Tourneur L, Michallet AS et al. Autologous peptides eluted from acute myeloid leukemia cells can be used to generate specific antileukemic CD4 helper and CD8 cytotoxic T lymphocyte responses in vitro. Haematologica 2005;90:1050-1062.
54. Delluc S, Hachem P, Rusakiewicz S et al. Dramatic efficacy improvement of a DC-based vaccine against AML by CD25 T cell depletion allowing the induction of a long-lasting T cell response. Cancer Immunol.Immunother. 2009;58:1669-1677.
55. Obeid M, Tesniere A, Ghiringhelli F et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat.Med. 2007;13:54-61.
56. Kono H, Rock KL. How dying cells alert the immune system to danger. Nat.Rev.Immunol. 2008;8:279-289.
57. Panaretakis T, Kepp O, Brockmeier U et al. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. EMBO J. 2009;28:578-590.
58. Fujii S, Fujimoto K, Shimizu K et al. Presentation of tumor antigens by phagocytic dendritic cell clusters generated from human $CD34^+$ hematopoietic progenitor cells: induction of autologous cytotoxic T lymphocytes against leukemic cells in acute myelogenous leukemia patients. Cancer Res. 1999;59:2150-2158.
59. Thomas-Kaskel AK, ves Portugal TG, Herchenbach D et al. Allogeneic HLA-Matched Donor Dendritic Cells Loaded with Patient Leukemic Blasts Preferentially Induce CD4-Positive Leukemia-Reactive Donor Lymphocytes. Acta Haematol. 2007;117:226-235.
60. Spisek R, Charalambous A, Mazumder A et al. Bortezomib enhances dendritic cell (DC) mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. Blood 2007;109:4839-4845.
61. Johansson U, Walther-Jallow L, Smed-Sorensen A, Spetz AL. Triggering of Dendritic Cell Responses after Exposure to Activated, but Not Resting, Apoptotic PBMCs. J.Immunol. 2007;179:1711-1720.
62. Sen P, Wallet MA, Yi Z et al. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. Blood 2007;109:653-660.
63. Blander JM, Medzhitov R. On regulation of phagosome maturation and antigen presentation. Nat.Immunol. 2006;7:1029-1035.
64. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nat. Rev.Immunol. 2009;9:353-363.
65. Spisek R, Dhodapkar MV. Towards a better way to die with chemotherapy: role of heat shock protein exposure on dying tumor cells. Cell Cycle 2007;6:1962-1965.

66. Smits EL, Ponsaerts P, Van de Velde AL et al. Proinflammatory response of human leukemic cells to dsRNA transfection linked to activation of dendritic cells. Leukemia 2007;21:1691-1699.
67. Lane JD, Allan VJ, Woodman PG. Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells. J.Cell Sci. 2005;118:4059-4071.
68. Fransen JH, Hilbrands LB, Ruben J et al. Mouse dendritic cells matured by ingestion of apoptotic blebs induce T cells to produce interleukin-17. Arthritis Rheum. 2009;60:2304-2313.
69. Iero M, Valenti R, Huber V et al. Tumour-released exosomes and their implications in cancer immunity. Cell Death.Differ. 2008;15:80-88.
70. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J.Exp.Med. 2000;191:411-416.
71. Sauter B, Albert ML, Francisco L et al. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J.Exp.Med. 2000;191:423-434.
72. Rock KL, Kono H. The inflammatory response to cell death. Annu.Rev.Pathol. 2008;3:99-126.
73. Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. Nat.Rev.Immunol. 2004;4:641-648.
74. Roskrow MA, Dilloo D, Suzuki N et al. Autoimmune disease induced by dendritic cell immunization against leukemia. Leuk.Res. 1999;23:549-557.
75. Schui DK, Singh L, Schneider B et al. Inhibiting effects on the induction of cytotoxic T lymphocytes by dendritic cells pulsed with lysates from acute myeloid leukemia blasts. Leuk. Res. 2002;26:383-389.
76. Galea-Lauri J, Wells JW, Darling D, Harrison P, Farzaneh F. Strategies for antigen choice and priming of dendritic cells influence the polarization and efficacy of antitumor T-cell responses in dendritic cell-based cancer vaccination. Cancer Immunol.Immunother. 2004;53:963-977.
77. Qiu J, Li GW, Sui YF et al. Heat-shocked tumor cell lysate-pulsed dendritic cells induce effective anti-tumor immune response in vivo. World J.Gastroenterol. 2006;12:473-478.
78. Xing D, Decker WK, Li S et al. AML-loaded DC generate Th1-type cellular immune responses in vitro. Cytotherapy. 2006;8:95-104.
79. Lee JJ, Park MS, Park JS et al. Induction of leukemic-cell-specific cytotoxic T lymphocytes by autologous monocyte-derived dendritic cells presenting leukemic cell antigens. J.Clin.Apher. 2006;21:188-194.
80. Lee JJ, Kook H, Park MS et al. Immunotherapy using autologous monocyte-derived dendritic cells pulsed with leukemic cell lysates for acute myeloid leukemia relapse after autologous peripheral blood stem cell transplantation. J.Clin.Apher. 2004;19:66-70.
81. Hsu AK, Kerr BM, Jones KL et al. RNA loading of leukemic antigens into cord blood-derived dendritic cells for immunotherapy. Biol.Blood Marrow Transplant. 2006;12:855-867.
82. Jarnjak-Jankovic S, Pettersen RD, Saeboe-Larssen S, Wesenberg F, Gaudernack G. Evaluation of dendritic cells loaded with apoptotic cancer cells or expressing tumour mRNA as potential cancer vaccines against leukemia. BMC.Cancer 2005;5:20-30.
83. Jarnjak-Jankovic S, Hammerstad H, Saeboe-Larssen S, Kvalheim G, Gaudernack G. A full scale comparative study of methods for generation of functional Dendritic cells for use as cancer vaccines. BMC.Cancer 2007;7:119.

84. Dissanayake SK, Tuera N, Ostrand-Rosenberg S. Presentation of endogenously synthesized MHC class II-restricted epitopes by MHC class II cancer vaccines is independent of transporter associated with Ag processing and the proteasome. J.Immunol. 2005;174:1811-1819.
85. Decker WK, Xing D, Li S et al. Double loading of dendritic cell MHC class I and MHC class II with an AML antigen repertoire enhances correlates of T-cell immunity in vitro via amplification of T-cell help. Vaccine 2006;24:3203-3216.
86. Ji H, Wang TL, Chen CH et al. Targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartment enhances the antitumor immunity of DNA vaccines against murine human papillomavirus type 16 E7-expressing tumors. Hum.Gene Ther. 1999;10:2727-2740.
87. Banat GA, Usluoglu N, Hoeck M et al. Dendritic cells fused with core binding factor-beta positive acute myeloid leukaemia blast cells induce activation of cytotoxic lymphocytes. Br.J.Haematol. 2004;126:593-601.
88. Klammer M, Waterfall M, Samuel K, Turner ML, Roddie PH. Fusion hybrids of dendritic cells and autologous myeloid blasts as a potential cellular vaccine for acute myeloid leukaemia. Br.J.Haematol. 2005;129:340-349.
89. Lei Z, Zhang GM, Hong M, Feng ZH, Huang B. Fusion of dendritic cells and $CD34^{+}CD38^{-}$ acute myeloid leukemia (AML) cells potentiates targeting AML-initiating cells by specific CTL induction. J.Immunother. 2009;32:408-414.
90. Weigel BJ, Panoskaltsis-Mortari A, Diers M et al. Dendritic cells pulsed or fused with AML cellular antigen provide comparable in vivo antitumor protective responses. Exp.Hematol. 2006;34:1403-1412.
91. Galea-Lauri J, Darling D, Mufti G, Harrison P, Farzaneh F. Eliciting cytotoxic T lymphocytes against acute myeloid leukemia-derived antigens: evaluation of dendritic cell-leukemia cell hybrids and other antigen-loading strategies for dendritic cell-based vaccination. Cancer Immunol.Immunother. 2002;51:299-310.
92. Kotera Y, Shimizu K, Mule JJ. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. Cancer Res. 2001;61:8105-8109.
93. Larmonier N, Merino D, Nicolas A et al. Apoptotic, necrotic, or fused tumor cells: an equivalent source of antigen for dendritic cell loading. Apoptosis. 2006;11:1513-1524.
94. Guy B. The perfect mix: recent progress in adjuvant research. Nat.Rev.Microbiol. 2007;5:505-517.
95. Amigorena S. Fc gamma receptors and cross-presentation in dendritic cells. J.Exp.Med. 2002;195:F1-F3.
96. Franki SN, Steward KK, Betting DJ et al. Dendritic cells loaded with apoptotic antibody-coated tumor cells provide protective immunity against B cell lymphoma in vivo. Blood 2007;111:1504-1511.
97. Castellino F, Boucher PE, Eichelberg K et al. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J.Exp.Med. 2000;191:1957-1964.
98. Shi H, Cao T, Connolly JE et al. Hyperthermia enhances CTL cross-priming. J.Immunol. 2006;176:2134-2141.
99. Gavin AL, Hoebe K, Duong B et al. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 2006;314:1936-1938.
100. Wilson NS, Behrens GM, Lundie RJ et al. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. Nat. Immunol. 2006;7:165-172.

101. Ten Brinke A., Karsten ML, Dieker MC, Zwaginga JJ, Van Ham SM. The clinical grade maturation cocktail monophosphoryl lipid A plus IFNgamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. Vaccine 2007;25:7145-7152.
102. Seya T, Akazawa T, Tsujita T, Matsumoto M. Role of Toll-like receptors in adjuvant-augmented immune therapies. Evid.Based.Complement Alternat.Med. 2006;3:31-38.
103. Stary, G., Bangert, C., Altrichter, S., Strohal, R., Kopp, T., and Stingle, G. Dendritic cells with cytotoxic potential after toll-like receptor 7/8-activation. Dendritic Cells 2006;9th International Conference:S02.03
104. Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat.Rev.Immunol. 2004;4:249-258.
105. Jarnicki AG, Conroy H, Brereton C et al. Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. J.Immunol. 2008;180:3797-3806.
106. Li L, Giannopoulos K, Reinhardt P et al. Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts. Int.J.Oncol. 2006;28:855-861.
107. Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Dendritic cell-based immunotherapy in acute and chronic myeloid leukaemia. Biomed.Pharmacother. 2007;61:306-314.
108. Oka Y, Tsuboi A, Taguchi T et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc.Natl.Acad. Sci.U.S.A 2004;101:13885-13890.
109. Rezvani K, Yong AS, Mielke S et al. Leukemia-associated antigen specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood 2007
110. Gao L, Bellantuono I, Elsasser A et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood 2000;95:2198-2203.
111. Bellantuono I, Gao L, Parry S et al. Two distinct HLA-A0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. Blood 2002;100:3835-3837.
112. Kitawaki T, Kadowaki N, Kondo T et al. Potential of dendritic cell immunotherapy for relapse after allogeneic hematopoietic stem cell transplantation, shown by WT1 peptide- and keyhole limpet hemocyanin-pulsed, donor-derived dendritic cell vaccine for acute myeloid leukemia. Am.J.Hematol. 2007;83:315-317.
113. Tacken PJ, de V, I, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. Nat.Rev.Immunol. 2007;7:790-802.
114. Rusakiewicz S, Madrigal A, Travers P, Dodi AI. BCR/ABL-specific $CD8^+$ T cells can be detected from CML patients, but are only expanded from healthy donors. Cancer Immunol. Immunother. 2009;58:1449-1457.
115. Westers TM, Houtenbos I, Snoijs NC, van de Loosdrecht AA, Ossenkoppele GJ. Leukemia-derived dendritic cells in acute myeloid leukemia exhibit potent migratory capacity. Leukemia 2005;19:1270-1272.
116. Feller N, van der Pol MA, van SA et al. MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. Leukemia 2004;18:1380-1390.

117. Candoni A, Tiribelli M, Toffoletti E et al. Quantitative assessment of WT1 gene expression after allogeneic stem cell transplantation is a useful tool for monitoring minimal residual disease in acute myeloid leukemia. Eur.J.Haematol. 2009;82:61-68.
118. Xu W, Celeridad M, Sankar S, Webb DR, Bennett BL. CC-4047 promotes Th1 cell differentiation and reprograms polarized human Th2 cells by enhancing transcription factor T-bet. Clin. Immunol. 2008;128:392-399.
119. Khong T, Sharkey J, Spencer A. The effect of azacitidine on interleukin-6 signaling and nuclear factor-kappaB activation and its in vitro and in vivo activity against multiple myeloma. Haematologica 2008;93:860-869.
120. Magner WJ, Kazim AL, Stewart C et al. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J.Immunol. 2000;165:7017-7024.
121. Moldenhauer A, Frank RC, Pinilla-Ibarz J et al. Histone deacetylase inhibition improves dendritic cell differentiation of leukemic blasts with AML1-containing fusion proteins. J.Leukoc.Biol. 2004;76:623-633.
122. Mullins DW, Sheasley SL, Ream RM et al. Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. J.Exp.Med. 2003;198:1023-1034.
123. Fong L, Brockstedt D, Benike C, Wu L, Engleman EG. Dendritic cells injected via different routes induce immunity in cancer patients. J.Immunol. 2001;166:4254-4259.
124. Butterfield LH, Ribas A, Dissette VB et al. Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clin.Cancer Res. 2003;9:998-1008.
125. Krause P, Bruckner M, Uermosi C et al. Prostaglandin E(2) enhances T-cell proliferation by inducing the costimulatory molecules OX40L, CD70, and 4-1BBL on dendritic cells. Blood 2009;113:2451-2460.
126. de Vries I, Lesterhuis WJ, Scharenborg NM et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin.Cancer Res. 2003;9:5091-5100.
127. von Bergwelt-Baildon MS, Popov A, Saric T et al. CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo: additional mechanisms of T-cell inhibition. Blood 2006;108:228-237.
128. Luft T, Jefford M, Luetjens P et al. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. Blood 2002;100:1362-1372.
129. Muthuswamy R, Urban J, Lee JJ et al. Ability of mature dendritic cells to interact with regulatory T cells is imprinted during maturation. Cancer Res. 2008;68:5972-5978.
130. Dauer M, Lam V, Arnold H et al. Combined use of toll-like receptor agonists and prostaglandin E(2) in the FastDC model: Rapid generation of human monocyte-derived dendritic cells capable of migration and IL-12p70 production. J.Immunol.Methods 2008;337:97-105.
131. Bedrosian I, Mick R, Xu S et al. Intranodal administration of peptide-pulsed mature dendritic cell vaccines results in superior $CD8^+$ T-cell function in melanoma patients. J.Clin.Oncol. 2003;21:3826-3835.
132. Verdijk P, Aarntzen EH, Lesterhuis WJ et al. Limited amounts of dendritic cells migrate into the T-cell area of lymph nodes but have high immune activating potential in melanoma patients. Clin.Cancer Res. 2009;15:2531-2540.

Priming of PRAME and WT1 specific CD8^{+} T cells in healthy donors but not in AML patients in first complete remission: implications for immunotherapy

W van den Ancker
JM Ruben
TM Westers
D Wulandari
HJ Bontkes
E Hooijberg
AGM Stam
SJAM Santegoets
GJ Ossenkoppele
TD de Gruijl
AA van de Loosdrecht

Oncoimmunology.
2013 Apr 1;2(4):e23971

Abstract

Active immunotherapy may prevent relapse of acute myeloid leukemia (AML) by inducing leukaemia-specific T cells. Here we investigated whether WT1 and PRAME specific T cells could be induced after priming of healthy donor and patient derived T cells with HLA-A2 matched, peptide-loaded allogeneic dendritic cells. AML-reactive, tetramer (Tm) binding, and interferon producing, cytotoxic T Lymphocytes specific for PRAME could be cultured readily from healthy donors. PRAME specific priming efficiencies were significantly higher than for WT1. Priming of T cells from patient derived T cells was proved near-impossible: no LAA-specific T cells could be primed from patients shortly after reaching CR (n=4); in only 1 out of 3 patients with sustained CR did we find low-frequency WT1 specific T cells. This finding points to differences in the functionality or repertoire of T cells between HD and AML patients in CR and may have repercussions for the implementation of active vaccination approaches for AML.

Introduction

Therapeutic vaccination with dendritic cells (DC) is regarded as a viable option for the treatment of minimal residual disease in (MRD) acute myeloid leukemia (AML). DC vaccination aims to elicit a leukemia associated antigen (LAA)-specific immune response, directed against residual leukemic cells, thus preventing relapse [1]. In this respect, peptides carrying defined LAA-epitopes are an attractive source of antigen. Amongst others, Wilms tumor 1 (WT1) and preferentially expressed antigen of melanoma (PRAME) are both LAA that are over-expressed in various hematological malignancies and share the potential for the induction of specific T cell responses [2;3]. Expression rates in AML of each of these LAA are high, ranging from 60 to 90% [4]. Both PRAME and WT1 are involved in leukemogenesis. PRAME influences disease progression by interfering with retinoic acid (RA) receptor signalling and gene transcription [5], while WT1 inhibits differentiation through as yet unknown mechanisms [6;7]. Their involvement in leukemogenesis and their expression in malignant stem cells make PRAME and WT1 attractive antigens for the detection of MRD and valuable targets for therapy [8-12]. CTL epitope sequences of WT1 and PRAME have been characterized for both HLA-A2 and -A24 molecules [13-16]. Moreover, T cells recognizing PRAME- and WT1-derived endogeneous peptides that are presented by HLA-A2 molecules on the surface of tumor cells have been reported in various malignancies including myeloid leukemia [2;17;18].

Here, we investigated the relative priming efficiencies of cytotoxic T lymphocytes (CTL) in peripheral blood (PB) from healthy donors (HD) and leucapheresis (LF) material or Peripheral Blood samples from AML patients in first complete remission (CR).

Material and methods

Patient material

Peripheral blood cells were drawn from 9 AML patients after obtaining the patients' informed consent. Peripheral blood mononuclear cells from 3 patients in first complete remission for

1.5 year or longer (long-term CR1, LT-CR1; table 2) were isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Peripheral blood mononuclear cells, containing >80% leukemic cells, from 2 patients at diagnosis were isolated using Ficoll-Paque (Amersham Pharmacia Biotech) and stored upon use for cytoxicity assays. Cells were cryopreserved at a controlled rate in liquid nitrogen using RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 20% heat-inactivated FCS (Greiner, Alphen a/d Rijn, The Netherlands) and 10% dimethylsulphoxide (Merck, Darmstadt, Germany). Autologous leucapheresis products from 4 patients (Table 2, AML-CR1) were cryopreserved at a controlled rate according to institutional guidelines. Before application cryopreserved material was rapidly thawed and washed twice in RPMI-1640 supplemented with 40% FCS. The cells were then resuspended in culture medium as described below.

Cell lines

The CD34$^+$ human acute myeloid leukemia cell line MUTZ-3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) was cultured in MEM-α medium containing ribonucleosides and deoxyribonucleosides (Life Technologies, Paisley, UK) supplemented with 20% Fetal calf serum (FCS, Perbio, Helsingborg, Sweden), 100 I.E./ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 µg/ml streptomycin sulfate (Radiumfarma- Fisiopharma, Naples, Italy), 2.0 mM L-glutamine (L-glut, Invitrogen, Breda, The Netherlands), 0.01 mM 2-mercaptoethanol (βME, Merck, Darmstadt, Germany) and 10% 5637-conditioned medium (CM) [13, 19]. The EBV-transformed B cell line JY (HLA-A2$^+$) was cultured in IMDM (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum, 100 I.E./ml sodium penicillin, 100 lg/ml streptomycin sulphate, 2.0 mM ,-glutamine and 0.01 mM 2-mercapoethanol (complete medium). The CML cell lines K562 (HLA-A2- and PRAME$^+$), K562-A2$^+$ (a kind gift from Dr Carl June, Philadelphia PA; PRAME$^+$ and HLA-A2$^+$), and ME1 (HLA-A2$^+$ and PRAME-) (all from ATCC, Manassas, VA) were cultured in IMDM complete medium (consisting of IMDM; 10% FCS, 100 I.E/ml Penicillin, 100µg/ml Streptomycin, 2.0mM L-glut and 0.01 mM βME); medium was replenished twice a week.

In vitro isolation of CD8$^+$ T cells from buffy coats and generation of MUTZ-3-derived DC

Buffy coats were obtained from healthy volunteers according to institutional guidelines in accordance with the Helsinki Declaration of 1975. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque. Subsequently, CD8$^+$ T cells were obtained by negative depletion using an untouched magnetic Microbead-based kit (Miltenyi Biotec GmbH Bergisch Gladbach, Germany). MUTZ-3 DC were generated as described [19]. Briefly, MUTZ-3 progenitors were cultured in 12 well tissue culture plates at a concentration of 1x10^5/ml in MEM-α medium without 5637-CM in the presence of 100 ng/ml GM-CSF (Strathmann Biotec, Hamburg, Germany), 1,000 U/ml IL-4 (Strathmann Biotec) and 2.5 ng/ml TNF-α (Strathmann Biotec) for 7 days. Every 3 days new cytokines were added. At day 7, maturation of MUTZ-3 DC was induced by adding Monocyte Conditioned Medium (MCM) at 30% over a period of 3 days. For preparation of MCM, see next paragraph.

Monocyte conditioned medium

Buffy coats were obtained from healthy volunteers according to institutional guidelines. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation

using Ficoll-Paque. Subsequently, monocytes were obtained by plating PBMC onto immunoglobulin (30µg/ml; Sanquin, Amsterdam) coated plastic culture plates. Non adherent cells were washed away. Adherent cells were incubated for 24h in IMDM culture medium supplemented with Penicilin, L-glutamine and 8%FCS. Supernatants, containing cytokines produced by monocytes were harvested, aliquoted and stored at -20C until usage. All cultures were performed at 37˚C and 5% CO2 in a humidified incubator.

Tetramers and flow cytometry

PE- and/or APC-labeled HLA-A2 tetramers (Tm) presenting the WT1 (126-134) or PRAME(100-108) (Sanquin) were used for flowcytometric analysis. As control HIV1 Tm was used (a kind gift from Ton Schumacher (NKI), Amsterdam, The Netherlands). Tetramer staining was performed in PBS for 15 min at 37ºC. Stained cells were analyzed on a FACScalibur (BD Biosciences) using Cell QuestPro software. To exclude dead cells in flow cytometric tetramer analysis, 0.5 lg/ml propidium iodide (ICN Biomedicals, Zoetermeer, The Netherlands) was used. Tetramer-guided flow sorting was performed on a FACSAria (BD Biosciences).

CTL induction

MUTZ3-DC represent a standardized source of allogeneic DC that can be used to generate fully functional tumour-specific T cells [19;20] In vitro antigen-specific CTL were generated as described previously 30. Mature MUTZ-3 DC, prepared as described above, were loaded with 10 µg/ml peptide in the presence of 3 µg/ml β2-microglobulin (Sigma-Aldrich, St. Louise, MO, USA) for 2–4 h at room temperature and irradiated (50 Gy). Peptides used were PRAME 100-108 (VLDGLDVLL), synthesized according to FMOC technology (IHB-LUMC, Leiden, the Netherlands) and WT1 126-134 (RMFPNAPYL) obtained from Sanquin. $1{\cdot}10^5$ peptide-loaded DC were cultured for 10 days with $1{\cdot}10^6$ $CD8^+$ cells and $1{\cdot}10^6$ irradiated (50 Gy) $CD14^-/CD8^-$ PBMC in Yssel's medium 43 supplemented with 1% human AB serum (ICN Biochemicals), 10 ng/ml IL-6 and 10 ng/ml IL-12 in a 24 well tissue-culture plate. At day 1, 10 ng/ml IL-10 (R&D Systems) was added. From day 10, CTL cultures were stimulated every week for 5 weeks with $1{\cdot}10^5$ fresh peptide-loaded antigen presenting cells (10ng/ml) in the presence of 5 ng/ml IL-7 (Strathmann Biotec). For the first restimulation MUTZ-3 DC were used, for the second and further restimulations $HLA\text{-}A2^+$ JY cells were used as antigen presenting cells. One day prior to each restimulation, a sample was taken and analyzed by flow cytometry using both PE- and APC labelled Tm presenting the relevant epitope. Two days after each restimulation, 10 U/ml IL-2 (Strathmann Biotec) was added. PRAME Tm^+ CTL were isolated by Tm^+ flow sorting and subsequently cloned by limiting dilution. For this purpose, CTL were weekly stimulated with irradiated feeder-mix consisting of allogeneic PBMC (1:10 ratio with CTL) and JY cells (1:100 ratio with CTL) in Yssel's medium supplemented with 100 ng/ml phytohemagglutin (PHA; Murex Biotech, Dartford, UK) and 20 U/ml IL-2. Human telomerase reverse transcriptase (hTERT) was introduced in the PRAME-specific CTL clone. PRAME-specific CTL, stimulated for 48 h with feeder-mix as described above, were transduced with retrovirus encoding LZRS-hTERT-IRES-DNGFR, in fibronectin-coated cell culture plates (Retronectin, Takara, Japan) in the presence of 100 U/ml IL-2. During transduction, the plates were centrifuged at 2,000·g for 90 min at 23ºC and subsequently incubated at 37ºC for 4.5h. Then, cells were washed and cultured overnight in Yssel's medium containing 20 U/ml IL-2. Next day, retroviral transduction was repeated. After 48h, transduction efficiency was determined by flow cytometric analysis of nerve growth factor receptor (NGFR) expression on the surface of the cell.

Intracellular IFN-γ detection
To determine the capacity of the CTL clones to produce IFN-γ upon recognition of a specific target, intracellular IFN-γ staining was performed. Target cells used included HLA-A2$^+$/PRAME$^+$, HLA-A2-/PRAME$^+$ tumour cell lines and JY cells or T2 cells pulsed with either relevant or irrelevant peptide. CTL were cultured with target cells at an effector:target cell (E:T) ratio of 2:1 in 96-well round-bottom plate and 0.5 µl of GolgiPlug (BD Biosciences) was added to each well. After 6 h, cells were harvested, washed, stained with PE-labeled tetramer and APC-labeled anti-CD8 mAb. After fixation with 4% paraformaldehyde (Merck) and permeabilization with BD Perm/wash solution (BD Biosciences), cells were labeled with FITC conjugated anti-IFN-γ Ab (BD Biosciences) and were analyzed on a flow cytometer.

FACS based cytotoxicity assay
Cytotoxic activity of the PRAME CTL clone was assessed by a flow cytometry based cytotoxicity assay as described previously [21]. In short, effector T cells were labelled with CFSE (1µM for 10 min, Molecular Probes, Eugene, OR) and subsequently co-cultured with 104 target cells (cell lines and patient samples) in ratios of 20:1, 10:1, and 5:1 in sterile round bottom polystyrene tubes (BD Biosciences). All E:T ratios were tested in duplicate. Cell suspension volume was adjusted to 100µl with IMDM complete medium. Separate control cultures of effector and target cells alone were performed to check spontaneous apoptosis and secondary necrosis. Co-cultures were kept for 6h in a 37ºC humidified incubator. At the end of cytotoxic contact, target cells were stained at 37°C with anti-CD34-PE, followed by staining for SYTO62 and 7-AAD [21] MHC-restriction was tested by addition of MHC class I-blocking antibodies (W6.32, 2.5 mg/ml, a kind gift of Dr. SM van Ham, Department of Immunopathology, Sanquin Research, Amsterdam, The Netherlands) or the appropriate isotype control (mouse IgG2a, 2.5 mg/ml, Sanquin).

Statistical analysis
Statistical significance of differences between the various cytotoxicity experiments was determined by the paired sample student's t-test (two-tailed). Fisher's Exact test was used to examine significance between different priming efficacies. P-values of <0.05 were regarded as significant.

Results

WT-1 and PRAME tetramer specific T cells can be primed in HD, but not in AML patients
Primed Tm$^+$ T cells were detectable in samples from HD for both tested LAA (figure 1). PRAME and WT1 Tm$^+$ T cells were induced in 3 out of 4 and in 2 out of 5 HD, respectively (table 1). Multiple parallel priming cultures of CD8$^+$ T cells were set up in separate wells with a fixed number of CD8$^+$ T cells per well (1 x 10^6 cells). WT1 Tm$^+$ CD8$^+$ T cells were detected in 4 out of a total of 104 cultures (*table I*; 3/28 for donor 1; 1/20 for donor 2). The priming efficiency of PRAME CD8$^+$ T cells in HD was significantly higher at 20 Tm$^+$ cultures out of a total of 51 (*tabe I*; 8/12 for donor1; 6/15 for donor 2; 6/9 for donor 3). Of note, the same healthy donor derived T cells were used for PRAME and WT1 priming experiments, WT1 and PRAME specific T cells were both detected in donor 1 and 2. Highest frequencies of PRAME or WT1 Tm$^+$ CD8$^+$ T cells were detected after 1 to 5 rounds of (re-)stimulation (*table I*). Tm$^+$

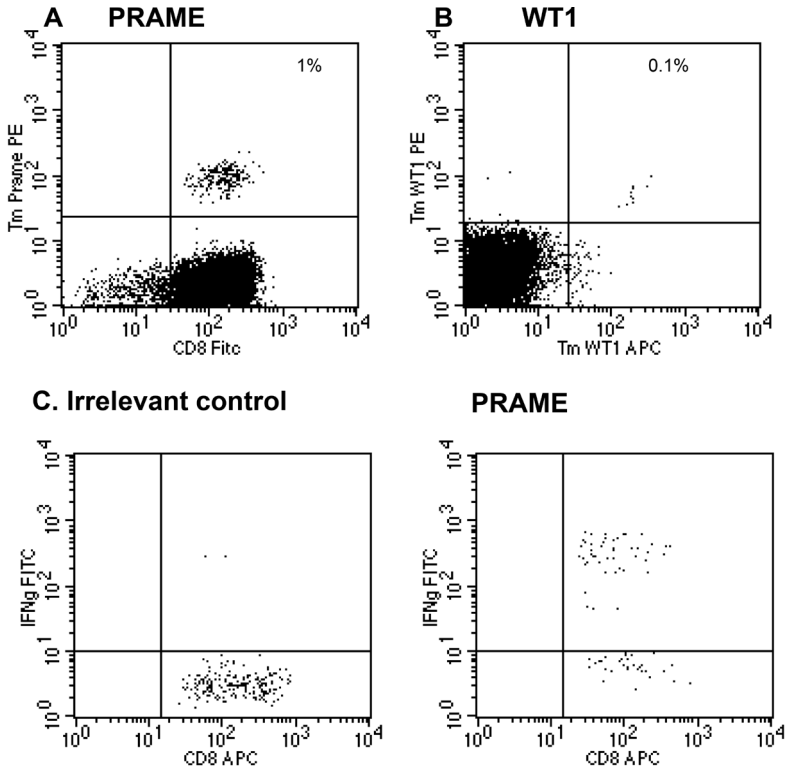


Figure 1.Detection of tetramer positive (Tm^+) T cells and interferon (IFNγ) production of Tm^+ T cells induced in healthy donor peripheral blood mononuclear cells. Plot A shows one representative example of PRAME (100-108) Tm+ T cells, plot B of WT1 (126-134) specific T cells. Plot C shows IFNγ production after incubation with T2 cells loaded with an irrelevant peptide or PRAME (100-108). The depicted cells were pre-gated on Tm positivity.

$CD8^+$ T cell frequencies ranged from 0.02 to 1.07% for the PRAME epitope and from 0.01 to 0.09% for the WT1 epitope.

In addition, we analysed the priming efficiencies of $CD8^+$ T cells obtained from patients directly after reaching CR or patients in sustained CR (*tabel 2*). No PRAME and WT1 Tm^+ T cells could be expanded from $CD8^+$ T cells obtained from patients shortly after achieving CR (1-6 months duration, Fisher's Exact test HD versus CR $p<0.001$ for PRAME) (table I) and in only one patient with a sustained CR WT1 specific T cells were detected at low frequencies in 2 out of 6 wells (UPN 1, table I).

Tetramer positive T cells produce IFNγ upon recognition of peptide

Induced Tm^+ $CD8^+$ T cells from HD produced IFNγ upon recognition of the PRAME or WT1 peptides (figure 1). For PRAME, expanded T cells were tested for intracellular IFNγ expression upon stimulation with the corresponding peptide and Tm^+ populations were able to produce IFNγ (in a range of 24-71% of Tm^+ T cells, for a representative result, see *figure 1C*). $CD8^+$ T cells from two HD were found to be Tm^+ for WT1 after priming (*table I*); IFNγ production

upon re-stimulation with WT1 peptide was tested in one HD, showing 27.7% IFNγ positive cells within the Tm$^+$ cell fraction (data not shown).

PRAME specific T cells lyse AML cell lines and leukemic cells from patients in a dose dependent manner

Tm$^+$ T cells recognizing the PRAME epitope were sorted from HD1, followed by limiting dilution cultures for the generation of a PRAME-specific CTL clone. A thus derived T cell clone was immortalized by retroviral hTERT transduction [22], after which its functionality was tested. In peptide titration experiments with intracellular IFNγ read-out, the clone was shown to be of intermediate functional avidity with half-maximal IFNγ production at 10 to 1 ng/ml PRAME peptide concentration. PRAME(100-108) peptide loaded JY cells were killed efficiently by the PRAME-specific T cell clone, whereas unloaded JY cells remained viable (*figure 2A*). Furthermore, HLA-A2 transduction of K562 cells (known to be PRAME$^+$ [23]), provoked specific IFNγ production (data not shown) and rendered the cells susceptible to specific kill by the PRAME-specific CTL clone as determined by a flow cytometry based cytotoxicity assay (*figure 2A*), demonstrating recognition of the endogenously expressed and processed PRAME epitope. HLA class I restriction of the observed K562 eradication was confirmed by the inclusion of a neutralizing monoclonal antibody (*figure 2B*).

To confirm the ability of the CTL clone to eliminate AML blasts, it was co-cultured with titrated amounts of HLA-A2$^+$ AML target cells and shown to kill both MUTZ-3 cells (the PRAME$^+$ AML precursors rather than differentiated DC, see *figure 2C*) and HLA-A2 matched allogeneic primary AML blasts (one representative example shown out of two tested patients, *figure 2D*), both in a dose dependent manner.

Table 1 Priming efficiencies of CD8+ cells from healty donors (HD), or leucapheresis products from AML patients during short-term (1-6 months) complete remission (AML-CR) or after long term CR (>1.5 years, AML-LT-CR) , all after stimulation with leukemia-associated antigen derived peptide loaded HLA-A2-matched MUTZ -3-DC.

		Tm$^+$ rates after co-culture (per no. of tested HD/patients)	Total no. of Tm$^+$ co-cultures#	Median % Tm$^+$ cells per positive culture (range)	Number of restimulations at maximum of Tm $^+$
PRAME(100-108)	HD	3/4	21/50	0.10% (0.02-1.07)	1-5
WT1(126-134)	HD	2/5	4/104	0.07% (0.03-0.09)	1-4
PRAME(100-108)	AML-CR1	0/4	0/44*	0.00%	-
WT1(126-134)	AML-CR1	0/3	0/38	0.00%	-
PRAME(100-108)	AML-LT-CR1	0/3	0/14	0.00%	-
WT1(126-134)	AML-LT-CR1	1/3	2/14	0.05%	4

*AML-CR1 vs HD, p<0.001 by Chi-Square test. AML-LT-CR1: AML in long term (>1.5years), first complete remission. AML-CR1: AML in first complete remission.

Multiple cultures of 1 million CD8$^+$ T cells per donor were started. The number of TM positive wells per total number of wells are shown. A well was counted positive when 0.01% or more Tm+ cells were found.

Discussion

T cells recognizing WT1 and proteinase 3 have previously been detected in AML at diagnosis by their reactivity in a very sensitive Elispot assay and by IFNγ production after specific stimulation [18]. However, detection of T cells through Tm analysis offers a means of isolation and more extensive functional characterization of specific CTL clones. Since T cells from HD have not encountered leukemic cells, outgrowth of PRAME and WT1-specific $CD8^+$ T cells should be the result of priming of naive T cells. PRAME-specific Tm^+ T cells could be readily detected after priming in HD, whereas the frequency of WT1-specific T cells were low. Others have studied priming with WT1 (126-134)-loaded DC and found that 10 out of 10 HD low-frequency Tm^+ T cells could be primed, with up to 10% Tm^+ T cells after extensive in vitro expansion [24]. This difference in efficiency compared to our data could be a result of differences in the employed protocols [25], most notably the use of 2-day "fast"

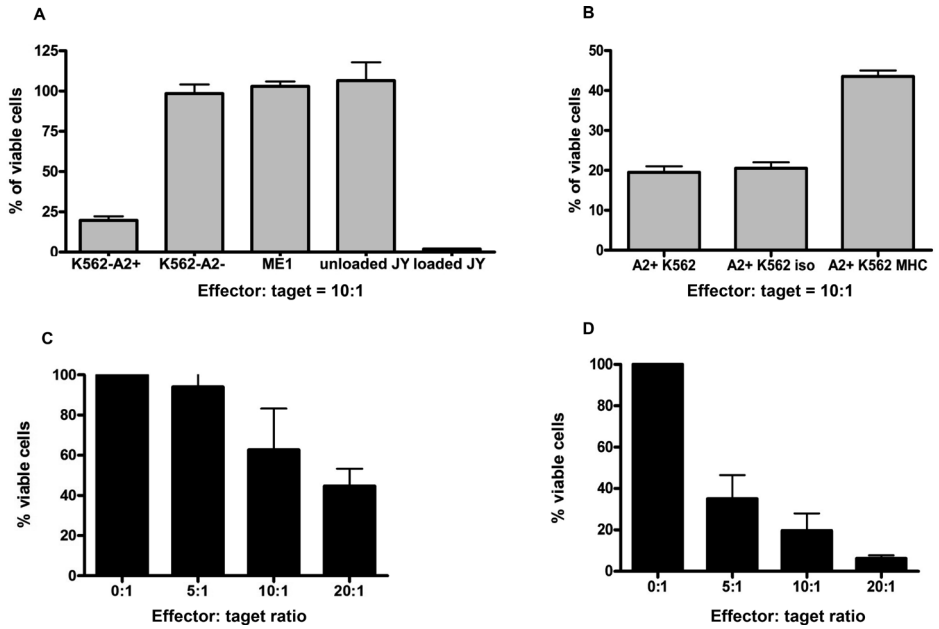


Figure 2. Cytotoxic activity of the PRAME CTL clone (derived from a healthy donor and specific for epitope 100-108) towards leukemic cell lines and patient samples. (A) In a flow cytometry-based assay cytotoxic activity against cell lines K562 (CML, PRAME+, HLA-A2 negative or transduced with HLA-A2), ME1 (AML, PRAME-, HLA-A2 positive), unloaded JY and PRAME(100-108) peptide loaded JY was tested in a 10:1 effector to target ratio, relative percentages of viable cells are depicted after incubation without or with T cells. In figure 2B percentage viable K562-A2+ cells is shown after incubation with PRAME specific T cells in presence of HLA class I-blocking antibody or isotype control. In figure (C) and (D) relative percentage of precursor MUTZ-3 cells and viable leukemic AML cells (one representative example of two HLA-A2 matched patients tested) respectively, is depicted after incubation with PRAME specific T cells in different effector-to-target ratios

Table 2. Patient characteristics of AML patients in long-term complete remission (LT-CR) and in patients in short-term complete remission, leukapheresis

	AML subtype	duration CR at start priming
AML-LT-CR1 UPN 1	MDS-RAEB	1.5 years
AML-LT-CR1 UPN 2	M1	12 years
AML-LT-CR1 UPN 3	M4	14 years
AML-CR1 UPN 1	AML, not further classified	6 months
AML-CR1 UPN 2	AML-M2	1 month
AML-CR1 UPN 3	Therapy related AML	1 month
AML-CR1UPN 4	AML-M5B	2 months

DC and PBMC in the expansion rounds. However, employing the same protocol as in the current study, we previously observed higher priming efficiencies of other tumor-associated antigens such as hTERT, EBP-1 and CEA[19]. Moreover, Quintarelli and co-workers found similar priming efficiencies from HD blood for the PRAME(100-108) epitope as we did [26]. The same group recently reported on the ability of high-avidity PRAME-specific CTL to eliminate CML lines and primary colony forming precursors; we now confirm these findings for AML [27]. Together, these data validate our methodology and the low CTL precursor frequencies we found for WT1.

Immunotherapeutic treatment strategies necessitate an adequately responding immune system. In AML it is not yet clear at what time point the immune system has sufficiently recovered from the disease and chemotherapy treatment. Therefore, we analysed the priming efficiencies from patients directly after reaching CR or patients in sustained CR. Remarkably, no PRAME and WT1 Tm^+ T cells could be expanded from $CD8^+$ T cells from patients in short CR. This failure to prime LAA-specific T cells can be explained by an immunosuppressed state or by a disturbed T cell repertoire with very low T cell numbers directly after chemotherapeutic treatment; it can take up to six months before the numbers of T cells are fully restored from the interfering effects of the abundance of AML blasts and chemotherapeutics[28;29]. Furthermore, G-CSF, given before performing leucapheresis in these patients can induce T cell tolerance and is likely to hamper priming efficiencies [30]. Similar observations have been made for CML patients [26]. These negative effects could be far less in patients that have sustained CR. Indeed, we were able to induce the outgrowth of WT1-specific $CD8^+$ T cells , but not of PRAME specific T cells, from patients in long CR, after stimulation with WT1 epitope loaded MUTZ-3 DC, in one out of three patients (table 1). Remarkably, no PRAME-specific T cells could be primed in any of the patients, suggesting that either no PRAME naive T cell were present or PRAME CTLs were anergic and not able to expand. Although not conclusive, since we could not expand sufficient T cell numbers for functional testing, these findings strongly suggest that even after longer CR AML-reactive T cells remain relatively dysfunctional (or their repertoire exhausted) and may never fully recover sufficiently to respond to WT1 and PRAME. It remains to be investigated whether this applies to a wider range of AML-derived antigens and epitopes. Of note, in case of allogeneic stem cell transplantation (SCT), the immunosuppressive effects of chemotherapy and AML will not have affected the donor-derived haematopoietic cell lineages and under

these circumstances T cells are thus likely to respond as HD derived unprimed T cells. Moreover, allo-reactive T cells specifically recognizing PRAME positive tumour cells might prove particularly powerful antitumour effector cells [31].
In conclusion, we show that expanding LAA-specific T cells remains a challenge in patients recovering from their disease and treatment and indicates that active immunotherapy may be more effective in a prophase of AML, for instance MDS. This may then be combined with treatment with demethylating agents such as 5-azacytidine which might augment PRAME expression and subsequent PRAME-specific recognition and lysis by CTL clones [32;33]. In addition, the ability of PRAME Tm^+ T cells derived from healthy donors to recognize and kill target cells efficiently indicates that PRAME is a promising candidate for adoptive T cell transfer, e.g. employing TCR gene transfer, and deserves further research for implementation in induction and consolidation regimens in the treatment of AML.

Reference List

1. van den Ancker W, van Luijn MM, Westers TM et al. Recent advances in antigen-loaded dendritic cell-based strategies for treatment of minimal residual disease in acute myeloid leukemia. Immunotherapy 2010;2:69-83.
2. Kessler JH, Beekman NJ, Bres-Vloemans SA et al. Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. J.Exp.Med. 2001;193:73-88.
3. Van Tendeloo VF, Van de Velde A, Van Driessche A et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc.Natl.Acad.Sci.U.S.A 2010;107:13824-13829.
4. Greiner J, Schmitt M, Li L et al. Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. Blood 2006;108:4109-4117.
5. Oehler VG, Guthrie KA, Cummings CL et al. The preferentially expressed antigen in melanoma (PRAME) inhibits myeloid differentiation in normal hematopoietic and leukemic progenitor cells. Blood 2009;114:3299-3308.
6. Greiner J, Bullinger L, Guinn BA, Dohner H, Schmitt M. Leukemia-associated antigens are critical for the proliferation of acute myeloid leukemia cells. Clin.Cancer Res. 2008;14:7161-7166.
7. Kerst G, Bergold N, Viebahn S et al. WT1 protein expression in slowly proliferating myeloid leukemic cell lines is scarce throughout the cell cycle with a minimum in G(0)/G(1) phase. Leuk.Res. 2008;32:1393-1399.
8. Bellantuono I, Gao L, Parry S et al. Two distinct HLA-A0201-presented epitopes of the Wilms tumor antigen 1 can function as targets for leukemia-reactive CTL. Blood 2002;100:3835-3837.
9. Candoni A, Tiribelli M, Toffoletti E et al. Quantitative assessment of WT1 gene expression after allogeneic stem cell transplantation is a useful tool for monitoring minimal residual disease in acute myeloid leukemia. Eur.J.Haematol. 2009;82:61-68.
10. Matsushita M, Ikeda H, Kizaki M et al. Quantitative monitoring of the PRAME gene for the detection of minimal residual disease in leukaemia. Br.J.Haematol. 2001;112:916-926.

11. Ostergaard M, Olesen LH, Hasle H, Kjeldsen E, Hokland P. WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients - results from a single-centre study. Br.J.Haematol. 2004;125:590-600.
12. Yong AS, Keyvanfar K, Eniafe R et al. Hematopoietic stem cells and progenitors of chronic myeloid leukemia express leukemia-associated antigens: implications for the graft-versus-leukemia effect and peptide vaccine-based immunotherapy. Leukemia 2008
13. Gao L, Bellantuono I, Elsasser A et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood 2000;95:2198-2203.
14. Greiner J, Dohner H, Schmitt M. Cancer vaccines for patients with acute myeloid leukemia--definition of leukemia-associated antigens and current clinical protocols targeting these antigens. Haematologica 2006;91:1653-1661.
15. Griffioen M, Kessler JH, Borghi M et al. Detection and functional analysis of $CD8^+$ T cells specific for PRAME: a target for T-cell therapy. Clin.Cancer Res. 2006;12:3130-3136.
16. Rosenfeld C, Cheever MA, Gaiger A. WT1 in acute leukemia, chronic myelogenous leukemia and myelodysplastic syndrome: therapeutic potential of WT1 targeted therapies. Leukemia 2003;17:1301-1312.
17. Rezvani K, Yong AS, Savani BN et al. Graft-versus-leukemia effects associated with detectable Wilms tumor-1 specific T lymphocytes after allogeneic stem-cell transplantation for acute lymphoblastic leukemia. Blood 2007;110:1924-1932.
18. Scheibenbogen C, Letsch A, Thiel E et al. CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. Blood 2002;100:2132-2137.
19. Santegoets SJ, Schreurs MW, Masterson AJ et al. In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. Cancer Immunol.Immunother. 2006;55:1480-1490.
20. Santegoets SJ, Masterson AJ, van der Sluis PC et al. A CD34(+) human cell line model of myeloid dendritic cell differentiation: evidence for a CD14(+)CD11b(+) Langerhans cell precursor. J.Leukoc.Biol. 2006;80:1337-1344.
21. Westers TM, Houtenbos I, Schuurhuis GJ, Ossenkoppele GJ, van de Loosdrecht AA. Quantification of T-cell-mediated apoptosis in heterogeneous leukemia populations using four-color multiparameter flow cytometry. Cytometry A 2005;66:71-77.
22. Hooijberg E, Ruizendaal JJ, Snijders PJ et al. Immortalization of human $CD8^+$ T cell clones by ectopic expression of telomerase reverse transcriptase. J.Immunol. 2000;165:4239-4245.
23. Greiner J, Ringhoffer M, Taniguchi M et al. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. Int.J.Cancer 2004;108:704-711.
24. Ho WY, Nguyen HN, Wolfl M, Kuball J, Greenberg PD. In vitro methods for generating $CD8^+$ T-cell clones for immunotherapy from the naive repertoire. J.Immunol.Methods 2006;310:40-52.
25. Rasaiyaah J, Noursadeghi M, Kellam P, Chain B. Transcriptional and functional defects of dendritic cells derived from the MUTZ-3 leukaemia line. Immunology 2009;127:429-441.
26. Quintarelli C, Dotti G, De Angelis B et al. Cytotoxic T lymphocytes directed to the Preferentially Expressed Antigen of Melanoma (PRAME) target chronic myeloid leukemia. Blood. 2008;112:1876-8
27. Quintarelli C, Dotti G, Hasan ST et al. High-avidity cytotoxic T lymphocytes specific for a new PRAME-derived peptide can target leukemic and leukemic-precursor cells. Blood 2011;117:3353-3362.

28. Szczepanski MJ, Szajnik M, Czystowska M et al. Increased frequency and suppression by regulatory T cells in patients with acute myelogenous leukemia. Clin.Cancer Res. 2009;15:3325-3332.
29. Ohnishi K, Yamanishi H, Naito K et al. Reconstitution of peripheral blood lymphocyte subsets in the long-term disease-free survivors of patients with acute myeloblastic leukemia. Leukemia 1998;12:52-58.
30. Rutella S. Granulocyte colony-stimulating factor for the induction of T-cell tolerance. Transplantation 2007;84:S26-S30.
31. Amir AL, van der Steen DM, van Loenen MM et al. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. Clin. Cancer Res. 2011;17:5615-5625.
32. Gutierrez-Cosio S, de la Rica L, Ballestar E et al. Epigenetic regulation of PRAME in acute myeloid leukemia is different compared to CD34$^+$ cells from healthy donors: effect of 5-AZA treatment. Leuk.Res. 2012;36:895-899.
33. Yan M, Himoudi N, Basu BP et al. Increased PRAME antigen-specific killing of malignant cell lines by low avidity CTL clones, following treatment with 5-Aza-2'-Deoxycytidine. Cancer Immunol.Immunother. 2011;60:1243-1255.

Procedures for the expansion of CD14^{+} precursors from acute myeloid leukemic cells to facilitate dendritic cell based immunotherapy

12

W van den Ancker
PGJB Wijnands
JM Ruben
TM Westers
B Punt
C Bachas
N Ravenshorst
S Wetering
AM Kruisbeek
HJ Bontkes
GJ Ossenkoppele
AA van de Loosdrecht
TD de Gruijl

Immunotherapy, accepted for publication

Abstract

Vaccination with Acute Myeloid Leukemia (AML)-derived Dendritic Cells (DC) is a promising immunotherapeutic approach to prevent relapse of AML. However, in clinical practice AML-derived DC culture is unfeasible in 40% of cases. Here, we demonstrate that AML cells can be expanded *in vitro* prior to differentiation with cocktails of cytokines with known myeloid growth-promoting effects. We show that 9 out of 13 initially $CD14^-$ samples gain *de novo* CD14 (>10%) expression (69% increment, p=0.01) after *in vitro* expansion. These expanded $CD14^+$ leukemic cells displayed a high probability (6 out of 6 initially $CD14^-$ samples tested) to differentiate into DC upon culture with GM-CSF, TNFα and IL-4. In conclusion, induction of CD14 on initially $CD14^-$ AML cells potentially increases the number of patients eligible for DC-based immunotherapy.

Introduction

After high-dose chemotherapy, 50-85% of patients with acute myeloid leukemia (AML) achieve complete remission. Despite intensive treatment, including allogeneic stem cell transplantation, about 50% of patients relapse due to persistence of leukemic cells [1]. Further intensification of treatment regimens is not possible due to increased (extra) medullary toxicity. Therefore, other treatment modalities focusing on controlling minimal residual disease (MRD) are under investigation. In this respect, active immunotherapy with antigen presenting cells (APC), such as dendritic cells (DC), is regarded as an attractive alternative [2]. Leukemic cells have been proven to be able to differentiate into DC-like APC [3, 4]. Importantly, AML-derived DC harbor known and unknown individual leukemia-associated antigens (LAA) and possibly immunogenic mutations and have a T cell activating phenotype [5, 6]. AML-derived DC-based vaccination regimens have been proven feasible in clinical settings and might provide a therapeutic option [7, 8]. In clinical practice, however, leukemic cells can be differentiated into DC in only 60% of patients; this can be predicted by expression of CD14, TNFαRI and FLT3-ITD as we described previously [3, 4, 9, 10]. Others have tried different leukemic cell-differentiation strategies, thereby increasing the number of patients eligible for DC-based immunotherapy [11, 12]. Nevertheless, AML-DC vaccine preparation is hampered by an inability to culture sufficient numbers of AML-derived DC or lack of enough precursor cells for differentiation [5]. This might be overcome by expansion of leukemic cells prior to differentiation into DC. In the present study we expanded leukemic cells with various cytokine cocktails in order to achieve sufficient numbers of DC precursor cells and tested their ability to differentiate into DC. Expansion of $CD14^-$ AML cells resulted in a gain of CD14, a major positive predictor for DC differentiation. In contrast, in a priori $CD14^+$ AML CD14 expression was down-regulated during expansion. These results indicate that expansion of $CD14^-$ AML cells and their subsequent gain of CD14 expression by the use of specific cytokine cocktails increases the number of patients eligible for AML-derived DC based vaccination.

Material & Methods

Patient samples

Peripheral blood or bone marrow mononuclear cells from 46 patients with AML were isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden); see Supplementary *table 1* for patient characteristics. Since acute promyelocyctic leukemias are a distinct subgroup with in general a good progonis, t(9;22)-positive promyelocyctic leukemia cases (n=5) were excluded from analysis. All samples were drawn after the patients' informed consent at the time of collection. Cells were cultured immediately, or cryopreserved at a controlled rate in liquid nitrogen using RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS, Greiner, Alphen a/d Rijn, The Netherlands) and 10% dimethylsulphoxide (Merck, Darmstadt, Germany). Cryopreserved material was rapidly thawed and washed twice in RPMI1640 supplemented with 40% FCS, the cells were then resuspended in culture medium as described below. Of note, DC differentiation after expansion was feasible both from fresh and cryopreserved samples.

Culture conditions of leukemic cells

Leukemic cells were suspended in culture medium consisting of MEM-α medium (Lonza; Basel, Switzerland), supplemented with 15% FCS (Hyclone, Logan, UT), β-Mercaptho-ethanol (Merck, Darmstadt, Germany), 100U/ml Penicillin (Astellas Pharma, Leiden, The Netherlands), 100 µg/ml Streptomycin (Fisiopharma, Palamonte, Italia), further referred to as complete culture medium. Cell numbers and viability were determined by Trypan blue dye exclusion (0.2 g/ml, Merck). Cells were plated in flat bottom 24-wells plates (Greiner) at 0.5x106 cells/ml per well at a maximum of $1x10^6$ cells per condition. Different cytokine cocktails were tested as shown in *table 1*; these cocktails consisted of various combinations of GM-CSF (10ng/ml; Berlex, Montville, NJ), G–CSF (10ng/ml; R&D Systems, Minneapolis, MN), IL-3 (10 ng/ml; R&D systems), FLT3-ligand (25ng/ml; Peprotech, Rocky Hill, CT) SCF (10ng/ml; Peprotech) and 5637-conditioned medium (see below). These cytokines have known growth promoting activity for myeloid DC precursors [13]. Cells were passaged every 3-4 days, only when cells had expanded at least 2-fold; maximum number of achieved passages was defined as the penultimate passage number at which cells did not expand at least 2-fold within 3-4 days. Culture viability and cell numbers were evaluated using trypan blue exclusion. To induce DC differentiation, expanded cells were plated at $0.2x10^6$ cells/ml in complete culture medium supplemented with GM-CSF (100ng/ml; Berlex), TNFα (2.5 ng/ml; Strathmann, Hamburg, Germany) and IL4 (1000U/ml; R&D Systems). At day 3 and 6 medium was replenished and fresh cytokines were added. Of note, this culture method, including the use of FCS, is comparable with methods used for preparation of clinically applied DC-based vaccines (www.DCPrime.nl)

5637-conditioned culture medium

The bladder carcinoma cell line 5637 (obtained from DSMZ, Germany) is a constitutive producer of a number of myeloid growth-promoting cytokines, with high GM-CSF concentrations and low in IL-3 [14]. Cells were plated in culture flasks at $0.3x10^6$/ml in IMDM supplemented with Penicillin, L-glutamine and 10% FCS. Once 80% of the culture flask was covered with cells, medium was replenished. Cells were incubated for 24h; supernatants

Table I. Composition of different cytokine cocktails used for expansion of AML cells

Culture Cocktail	GM-CSF	G-CSF	IL-3	FLT3-Ligand	SCF	5637CM
1	10ng/ml	10 mg/ml	-	-	-	-
2	-	-	10ng/ml	-	-	-
3	-	-	-	25ng/ml	25ng/ml	-
4	-	-	-	25ng/ml	25ng/ml	-
5	10ng/ml	10 mg/ml	-	25ng/ml	25ng/ml	-
6	10ng/ml	10 mg/ml	-	-	-	10%
7	-	-	10ng/ml	-	-	10%
8	-	-	-	25ng/ml	25ng/ml	10%
9	-	-	10ng/ml	25ng/ml	25ng/ml	10%
10	10ng/ml	10 mg/ml	-	25ng/ml	25ng/ml	10%

containing cytokines produced by 5637 cells, were harvested and stored at -20˚C until usage.

Immunophenotypic analysis

Before and after expansion of AML cells, four-color flow cytometric analysis (FACS-Calibur flow cytometer, Becton Dickinson (BD), San Jose, CA) was performed; surface marker expression was examined and included differentiation related and growth promoting receptors with the following antibodies: CD14FITC, CD14PerCP (BD), CD34APC (Pharmingen), CD34PE (Sanquin, Amsterdam, The Netherlands), CD11cPE (Immunotech, Prague, Czech Republic), CD123PE (IL3R) (BD), CD124PE (IL4R, Immunotech), CD114PE (G-CSFR; BD), CD116FITC (GM-CSFR; BD), CD117PE (SCFR; BD), CD120aFITC (TNFαRI; R&D systems, Mineapolis, MN), CD120bPE (TNFαRII; R&D systems), CD135PE (FLT3PE, Immunotech) and CD19PE (BD). Isotype controls used were IgG1FITC (BD), IgG1PE (BD), IgG1PerCP (DAKO, Glostrup, Denmark) and IgG1 APC (BD). Before and after differentiation, cells were analyzed using the following markers: CD14, CD34 as described above, CD1aPE (BD Pharmingen), DC-SIGN, CD40FITC, (Pharmingen) CD80PE (BD), CD83PE (BD), CD86PE (Pharmigen) and HLA-DRPE, (BD).

Results were calculated as percentage positive cells relative to the appropriate isotype control. Results were analyzed with CellQuestPro software (BD). In regular diagnostic procedures, myeloid origin of leukemic cells was confirmed following WHO2008 guidelines [15]. Percentages of marker positive cells were calculated on leukemic cells, defined as those cells with CD45dim expression combined with low sideward light scatter and immature markers such as CD34 and/or CD117. We considered DC differentiation successful when low expression (<10%) of CD14 and CD34 and increased expression (>10%) of all of the following markers was found in comparison with the corresponding progenitor cells: CD1a DC-SIGN, CD40, CD80, CD86 and HLA-DR.

RT-qPCR for PRAME, RHAMM and WT1

To ensure persistence of cells in the expansion cultures from leukemic origins carrying relevant antigens for AML vaccination we screened by qPCR for mRNA expression of the LAA Preferentially Expressed Antigen in Melanoma (PRAME), Receptor for Hyaluronan-Mediated

Motility (RHAMM) and Wilms' Tumor 1 (WT1). The human chronic myeloid leukemia (CML) cell line K562 (RHAMM$^+$ and PRAME$^+$) and the human AML cell line HL60 (WT1$^+$), both obtained from ATCC (LGC Standards, Teddington, UK) were used as positive controls. AML samples and cell lines were pelleted and freeze-dried; RNA was isolated and subsequently cDNA was generated employing a standard RNA isolation kit and cDNA preparation kit (both Applied Biosciences, Carlsbad CA). cDNA was synthesized from 1µg total RNA using M-MLV Reverse Transcriptase and random hexamer primers in a 40µl reaction according to protocol. For expression of PRAME, RHAMM and WT1, standard TaqMan Gene Expression Assays were used (Hs01022301; Hs00234864; Hs0110349 respectively, Roche Applied Biosystems, Almere, the Netherlands) following product guidelines. Target gene expression was normalized against the expression of the housekeeping gene GUS to adjust for variations in RNA quality and efficiencies of cDNA synthesis (Biolegio, Nijmegen, The Netherlands) qPCR was run on the ABI/PRISM 7500 Sequence Detection System (Applied Biosystem, Carlsbad, CA); all qPCR experiments were performed in duplicate. Mean Ct value for each sample was used for relative quantification using the ΔΔCt method [16]. mRNA expression of the antigen was classified as either not detectable (-; 2^-ΔΔCt = 0), very low but detectable mRNA expression in up to 33 cycles (+; 2^-ΔΔCt >0 and <0.5), expression comparable with housekeeping gene (++; 2^-ΔΔCt >0.5; <1.5), and higher than the housekeeping gene (+++; 2^-ΔΔCt >1.5).

Statistical analysis

Relationships between variables were analyzed using Spearman's correlation coefficient. Changes in marker expression during expansion were analyzed with a paired t-test, differences in maximum passage numbers were evaluated with Mann Whitney U-test. P values were regarded as significant at $p<0.05$.

Results

Low expression of TNFαRI and high expression of TNFαRII predicts ability of AML to expand in *in vitro* cultures

Mononuclear cell fractions from 41 patients with AML were cultured in various conditions and expanded through various passages (mean 15, range 1-66). No correlation could be found between growth capacity (in number of attained passages) and different types of AML according to WHO2008 criteria (for a complete overview, see *Supplementary table I*). In leukemic cells from 25 patients, 10 different cytokine cocktails (see *table I*) were tested simultaneously; in these cultures no significant differences in maximum passage numbers between the various cytokine combinations were observed (see *figure 1a*). No significant correlation was found between the expression levels of any of the tested cytokine receptors on pre-expansion AML cells and the observed expansion rate in the corresponding AML cultures. TNFαRI and TNFαRII have previously been associated with a limited and large proliferative capacity of leukemic cells, respectively [17]. Independent of the applied cytokine expansion cocktail, we observed a prolonged expansion capacity for a TNFαRI/TNFαRII ratio below 2 (based on percentage positivity on leukemic cells, on average 10 vs.18 passages, p=0.02, *figure 1b*).

Table2. Tumor type, phenotypic and culture characteristics, and LAA expression in expanded AML cells

Leukemic cells able to differentiate into immature leukemic-DCs after expansion						
UPN*	Nr of passages at time of DC diff†	culture probability at diagnosis‡	WHO2008 classification	PRAME	WT1	RHAMM
11	15	16%	AML without maturation	ND	ND	ND
15	9	76%	AML with maturation	Neg	Neg	pos
23	14	0.6%	Acute myelomonocytic leukemia	ND	ND	ND
28	10	No FLT3-ITD known	Acute monoblastic leukemia	Neg	Pos	Pos
25	8	16%	Acute monoblastic leukemia	Neg	Neg	Pos
38	8	0.6%	AML with inv(16)	ND	ND	ND
Leukemic cells not able to differentiate into immature leukemic-DCs after expansion						
UPN	Nr of passages at time of DC diff	culture probability at diagnosis	WHO2008 classification	PRAME	WT1	RHAMM
14	20	16%	AML with maturation	Pos	Pos	Pos
17	15	76%	AML with maturation	ND	ND	ND
18	15	99%	AML without maturation	ND	ND	ND
27	8	84%	Acute monoblastic leukemia	Neg	Neg	Pos
34	8	76%	AML with 11q23 (MLL) abnormalities	ND	ND	ND

UPN, Unique Patient Number; † DC diff, dendritic cell differentiation; ‡culture probability at diagnosis is based on expression of CD14, FLT3-ITD and TNFR-alpha and calculated as described previously in REF8. FLT3-ITD, fms-like tyrosine kinase receptor-3 internal tandem duplication; ND, Not Done

Leukemic cells acquire CD14 expression upon cytokine-induced expansion and differentiate into DC

Upon culture with various cytokine cocktails marker expression on leukemic cells changed. Expression levels of GM-CSFR, G-CSFR, and IL-3R decreased (data not shown). Furthermore, we observed a significant decrease in CD34 expression on leukemic cells (p= 0.03, pre- vs post-expansion; n=12, data not shown). We did not observe any significant alterations in expression of either TNFαRI or -II upon culture (paired t- test: p= 0.8; n=6 and p= 0.9; n=6, respectively), moreover, no correlation was seen between post-culture TNFαRI/II ratio and DC differentiation ability (MWU: p=0.3; n=10). In contrast, expression levels of the known positive predictor for DC differentiation, CD14 [4,9], increased upon expansion of cells with originally low expression levels (≤5%) (*figure 2*). For one representative patient the cumulative number of $CD14^+$ cells in culture is shown over the course of expansion in

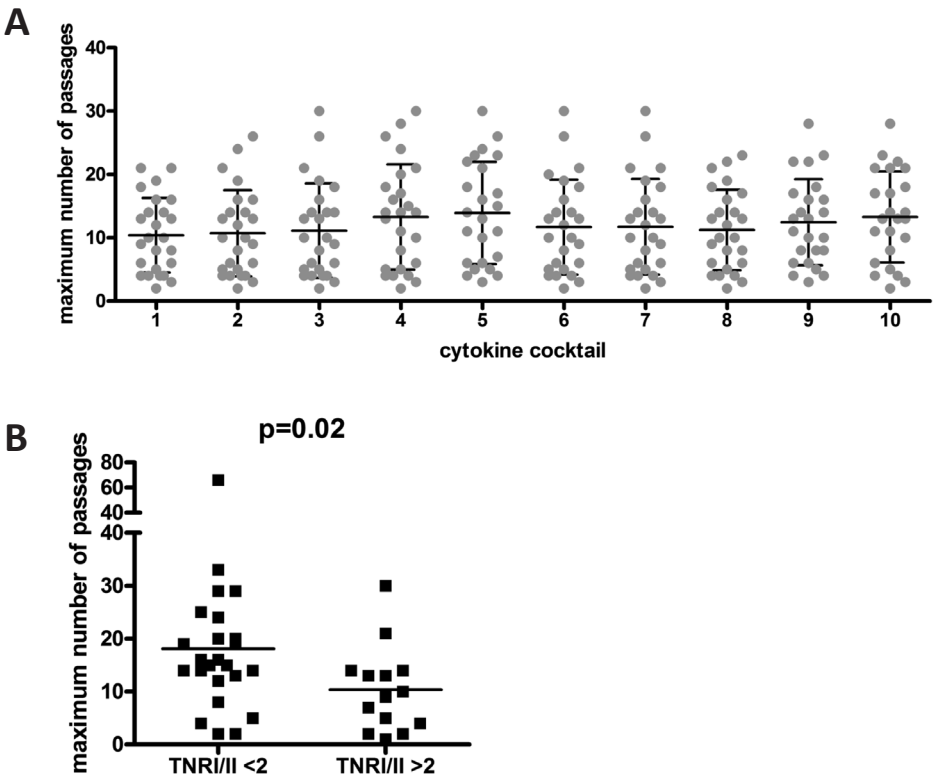


Figure 1. Markers predictive for AML expansion capacity.(A) Maximum number of passages attained during AML expansion, also in relation to (B) pre-expansion AML TNFαRI/TNFαRII expression ratio (above and below 2; ratio was calculated by dividing percentage of leukemic cells positive for these markers).

supplemental figure 2A. CD14 expression increased significantly upon expansion in 11 out of 17 cases tested; mean expression after culture 68% (range 0-89%); p<0.01, see *figure 2B*. In contrast, CD14 expression was down-regulated in 2/3 cultures with high baseline expression levels (>40%, see *figure 2B*). Acquired CD14 expression was associated with the ability of expanded AML cells to differentiate into DC in response to GM-CSF, TNFα and IL4; 6 out of the 7 tested samples were able to differentiate into DC (*figure 2B*; DC differentiation defined as down-regulated, low expression of CD14 (<10%) and CD34 (<10%) and increased expression of CD1a (>10%), DC-SIGN, CD40, CD80, CD86 and HLA-DR). In contrast, both cases with down-regulated CD14 expression were unable to differentiate into DC after expansion.

Presence of RNA encoding for LAA in leukemic cells after expansion

To assess leukemic origin of the expanded cells we screened for the presence of transcripts of the LAA PRAME, RHAMM and WT1 [17]. In all five samples screened for LAA, expression of at least one LAA could be found (RHAMM was detected in all samples tested); three of these were able to differentiate into leukemia-derived DC (*table I*).

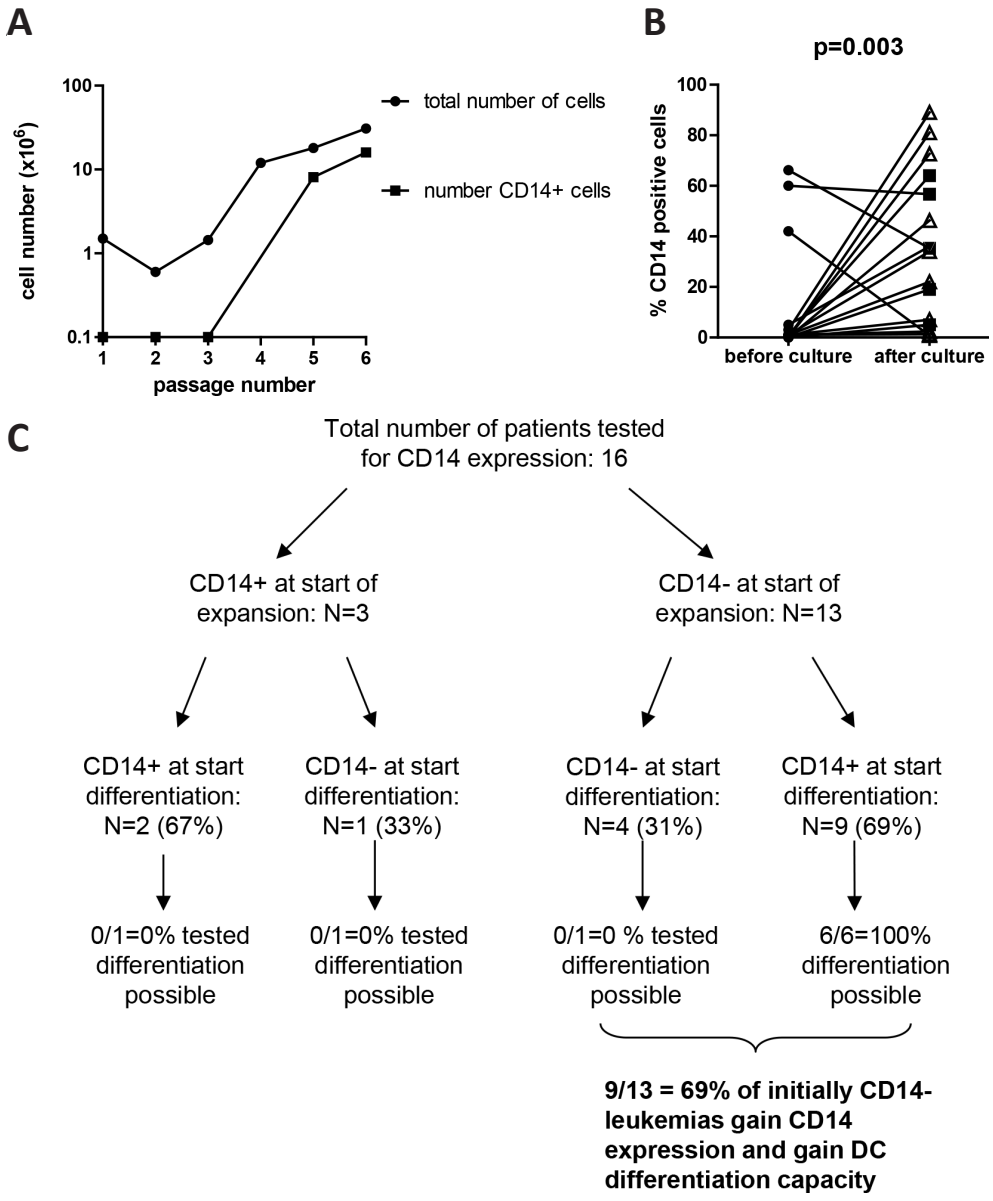


Figure 3. Expansion capacity and post-expansion differentiation ability. (A) One representative example of total and CD14+ cell numbers during expansion of a CD14-converting AML sample with a cytokine cocktail consisting of GM-CSF, G-CSF, FLT3-Ligand and SCF. (B) Changes in CD14 marker expression on leukemic cells after expansion culture. Open symbols (Δ, n=6) indicate that post-expansion DC differentiation could be achieved, i.e. low expression of CD14 (<10%) and CD34 (<10%) and increased expression of CD1a (>10%), DC-SIGN, CD40, CD80, CD86 and HLA-DR; open triangles denote successful DC differentiation whereas closed inverted triangles (n=5) indicate that AML-derived DC could not be generated; "■": post-expansion DC-differentiation capacity was not tested. (C) Schematic overview of numbers of patients in relation to CD14 expression and tested DC differentiation capacity over the course of expansion.

Discussion

In clinical practice, AML-derived DC based immunotherapy is hampered by low numbers of DC precursors and the inability of a considerable proportion of AML to differentiate into DC. To overcome these limitations we evaluated the expansion of leukemic cells prior to induction of differentiation into DC. We show that leukemic cells can indeed be efficiently expanded *in vitro.* No major differences in expansion efficiency were found between the tested cytokine expansion cocktails; consistently good results were obtained with a cytokine mix consisting of GM-CSF, G-CSF, FLT3-L and SCF (Cocktail 5, see *table 1*).

Although antigen-pulsed monocyte-derived DC or allogeneic DC vaccines have also been explored (by others and by us [19]), the use of whole-cell autologous leukemic cell products, such as AML-derived DC, for immunotherapy is of particular interest because they harbour both known and as yet unidentified LAA as well as individual immunogenic mutations. Previous studies have shown the potency of leukemic cell-derived DC in terms of generating an anti-leukemia T cell response [20]. Such personalized AML-derived DC vaccines require conservation of LAA expression. In all of the five post-expansion AML samples tested we confirmed the presence of mRNA encoding LAA derived peptide sequences (i.e. PRAME, RHAMM and WT1). Especially the presence of PRAME transcripts demonstrated the presence of bona fide AML cells since these transcripts are not found in normal mononuclear cells or non-malignant $CD34^+$ cells isolated from bone marrow. For RHAMM however, activated T cells have also been reported to express this protein, thereby potentially inducing T cell fractricide [21].

During expansion we observed a decrease in expression of GM-CSFR, G-CSFR, and IL-3R. This decrease may either be due to receptor occupancy, internalization of the receptors or differentiation of the AML blasts. The latter appears most likely in view of the observed simultaneous up-regulation of CD14. It was previously shown that cytokines such as SCF and FLT3-L can induce differentiation from normal $CD34^+$ precursor cells into $CD14^+$ DC precursors [22]. Furthermore, CD14 up-regulation in leukemic cell lines has been induced by incubation with human bone marrow stromal cell lines or with iron chelators and vitamin D analogues [23, 24]. This development of AML cells into a monocytoid precursor is in keeping with their subsequent propensity to differentiate into leukemic DC [25]. Indeed, CD14 is a recognized predictive marker for DC differentiation capacity of precursors in AML samples [4,9]. In keeping with this, we found that *de novo* gain of CD14 expression after cytokine-mediated expansion was associated with cytokine-induced DC-differentiation ability (see *figure 1B*), while loss of CD14 expression was associated with a subsequent inability to differentiate into DC. Based on our previous and current findings, the percentage of $CD14^-$ AML samples with cytokine-based DC differentiation ability can now be raised from 26% [4] to approximately 70% (69% in *figure 2C*) through prior cytokine-mediated expansion and CD14 induction.

In summary, for $CD14^-$ AML samples, expansion prior to DC differentiation overcomes two of the major limitations for DC vaccination, i.e. low numbers of DC precursors and the inability of leukemic cells to develop into DC. Specifically, we suggest that expansion of $CD14^-$ AML cells with a cocktail of GM-SCF, G-CSF, FLT3-L and SCF facilitates their acquisition of CD14 surface expression and DC-differentiation ability in the presence of commonly used cytokine cocktails containing GM-CSF, TNFα and IL4. Importantly, a priori $CD14^+$ leukemic cells did not profit from expansion, but these $CD14^+$ cells already have a good chance of DC differentiation with a standard combined expansion and differentiation cytokine cocktail [4].

Conclusion

In conclusion, we demonstrate that AML cells can be expanded *in vitro* prior to differentiation with cocktails of cytokines with known myeloid growth-promoting effects (i.e. GM-CSF, G-CSF, SCF and FLT3-L). CD14 negative leukemic cells can gain CD14 expression upon expansion which is a positive predictor for differentiation. In this study we confirm that $CD14^+$ expanded leukemic cells are able to differentiate into DC. This approach will result in an increase the number of patients eligible for AML-DC vaccination in whom previously AML-DC vaccination was hampered due to low cell numbers at diagnosis.

Future perspectives

By expansion of $CD14^-$ leukemic cells prior to DC preparation, the number of patients eligible for DC-based immunotherapy based on cytokine-induced DC differentiation capacity will be increased (based on our previous and current findings an estimated increase from 60% to about 80% of all AML cases) and thereby the overall feasibility of this experimental approach to the treatment of residual AML. Future trials are needed to confirm the general applicability of AML-DC vaccination in clinical practice.

References

1. Provan D, Singer CRJ, Baglin T, Lilleyman J. Acute myeloblastic leukaemia (AML). 2004; 149-157.
2. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat.Rev.Cancer 2012; 4: 265-277.
3. Houtenbos I, Westers TM, de Gruijl TD, et al. TNF-alpha receptor 1 expression on acute myeloid leukemic blasts predicts differentiation into leukemic dendritic cells. Leukemia 2004; 6: 1149-1153.
4. Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Identification of CD14 as a predictor for leukemic dendritic cell differentiation in acute myeloid leukemia. Leukemia 2003; 8: 1683-1684.
5. Westers TM, Stam AM, Scheper RM, et al. Rapid generation of antigen-presenting cells from leukaemic blasts in acute myeloid leukaemia. Cancer Immunol.Immunother. 2003; 1: 17-27.
6. Li L, Reinhardt P, Schmitt A, et al. Dendritic cells generated from acute myeloid leukemia (AML) blasts maintain the expression of immunogenic leukemia associated antigens. Cancer Immunol.Immunother. 2005; 7: 685-693.
7. Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Feasibility of clinical dendritic cell vaccination in acute myeloid leukemia. Immunobiology 2006; 6-8: 677-685.
8. Roddie H, Klammer M, Thomas C, et al. Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia. Br.J.Haematol. 2006; 2: 152-157.
9. Houtenbos I, Westers TM, Hess CJ, et al. Flt-3 internal tandem duplication hampers differentiation of AML blasts towards leukemic dendritic cells. Leukemia 2006; 10: 1892-1895.

10. Mohty M, Isnardon D, Blaise D, et al. Identification of precursors of leukemic dendritic cells differentiated from patients with acute myeloid leukemia. Leukemia 2002; 11: 2267-2274.
11. Nourizadeh M, Masoumi F, Memarian A, et al. Synergistic effect of Toll-like receptor 4 and 7/8 agonists is necessary to generate potent blast-derived dendritic cells in Acute Myeloid Leukemia. Leuk.Res. 2012; 9: 1193-1199.
12. Kremser A, Dressig J, Grabrucker C, et al. Dendritic cells (DCs) can be successfully generated from leukemic blasts in individual patients with AML or MDS: an evaluation of different methods. J.Immunother. 2010; 2: 185-199.
13. Bontkes HJ, de Gruijl TD, Schuurhuis GJ, et al. Expansion of dendritic cell precursors from human CD34(+) progenitor cells isolated from healthy donor blood; growth factor combination determines proliferation rate and functional outcome. J.Leukoc.Biol. 2002; 2: 321-329.
14. Quentmeier H, Zaborski M, Drexler HG. The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. Leuk.Res. 1997; 4: 343-350.
15. Swerdlow SH, Campo E, Harris NL, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition. 2008.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 4: 402-408.
17. Carter A, Haddad N, Draxler I, et al. Expression and role in growth regulation of tumour necrosis factor receptors p55 and p75 in acute myeloblastic leukaemia cells. Br.J.Haematol. 1996; 1: 116-126.
18. Greiner J, Ringhoffer M, Taniguchi M, et al. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. Int.J.Cancer 2004; 5: 704-711.
19. de Gruijl TD, van den Eertwegh AJ, Pinedo HM, Scheper RJ. Whole-cell cancer vaccination: from autologous to allogeneic tumor- and dendritic cell-based vaccines. Cancer Immunol. Immunother. 2008; 10: 1569-1577.
20. Choudhury BA, Liang JC, Thomas EK, et al. Dendritic cells derived in vitro from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. Blood 1999; 3: 780-786.
21. Snauwaert S, Vanhee S, Goetgeluk G, et al. RHAMM/HMMR (CD168) is not an ideal target antigen for immunotherapy of acute myeloid leukemia. Haematologica 2012; 10: 1539-1547.
22. Arrighi JF, Hauser C, Chapuis B, Zubler RH, Kindler V. Long-term culture of human CD34(+) progenitors with FLT3-ligand, thrombopoietin, and stem cell factor induces extensive amplification of a CD34(-)CD14(-) and a CD34(-)CD14(+) dendritic cell precursor. Blood 1999; 7: 2244-2252.
23. Callens C, Coulon S, Naudin J, et al. Targeting iron homeostasis induces cellular differentiation and synergizes with differentiating agents in acute myeloid leukemia. J.Exp.Med. 2010; 4: 731-750.
24. Liang R, Huang GS, Wang Z, et al. Effects of human bone marrow stromal cell line (HFCL) on the proliferation, differentiation and apoptosis of acute myeloid leukemia cell lines U937, HL-60 and HL-60/VCR. Int.J.Hematol. 2008; 2: 152-166.
25. Rasaiyaah J, Yong K, Katz DR, Kellam P, Chain BM. Dendritic cells and myeloid leukaemias: plasticity and commitment in cell differentiation. Br.J.Haematol. 2007; 3: 281-290.

Supplemental material

Supplementary table 1 legend: Patient characteristics. Abbreviations: UPN, Unique patient number; SCF-R, stem cell factor receptor; GCSF-R granulocyte colony stimulating factor receptor; GMCSF-R, granulocyte, macrophage colony stimulating factor receptor; TNF-R, ratio of tumor necrosis factor receptor; FLT3-ITD, fms-like tyrosine kinase receptor-3 internal tandem duplication: "0" not detectable, "1" detectable "-" not done; Diff y/n, DC differentiation yes or no; "-", not done.

UPN	WHO-classification	Cytogenetics	% blasts	CD 14	CD 34	CD 11c
1	AML not further classified	not analysed	41%	-	-	81
2	AML not further classified	not analysed	9%	-	-	
3	AML not further classified	not analysed	89%	60	0	63
4	AML not further classified	no metaphases	74%	0	25	3
5	AML not further classified	not analysed	74%	1	1	-
6	AML not further classified	no metaphases	95%	4	84	-
7	AML not further classified	not analysed	95%	0	0	-
8	AML minimally differentiated	46,XY	83%	0	98	2
9	AML without maturation	not analysed	71%	5	3	5
10	AML without maturation	not analysed	97%	-	-	8
11	AML without maturation	not analysed	95%	5	0	58
12	AML with maturation	not analysed	64%	-	-	5
13	AML with maturation	no metaphases	86%	0	15	36
14	AML with maturation	46,XY	35%	1	2	68
15	AML with maturation	46,XY,+t(17;17)(p1?3;q2?3)[5]	73%	5	90	46
16	AML with maturation	46,XY	40%	-	0	3
17	AML with maturation	46,XY	21%	1	1	33
18	AML without maturation	46,XX	70%	42	1	66
19	Acute myelomonocytic leukemia	not analysed	65%	2	95	2
20	Acute myelomonocytic leukemia	not analysed	40%	-	-	9
21	Acute myelomonocytic leukemia	not analysed	69%	2	0	15
22	Acute myelomonocytic leukemia	46,XX	46%	-	-	41
23	Acute myelomonocytic leukemia	not analysed	80%	0	0	4
24	Acute myelomonocytic leukemia	45,X	33%	52	1	75
25	Acute monoblastic and monocytic leukemia	47,XY,+8[10]	89%	0	11	5
26	Acute monoblastic and monocytic leukemia	46,XY	84%	0	27	66

UPN	SCF-R	GCSF-R	GM CSF-R	TNFa RI/RII	FLT3-ITD	Diff y/n	T(9;22)	T(8;21)	T(15;7)	MLL rearrangement
1	49	15	44	0,64	-	-	-	-	-	-
2	47	62	54	0,63	-	-	-	-	-	-
3	23	15	20	0,66	-	-	neg	neg	neg	neg
4	36	19	11	8,91	Y	-	neg	neg	neg	neg
5	-	-	-	-	Y	-	neg	neg	neg	neg
6	-	-	-	-	Y	-	neg	neg	neg	neg
7	-	-	-	-	Y	-	neg	neg	neg	neg
8	91	5	3	0,46	-	-	neg	neg	neg	neg
9	9	26	82	9,74	Y	-	neg	neg	neg	neg
10	84	40	20	0,86	-	-	-	-	-	-
11	11	54	35	0,28	Y	Y	neg	neg	neg	neg
12	85	65	5	0,56	-	-	-	-	-	-
13	24	46	8	2,28	-	-	-	-	-	-
14	52	39	26	0,37	N	N	neg	neg	neg	neg
15	80	60	15	0,15	N	Y	neg	neg	neg	neg
16	61	9	4	0,51	Y	-	neg	neg	neg	neg
17	28	3	75	0,77	N	N	neg	neg	neg	neg
18	11	39	86	0,42	N	N	neg	neg	neg	neg
19	98	27	16	2,24	N	-	neg	neg	neg	neg
20	97	53	34	0,42	-	-	-	-	-	-
21	58	44	22	0,96	Y	-	neg	neg	neg	neg
22	14	5	16	10,14	-	-	-	-	-	-
23	2	5	67	0,16	Y	Y	neg	neg	neg	neg
24	22	63	89	0,45	N	-	neg	neg	neg	neg
25	89	61	8	2,24	Y	Y	neg	neg	neg	neg
26	94	43	10	0,80	Y	-	neg	neg	neg	neg

Supplementary table 1, continued

UPN	WHO-classification	Cytogenetics	% blasts	CD 14	CD 34	CD 11c
27	Acute monoblastic and monocytic leukemia	46,XX,der(8)t(8;16)(p11;p13) dup(8)(q11q24),der(16) t(8;16)	94%	1	1	76
28	Acute monoblastic and monocytic leukemia	not analysed	91%	9	56	57
29	Acute monoblastic and monocytic leukemia	not analysed	93%	0	0	3
30	Acute monoblastic and monocytic leukemia	not analysed	30%	7	0	69
31	Acute monoblastic and monocytic leukemia	46,XY	84%	0	27	49
32	Acute monoblastic and monocytic leukemia	46,XX	23%	0	1	32
33	Acute erythroid leukemia	not analysed	16%	4	82	22
34	AML with 11q23 (MLL) abnormalities	11q23 mutation present	65%	66	13	51
35	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1	nuc ish (ETOx2, AML1x3) [44/200]	24%	2	91	31
36	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1	not analysed	15%	2	80	2
37	AML with inv (16)(p13.1q22); CBFB-MYH11	46,XX,inv(16)(p13q22)[11]	95%	41	55	56
38	AML with inv (16)(p13.1q22); CBFB-MYH11	46,XX,inv(9)(p22q13)c	52%	3	84	83
39	Acute leukemia of ambigious lineage: MPAL	Cytogenetic changes on chromosome 3	23%	9	88	6
40	Acute leukemia of ambigious lineage: MPAL	46,XX,t(2;3)(p23;q26),-7,+mar[10].ish del(7)(q11.2) (D7Z1+,D7S486-)	53%	3	94	37
41	Acute leukemia of ambigious lineage: MPAL	45,XY,-7	23%	1	99	11

UPN	SCF-R	GCSF-R	GM CSF-R	TNFa RI/RII	FLT3-ITD	Diff y/n	T(9;22)	T(8;21)	T(15;7)	MLL rearrangement
27	5	57	7	0.48	N	N	neg	neg	neg	neg
28	33	32	69	12,39	-	Y	-	-	-	-
29	91	46	90	0,27	-	-	-	-	-	-
30	33	32	15	8,42	-	-	-	-	-	-
31	98	31	21	6,28	Y	-	neg	neg	neg	neg
32	24	4	18	2,13	Y	-	neg	neg	neg	neg
33	78	18	34	1,83	N	-	neg	neg	neg	neg
34	1	1	6	0,05	-	N	neg	neg	neg	POS
35	1	94	27	0,32	N	-	neg	POS	neg	neg
36	49	3	8	4,32	N	-	neg	POS	neg	neg
37	96	56	64	0,21	N	-	neg	neg	neg	neg
38	5	72	85	86,36	Y	Y	neg	neg	neg	neg
39	27	6	6	4,17	-	-	neg	neg	neg	neg
40	69	26	14	1,08	N	-	neg	neg	neg	neg
41	94	20	72	7,16	N	-	neg	neg	neg	neg

CML lysate-loaded dendritic cells induce specific T cell responses towards leukemia progenitor cells indicating their potency for application in active specific immunotherapy

13

TM Westers
W van den Ancker
HJ Bontkes
JJWM Janssen
AA van de Loosdrecht
GJ Ossenkoppele

Immunotherapy. 2011 Apr;3(4):569-76

Abstract

Treatment of chronic myeloid leukemia (CML) with tyrosine kinase inhibitors such as Imatinib mesylate, dasatinib and nilotinib results in high rates of cytogenetic and molecular responses. However, in many patients minimal residual disease (MRD) is detected by molecular techniques. Since CML cells are particularly good targets for immune surveillance mechanisms, we explored active specific immunotherapy using leukemia lysate-loaded dendritic cells (DCs) in vitro. Our data show the potency of DC-based vaccination strategies for the induction of allogeneic anti-leukemia T cell-mediated responses to eradicate MRD.

Background

Treatment of chronic myeloid leukemia (CML) with Imatinib mesylate or other tyrosine kinase inhibitors (TKI), such as dasatinib and nilotinib, results in high rates of cytogenetic and molecular responses. Nevertheless, in many patients minimal residual disease (MRD) is detected by highly sensitive molecular techniques [1-4]. BCR-ABL, the target of the TKI, is not only a critical oncogene, but a CML-specific antigen as well. Amino-acid sequences of the fusion protein BCR-ABL can be processed and presented by HLA-molecules on the surface of leukemic cells [5-8]. Thus, CML cells are particularly good targets for immunological surveillance mechanisms; this has been demonstrated by the curative potential of allogeneic stem cell transplantation (SCT) and the re-induction of complete remission upon donor lymphocyte infusion in patients with relapsed CML [9;10]. Dendritic cells (DCs) are better inducers of immune responses than leukemic cells [11;12]. Therefore, active specific DC-based vaccination strategies may eradicate MRD when applied in combination with TKI or supplementary in patients resistant to TKI. In a small phase I study, we have demonstrated that vaccination with *in vitro* generated autologous CML-derived DCs in patients in late chronic phase CML induced an anti-leukemia immune response, evidenced by strong delayed type of hypersensitivity (DTH) [13]. A phase I/II vaccination trial, also based on autologous CML-derived DCs, in 10 patients that failed to achieve cytogenetic responses after treatment with α-interferon or Imatinib mesylate, showed similar results [14]. CML-derived DCs enable presentation of both known (e.g. BCR-ABL and WT-1) and unknown tumor-associated antigens [15]. Since CML-derived DCs are hampered by functional defects resulting in inferior migratory capacity and antigen presentation which may negatively influence the outcome of DC-based immunotherapy normal, non-leukemic monocyte-derived DCs (MoDCs) can serve as an alternative [16-18]. Similar to CML-derived DCs, MoDCs loaded with whole cell CML lysates will present the full range of known and unknown tumor-associated antigens. In AML, MoDCs loaded with leukemia cell-derived lysates have been exploited *in vivo* and results were encouraging [19]. In mice, the combination of CML lysate-loaded DCs and Imatinib mesylate resulted in activation of antigen-specific cells and superior anti-tumor activity against bcr-abl$^+$ leukemic cells as compared to lysate-loaded DCs or Imatinib mesylate alone. [20] This points toward a role for immunotherapeutic strategies complementary to standard treatment protocols. In this report we evaluated the potency of normal allogeneic MoDCs pulsed with whole cell CML lysates to stimulate T cell-mediated immune responses towards CML progenitor cells.

Materials and Methods

Generation of dendritic cells and leukemia-lysate preparation

Mononuclear cells were isolated by Ficoll-density centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) from bone marrow samples of three CML patients in chronic phase and buffy coats of two HLA-A2$^+$ donors. All samples were drawn after informed consent; the study was approved by the local Medical Ethics Committee. MoDCs and lysates were prepared as described previously [21]. In short: CML lysates were prepared by four repetitive cycles of freeze-thawing. CML CD34$^+$ progenitor cells were isolated by CD34 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instruction using an AutoMACS device (Miltenyi Biotec). Donor monocytes and CD8$^+$ T cells were isolated by CD14 and CD8 Microbeads (both Miltenyi Biotec), respectively. Immature MoDCs were cultured from CD14$^+$ cells in 5 days. Half of the amount of MoDCs was incubated with CML lysate for 2h. in a DC to CML ratio of 1:1; the other half was left untouched. This ratio was determined based on the literature on other DC loading strategies (e.g. AML) [19;22]. Thereafter, unloaded and lysate-loaded DCs (LL-MoDCs) were matured for 48h. with a standard mixture of inflammatory cytokines TNF-α (10ng/ml), IL-1β (10ng/ml, specific activity 107U/mg protein, Strathmann Biotec, Hamburg, Germany), IL-6 (10ng/ml, R&D Systems, Abingdon, UK) and prostaglandin E2 (1μg/ml, Sigma, St. Louis, MO) and characterized by flow cytometry as described previously [17].

Analysis of T cell subpopulations during co-culture of T cells and dendritic cells

During 4-6 weeks, donor CD8$^+$ T cells were weekly stimulated with irradiated MoDCs or LL-MoDCs at a 5:1 ratio in the presence of irradiated CD4$^+$ lymphocytes as feeder cells. RhIL-7 (5 ng/ml, specific activity 5*10^{7}U/ml, Strathmann Biotech GmbH, Hannover, Germany) was supplied from culture start, whereas rhIL-2 (10 U/ml, specific activity 10^{7}U/mg, Strathmann Biotech) was added from the second day after (re)stimulation. For every CML patient co-cultures were initiated using one donor; one donor was used in two separate experiments. A flow-chart of the study design is shown in *figure 1*.

Proliferative activity, cytokine production and proportion of naïve, memory and effector CD8$^+$ subpopulations were assessed weekly. T cell subpopulations were characterized by flow cytometry based on expression patterns of CD27 (Immunotech, Marseille, France) and CD45RA (Sanquin, Amsterdam, The Netherlands). Cell free supernatants of the co-cultures (24h. after re-stimulation) were used for evaluation of IFN-γ and IL-4 production by ELISA according to the manufacturer's instructions (Sanquin). Detection limit of both assays was 1 pg/ml. The percentage of cytokine-producing T cells was assessed as described previously [23]. After stimulation by PMA (25 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) for 6 h. at 37ºC. Brefeldin A (10 μg/ml, Sigma) was added for the last 4 h. of incubation; subsequently the percentage IFN-γ and IL-4 positive T cells were determined. Skewing of CD8$^+$ T cells was analyzed by flow cytometry using a panel of monoclonal antibodies representing the most frequently expressed Vβ chains in the normal Vβ repertoire (BeckmanCoulter, Immunotech). Increase above mean plus 2 times the standard deviation according to the manufacturer's reference chart was regarded as outgrowth of a population expressing a certain TCR-Vβ subtype.

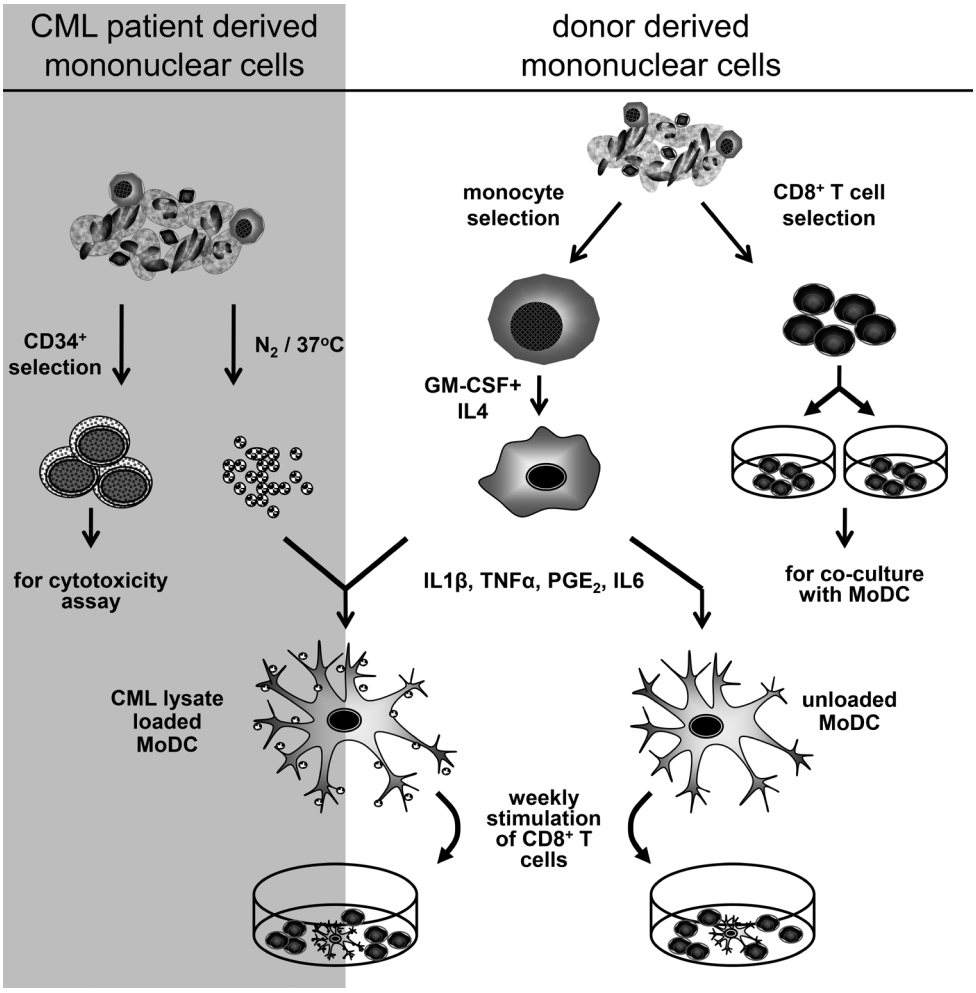


Figure 1. Schematic representation of the study design. The grey panel depicts preparation of lysate and selection of $CD34^+$ cells from CML samples. $CD34^+$ cells were used for testing of cytotoxicity. The white panel depicts the preparation of allogeneic MoDCs after $CD14^+$ cell selection; these were cultured into immature MoDCs, and loaded with CML lysate (border of grey and white panel) or left untouched; subsequently maturation was induced with IL1-β, TNFα, PGE2 and IL6. $CD8^+$ cells from the same donor were isolated and used for co-culture with lysate-loaded or unloaded MoDCs.

Analysis of cytolytic potential of T cells

At the end of co-culture $CD8^+$ T cells were used as effector cells in a cytotoxicity assay as previously described [24]. CML $CD34^+$ progenitor cells derived from the same patient as the CML lysate were used as target cells. In short, CML $CD34^+$ progenitor cells and stimulated allogeneic $CD8^+$ T cells were co-cultured for 6h., stained by a progenitor marker (CD34-APC) and a specific T cell marker (CD3-PE), respectively, followed by a SYTO-16/7-AAD viability staining. To investigate whether the cytotoxic activity was MHC-restricted the cytotoxicity assay was performed in the presence and absence of MHC class I-blocking antibodies or the appropriate isotype control. [23]

Results

Preserved phenotypic characteristics of lysate-loaded MoDC

To investigate whether loading of CML lysate affects DCs, immunophenotype of DCs was analyzed by flow cytometry. Maturation of immature MoDCs in the presence of CML lysate induced similar expression levels of costimulatory molecules CD40, CD80 and CD86 as compared to maturation without lysate (*figure 2*). Maturation marker CD83 was expressed in 85% of the DCs; the mean fluorescence intensity was approximately two-fold higher after lysate-loading. Expression of CCR7, a receptor important for lymph node homing of DCs, decreased from 79 towards 54%. Interestingly, maturation in the presence of CML lysate increased intensity of HLA-DR expression two-fold and HLA-ABC expression 1.3 fold indicating improved antigen presentation by lysate-loaded DCs and possibly enhanced T cell stimulation.

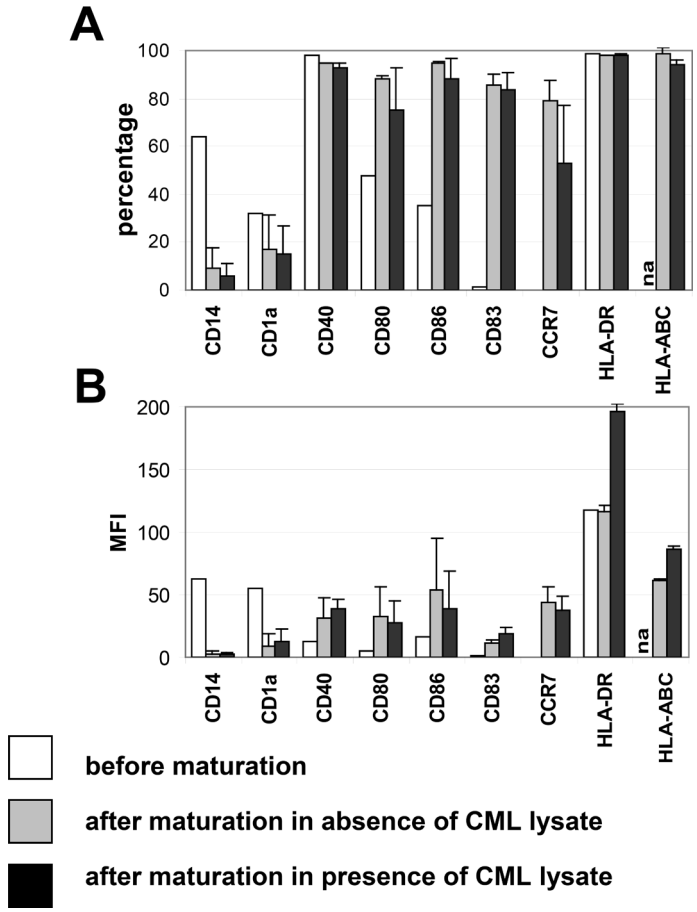


Figure 2. Immunophenotype of CML lysate-pulsed MoDCs and unloaded MoDCs. Panel A. shows percentage positivity for the displayed antigens of MoDCs before maturation (white bars) and after maturation in the absence or presence of CML lysate (grey and black bars respectively); panel B depicts mean fluorescence intensities. HLA-ABC results are not available (na) in immature DCs.

Lysate-loaded MoDCs induce differentiation and increased IFN-γ production in allogeneic CD8⁺ T cells.

Before co-culture, the majority of the T cells had a naïve or central memory phenotype ($CD45RA^+CD27^+$ and $CD45RA^-CD27^+$, mean 25% and 55%, respectively (n=2)). Upon stimulation with LL-MoDCs as well as with unloaded MoDCs a preferential outgrowth of effector-memory cells was observed (three separate co-culture experiments: from 10-15% CD45RA-CD27- at start of co-cultures up to 60-95% after 4-6 weeks). LL-MoDC stimulation resulted in highest percentages of effector T cells ($CD45RA^+CD27^-$: mean 4.6% versus 1.5% in unloaded MoDC co-cultures, *figure 3A*). Of note, $CD8^+$ T cells co-cultured with LL-MoDCs showed a less pronounced proliferation as compared to those co-cultured with unloaded

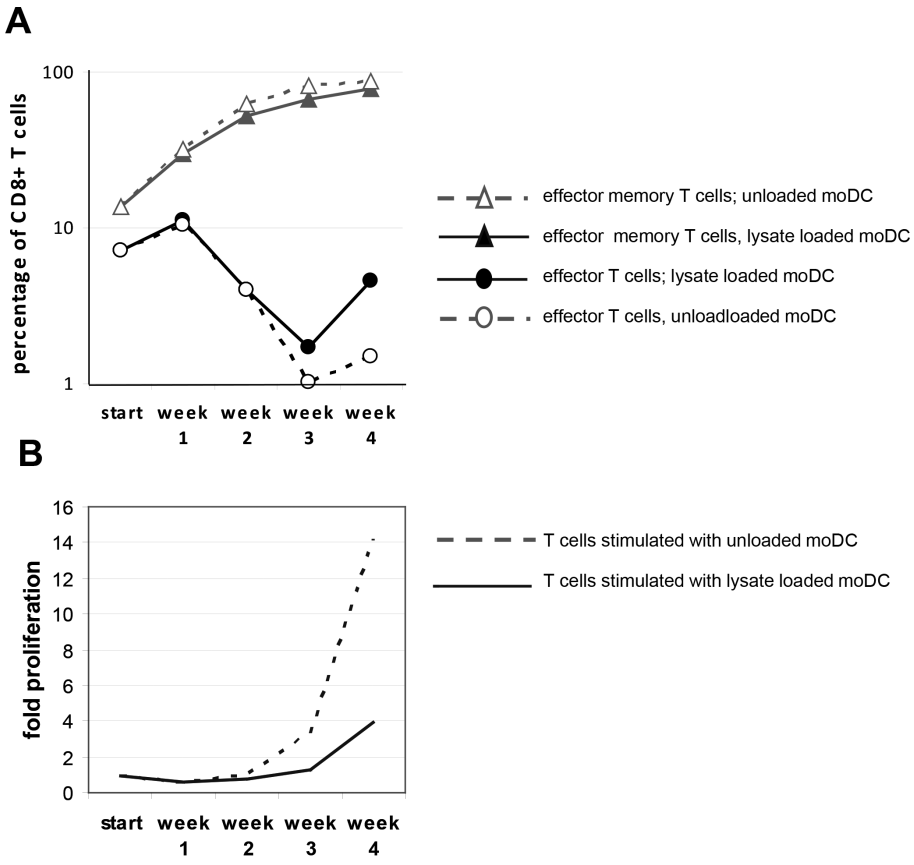


Figure 3. Distribution of T cell subpopulations and proliferation of $CD8^+$ T cells after co-culture with CML lysate-pulsed MoDCs and unloaded MoDCs. Panel A. depicts distribution of effector memory T cells (grey, triangles) and effector T cells (black, circles) after unloaded (dashed lines and open symbols) and CML lysate-loaded DC stimulation (solid lines and closed symbols). Panel B. depicts a representative example of proliferation of $CD8^+$ T cells after co-culture with unloaded MoDCs (dashed line) and LL-MoDCs (solid line).

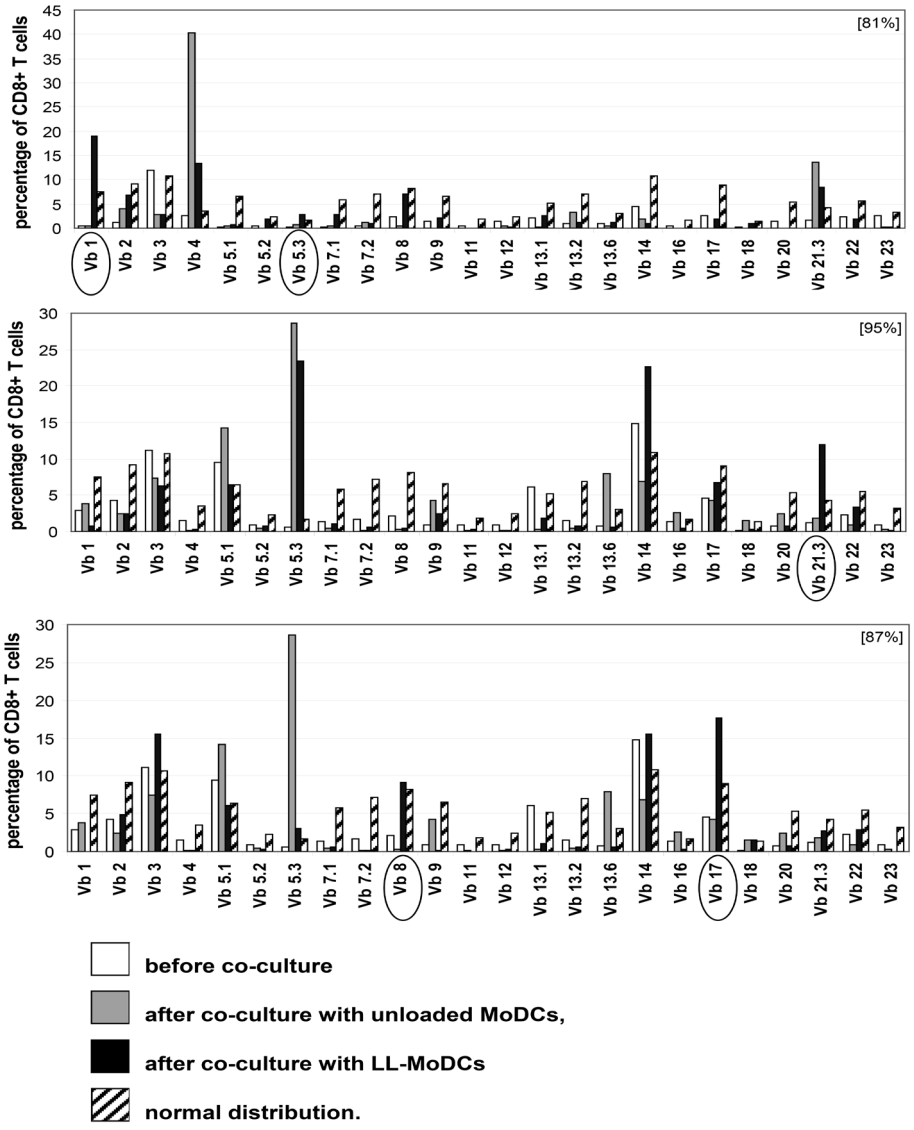


Figure 4. TCR Vβ analysis before and after co-culture with CML lysate-pulsed MoDCs and unloaded MoDCs. Results obtained in three separate experiments are depicted; for every CML patient co-cultures were initiated using one donor; one donor was used in two separate patients. White bars represent TCR Vβ repertoire before co-culture, grey bars represent results after co-culture with unloaded MoDCs, black bars after co-culture with LL-MoDCs and striped bars represent normal distribution. Increase above mean plus 2 times the standard deviation according to the manufacturer's reference chart was regarded as outgrowth of a population expressing a certain TCR-Vβ subtype. Skewing towards a certain Vβ type observed after both LL-MoDC and unloaded MoDC stimulation was considered non-specific. Circles indicate outgrowth only observed after co-culture with LL-MoDCs, this might indicate potentially leukemia-specific skewing. Inserted percentages in the upper right corner of every panel indicate the percentage of the T cell population characterized with the antibody panel after co-culture with LL-MoDCs.

MoDCs (*figure 3B*). Effector-memory and effector T cells are able to produce IFN-γ. At the end of co culture with LL-MoDCs, the number of IFN-γ-producing T cells as measured by flow cytometry was median 25%, i.e. 2.8 fold higher as compared to stimulation with unloaded MoDCs (median 8.9%). The same holds true for IFN-γ as detected in cell free culture supernatants: median 1306 and 268 pg/ml upon LL-MoDC and unloaded MoDC stimulation, respectively. IL-4 production was low for both LL-MoDC and unloaded MoDC stimulation (below 7pg/ml).

Skewing of allogeneic T cells upon lysate-loaded MoDC stimulation

When CML-specific T cells expand after stimulation, a skewing of the T cell repertoire is expected. Therefore, a TCR-Vβ analysis was performed comparing the TCR-Vβ repertoire of T cells before culture and after co-culture with either unloaded MoDCs or LL-MoDCs (*figure 4*). T cells expressing certain TCR-Vβ subtypes preferentially expanded after LL-MoDC stimulation; these populations represented median 16% of the $CD8^+$ T cell population. This might indicate leukemia-specific skewing.

Lysate-loaded MoDCs induce MHC-restricted T cell-mediated cytotoxicity against CML progenitor cells.

To assess whether these allogeneic T-cells were functional, cytotoxic activity of stimulated T cells towards CML $CD34^+$ progenitor cells was analyzed by flow-cytometry. Indeed incubation with LL-MoDC-stimulated T cells resulted in a higher percentage of apoptotic and secondary necrotic $CD34^+$ CML progenitor cells as compared to unloaded MoDC-stimulated T cells (84% versus 36%, 36% versus 29% and 42% versus 29% at E:T=10:1 for LL-MoDC-stimulated and unloaded MoDC-stimulated T cells, respectively, *figure 5A*). In two out of three cases, induction of apoptosis/necrosis in CML progenitors by LL-MoDC-stimulated T cells was abrogated in the presence of MHC class I antibody, indicating MHC class I-restriction (*figure 5B*).

Discussion

Specific T cell responses against CML progenitor cells might effectively eliminate MRD after treatment with TKI. LL-MoDC-based vaccines in CML may offer an advantage over single antigen-derived peptide vaccination (i.e. BCR-ABL and WT1) as they have the ability to present a broad spectrum of potential leukemia-associated antigens resulting in a broader T cell response. Moreover, the use of leukemia lysate as an antigen source for vaccine preparation circumvents the cumbersome need for viable, fresh leukemia cells for leukemic DC-based vaccines [25]. In this study we showed that MoDCs can be loaded with CML lysates and that these LL-MoDCs have the potency to induce, at least *in vitro*, anti-leukemic T cell responses as demonstrated by TCR-Vβ skewing and cytolytic activity. A limitation of the use of lysates may be that they contain many non-malignancy associated peptides which might increase the risk of auto-immunity [26]. Nevertheless, in clinical trials with CML-derived DCs, AML-derived DCs and MoDCs loaded with AML lysates no or only minor auto-reactivity was reported [13;19;22;27]. Moreover, cell lysates may impair DC function because they contain intracellular proteases with potential inhibitory activity, and immunosuppressive cytokines, such as TGF-β, which might impair immune responses [28-30]. In fact, our data

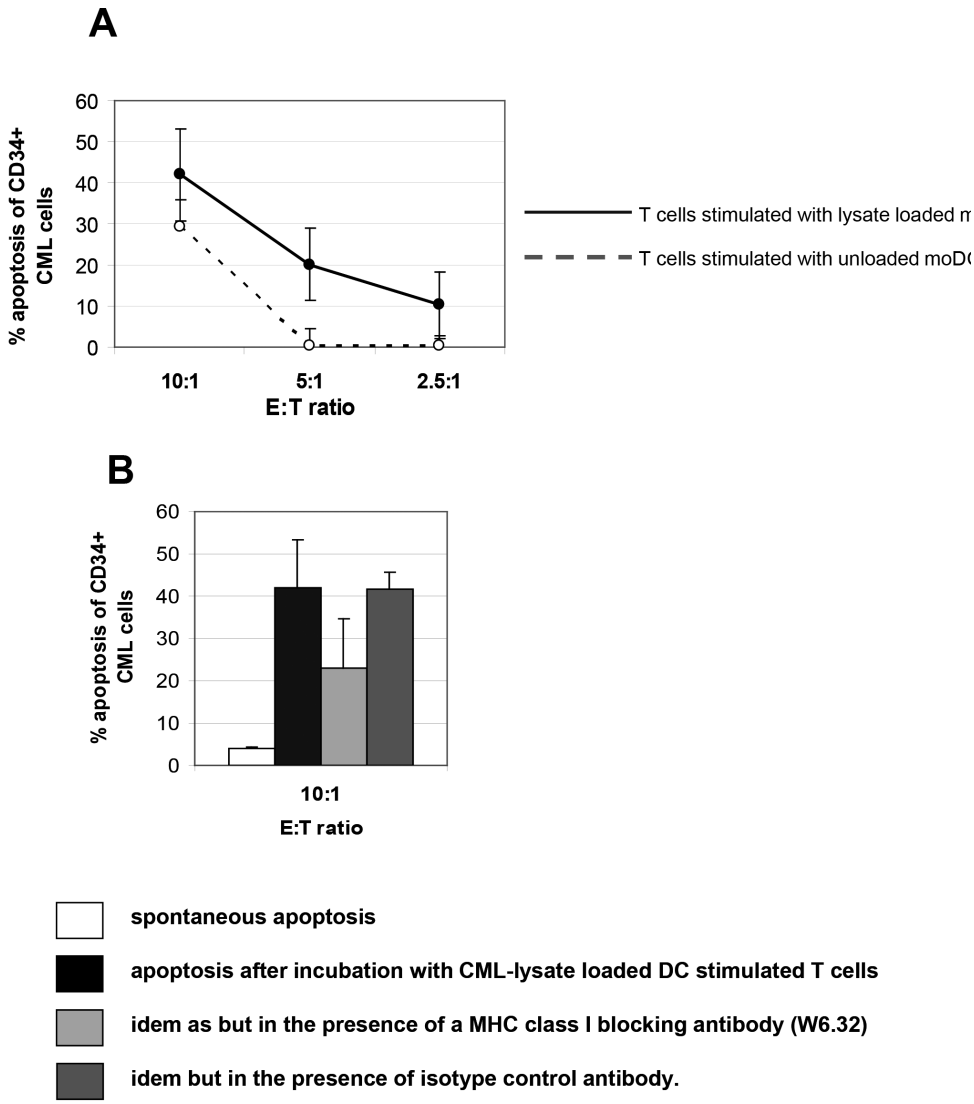


Figure 5. MHC-restricted cytotoxicity towards CD34⁺ CML progenitor cells induced by lysate-loaded MoDC-stimulated CD8⁺ T cells. A representative example of the analysis of cytotoxic activity of DC-stimulated T cells is shown in panel A; cytotoxic activity is depicted at several effector to target ratios (E:T). The solid line represents apoptosis/secondary necrosis of target cells after 6 hours of co-culture with CD8⁺ T cells stimulated by LL-MoDCs; the dashed line represents apoptosis/secondary necrosis of target cells after 6 hours of co-culture with CD8⁺ T cells stimulated by unloaded MoDCs. In panel B, analysis of MHC class I restriction of cytotoxic activity is depicted. White bar represents spontaneous apoptosis of CD34⁺ CML progenitor cells, black bar represents apoptosis of CD34⁺ CML progenitor cells after co-culture with LL-MoDC-stimulated CD8⁺ T cells, light grey bar after co-culture with LL-MoDC-stimulated CD8⁺ T cells in the presence of a MHC class I blocking antibody (W6.32) and the dark grey bar after co-culture with LL-MoDC-stimulated CD8⁺ T cells in the presence of isotype control antibody.

showed less proliferation of T cells in co-cultures with LL-MoDCs; an alternative explanation of this phenomenon might be antigen-induced cell death due to overstimulation by CML lysate-derived antigens. Indeed, LL-MoDCs showed increased expression of MHC class I and II molecules, indicating potentially increased presentation of LAA-derived peptides and hence improved immunogenicity. In contrast, LL-MoDCs showed decreased expression of CCR7, potentially indicating decreased migratory capacity. Whether this decrease has any clinical impact *in vivo* is not clear, since in general, only low numbers of DCs reach the lymph node after vaccination [31].

In CML, the leukemic population is rather heterogeneous with regard to maturation and differentiation of the myeloid cells, which may influence the susceptibility to different cytotoxic modalities [32]. This was confirmed in this study by partial killing of CML-derived $CD34^+$ progenitor cells *in vitro*. Escape of CML progenitor cells from immunotherapeutic intervention causes persistence of MRD. MRD cells in CML are thought to be quiescent progenitor cells mainly residing in the bone marrow; these cells are more resistant to TKI than their cycling counterparts [33-36]. One way to target this specific subpopulation is to load DCs with lysates of leukemic stem cells. However, some reports argue that RNA- and protein-pulsed DCs are more effective in inducing CML-specific T cell responses *in vitro* than lysate-loaded DCs [37;38]. An advantage of RNA is that it can be amplified from small populations such as stem cells, moreover, as with whole cell lysates, it covers both known and unknown antigens. Leukemic stem cells in CML, defined as $CD34^+CD38^-$ or $CD34^-lin^-$, can be distinguished by flow cytometry from normal stem cells by scatter profile and aberrant antigen expression [39;40]. These characteristics can be used to sort leukemic stem cells for application in DC-based vaccine preparation.

Conclusion

Here we provide proof of principle that MoDCs loaded with CML lysates were able to induce T cell proliferation and IFN-γ production by $CD8^+$ T cells. Importantly, these allogeneic T cells were able to recognize and lyse CML cells *in vitro*. Hence, our data show the potency of DC-based vaccination strategies in the induction of anti-leukemia responses and offer a basis for further optimalization of MoDC-based vaccination strategies.

Future perspectives

CML patients that have residual disease after conventional treatments with TKIs are at high risk for relapse and might benefit from additional therapeutic strategies. In these cases, DC-based immunotherapy might provide an additional therapeutic option. The data shown in this manuscript provide a starting point for further research in this respect. Our results are a first step down the road towards DC vaccination after allogeneic SCT. Moreover, the data prelude application of DCs specifically directing T cell responses towards the eradication of leukemic stem cells thereby reducing the risk of relapse.

Reference List

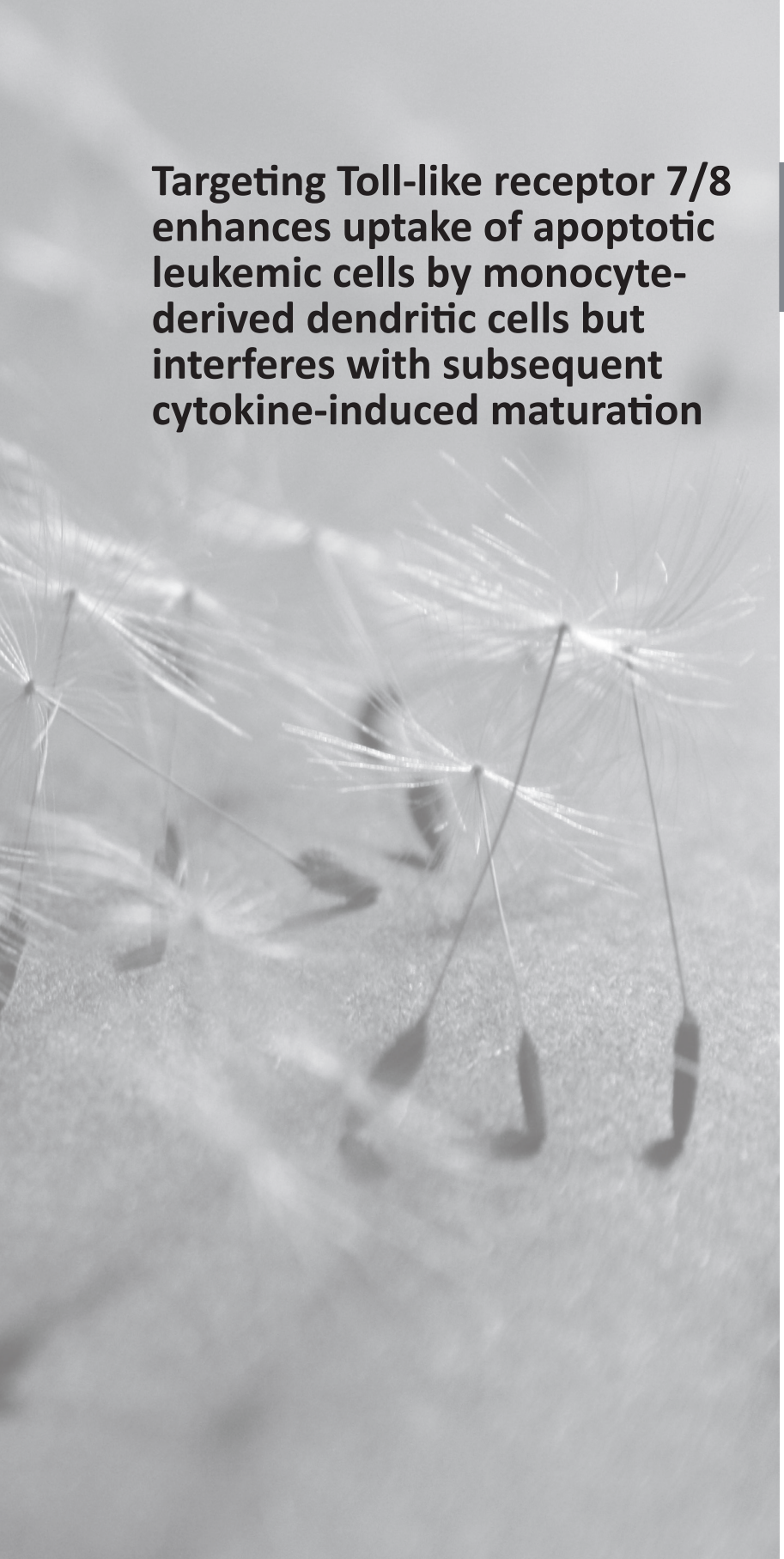
1. Kantarjian HM, Cortes JE, O'Brien S et al. Long-term survival benefit and improved complete cytogenetic and molecular response rates with imatinib mesylate in Philadelphia chromosome-positive chronic-phase chronic myeloid leukemia after failure of interferon-alpha. Blood. 2004;104:1979-1988.
2. Druker BJ, Guilhot F, O'Brien SG et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N.Engl.J.Med. 2006;355:2408-2417.
3. Kantarjian H, Giles F, Wunderle L et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N.Engl.J.Med. 2006;354:2542-2551.
4. Hochhaus A, Kantarjian HM, Baccarani M et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. Blood. 2007;109:2303-2309.
5. Clark RE, Dodi IA, Hill SC et al. Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. Blood 2001;98:2887-2893.
6. Makita M, Azuma T, Hamaguchi H et al. Leukemia-associated fusion proteins, dek-can and bcr-abl, represent immunogenic HLA-DR-restricted epitopes recognized by fusion peptide-specific $CD4^+$ T lymphocytes. Leukemia 2002;16:2400-2407.
7. Wagner WM, Ouyang Q, Pawelec G. The abl/bcr gene product as a novel leukemia-specific antigen: peptides spanning the fusion region of abl/bcr can be recognized by both $CD4^+$ and $CD8^+$ T lymphocytes. Cancer Immunol.Immunother. 2003;52:89-96.
8. Rojas JM, Knight K, Wang L, Clark RE. Clinical evaluation of BCR-ABL peptide immunisation in chronic myeloid leukaemia: results of the EPIC study. Leukemia. 2007;21:2287-2295.
9. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood. 2008;112:4371-4383.
10. Smit WM, Rijnbeek M, van Bergen CA et al. T cells recognizing leukemic CD34(+) progenitor cells mediate the antileukemic effect of donor lymphocyte infusions for relapsed chronic myeloid leukemia after allogeneic stem cell transplantation. Proc.Natl.Acad.Sci.U.S.A. 1998;95:10152-10157.
11. Grabrucker C, Liepert A, Dreyig J et al. The quality and quantity of leukemia-derived dendritic cells from patients with acute myeloid leukemia and myelodysplastic syndrome are a predictive factor for the lytic potential of dendritic cells-primed leukemia-specific T cells. J.Immunother. 2010;33:523-537.
12. Liepert A, Grabrucker C, Kremser A et al. Quality of T-cells after stimulation with leukemia-derived dendritic cells (DC) from patients with acute myeloid leukemia (AML) or myeloid dysplastic syndrome (MDS) is predictive for their leukemia cytotoxic potential. Cell Immunol. 2010;265:23-30.
13. Ossenkoppele GJ, Stam AG, Westers TM et al. Vaccination of chronic myeloid leukemia patients with autologous in vitro cultured leukemic dendritic cells. Leukemia 2003;17:1424-1426.
14. Westermann J, Kopp J, van LA et al. Vaccination with autologous non-irradiated dendritic cells in patients with bcr/abl+ chronic myeloid leukaemia. Br.J.Haematol. 2007;137:297-306.
15. Schmitt M, Li L, Giannopoulos K et al. Chronic myeloid leukemia cells express tumor-associated antigens eliciting specific $CD8^+$ T-cell responses and are lacking costimulatory molecules. Exp.Hematol. 2006;34:1709-1719.

16. Dong R, Cwynarski K, Entwistle A et al. Dendritic cells from CML patients have altered actin organization, reduced antigen processing, and impaired migration. Blood 2003;101:3560-3567.
17. Westers TM, Janssen JJ, Houtenbos I et al. Maintained immunogenicity of chronic myeloid leukemia-derived dendritic cells in the presence of Imatinib mesylate: implication for vaccination regimens. Leukemia 2006;20:154-157.
18. Eisendle K, Lang A, Eibl B et al. Phenotypic and functional deficiencies of leukaemic dendritic cells from patients with chronic myeloid leukaemia. Br.J.Haematol. 2003;120:63-73.
19. Lee JJ, Kook H, Park MS et al. Immunotherapy using autologous monocyte-derived dendritic cells pulsed with leukemic cell lysates for acute myeloid leukemia relapse after autologous peripheral blood stem cell transplantation. J.Clin.Apheresis. 2004;19:66-70.
20. Zeng Y, Graner MW, Feng H, Li G, Katsanis E. Imatinib mesylate effectively combines with chaperone-rich cell lysate-loaded dendritic cells to treat bcr-abl$^+$ murine leukemia. Int.J.Cancer 2004;110:251-259.
21. van den Ancker W, van Luijn MM, Ruben JM et al. Targeting Toll-like receptor 7/8 enhances uptake of apoptotic leukemic cells by monocyte-derived dendritic cells but interferes with subsequent cytokine-induced maturation. Cancer Immunol.Immunother. . 2011;60:37-47.
22. Weigel BJ, Panoskaltsis-Mortari A, Diers M et al. Dendritic cells pulsed or fused with AML cellular antigen provide comparable in vivo antitumor protective responses. Exp.Hematol. 2006;34:1403-1412.
23. Westers TM, Houtenbos I, van de Loosdrecht AA, Ossenkoppele GJ. Divergent autologous T cell responses to leukaemic dendritic cells during remission in acute promyelocytic leukaemia. Cell Oncol. 2005;27:261-266.
24. Westers TM, Houtenbos I, Schuurhuis GJ, Ossenkoppele GJ, van de Loosdrecht AA. Quantification of T-cell-mediated apoptosis in heterogeneous leukemia populations using four-color multiparameter flow cytometry. Cytometry A 2005;66:71-77.
25. van den Ancker W, van Luijn MM, Westers TM et al. Recent advances in antigen-loaded dendritic cell-based strategies for treatment of minimal residual disease in acute myeloid leukemia. Immunotherapy. 2010;2:69-83.
26. Roskrow MA, Dilloo D, Suzuki N et al. Autoimmune disease induced by dendritic cell immunization against leukemia. Leuk.Res. 1999;23:549-557.
27. Li L, Giannopoulos K, Reinhardt P et al. Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts. Int.J.Oncol. 2006;28:855-861.
28. Schui DK, Singh L, Schneider B et al. Inhibiting effects on the induction of cytotoxic T lymphocytes by dendritic cells pulsed with lysates from acute myeloid leukemia blasts. Leuk. Res. 2002;26:383-389.
29. Galea-Lauri J, Wells JW, Darling D, Harrison P, Farzaneh F. Strategies for antigen choice and priming of dendritic cells influence the polarization and efficacy of antitumor T-cell responses in dendritic cell-based cancer vaccination. Cancer Immunol.Immunother. 2004;53:963-977.
30. Motta JM, Nascimento CR, Rumjanek VM. Leukemic cell products down-regulate human dendritic cell differentiation. Cancer Immunol.Immunother. 2010;59:1645-1653.
31. de Vries IJ, Lesterhuis WJ, Barentsz JO et al. Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy. Nat.Biotechnol. 2005;23:1407-1413.
32. Sloma I, Jiang X, Eaves AC, Eaves CJ. Insights into the stem cells of chronic myeloid leukemia. Leukemia. 2010;24:1823-1833.

33. Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood. 1999;94:2056-2064.
34. Graham SM, Jorgensen HG, Allan E et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 2002;99:319-325.
35. Copland M, Hamilton A, Elrick LJ et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood. 2006;107:4532-4539.
36. Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in $CD34^+$ CML cells. Blood. 2007;109:4016-4019.
37. Syme R, Bryan T, Duggan P et al. Priming with dendritic cells can generate strong cytotoxic T cell responses to chronic myelogenous leukemia cells in vitro. Stem Cells Dev. 2004;13:211-221.
38. Decker WK, Xing D, Li S et al. Double loading of dendritic cell MHC class I and MHC class II with an AML antigen repertoire enhances correlates of T-cell immunity in vitro via amplification of T-cell help. Vaccine. 2006;24:3203-3216.
39. Lemoli RM, Salvestrini V, Bianchi E et al. Molecular and functional analysis of the stem cell compartment of chronic myelogenous leukemia reveals the presence of a CD34- cell population with intrinsic resistance to imatinib. Blood. 2009;114:5191-5200.
40. Janssen JJ, Deenik W, Smolders KG et al. Residual normal stem cells can be detected in newly diagnosed chronic myeloid leukemia patients by a new flow cytometric approach and predict for optimal response to imatinib. Leukemia. 2012;26:977-84

Targeting Toll-like receptor 7/8 enhances uptake of apoptotic leukemic cells by monocyte-derived dendritic cells but interferes with subsequent cytokine-induced maturation

14



W van den Ancker
MM van Luijn
JM Ruben
TM Westers
HJ Bontkes
GJ Ossenkoppele
TD de Gruijl
AA van de Loosdrecht

Cancer Immunol Immunother. 2011 Jan;60(1):37-4

Abstract

Therapeutic vaccination with dendritic cells (DC) is an emerging investigational therapy for eradication of minimal residual disease in acute myeloid leukemia (AML). Various strategies are being explored in manufacturing DC vaccines ex vivo, e.g. monocyte-derived DC (MoDC) loaded with leukemia-associated antigens (LAA). However, the optimal source of LAA and the choice of DC-activating stimuli are still not well defined. Here, loading with leukemic cell preparations (harboring both unknown and known LAA) was explored in combination with a DC maturation-inducing cytokine cocktail (CC; IL-1β, IL-6, TNF-α and PGE_2) and Toll-like receptor ligands (TLR-L) to optimize uptake. Since heat shock induced apoptotic blasts were more efficiently taken up than lysates we focused on uptake of apoptotic leukemic cells. Uptake of apoptotic blast was further enhanced by the TLR-7/8-L R848 (20-30%); in contrast, CC-induced maturation inhibited uptake. CC, and to a lesser extent R848, enhanced the ability of MoDC to migrate and stimulate T cells. Furthermore, class II-associated invariant chain peptide (CLIP) expression was down-modulated after R848- or CC-induced maturation, indicating enhanced processing and presentation of antigenic peptides. To improve both uptake and maturation, leukemic cells and MoDC were co-incubated with R848 for 24h followed by addition of CC. However, this approach interfered with CC-mediated MoDC maturation as indicated by diminished migratory and T cell stimulatory capacity and the absence of IL-12 production. Taken together, our data demonstrate that even though R848 improved uptake of apoptotic leukemic cells, the sequential use of R848 and CC is counter-indicated due to its adverse effects on MoDC maturation.

Introduction

Acute myeloid leukemia (AML) is characterized by proliferation of clonal neoplastic myeloid hematopoietic precursor cells and impaired normal hematopoiesis. Although 70-80% of patients (<60 years) achieve complete remission after intensive chemotherapy, AML frequently relapses due to the persistence of minimal residual disease (MRD) [1]. Escape of leukemic cells from immune surveillance has been associated with poor clinical outcome. For instance, a high occupancy of HLA-DR molecules with the class II-associated invariant chain peptide (CLIP) instead of an antigenic peptide is correlated with a shortened disease free and overall survival [2;3]. In contrast, immune control of leukemia, as shown for instance by the graft-versus-leukemia effect induced by allogeneic stem cell transplantation (SCT) or the reinduction of complete remission after donor lymphocyte infusion following allogeneic SCT, demonstrates the potential of exploiting the immune system in aid of anti-AML therapy [1].

The induction, regulation and maintenance of primary immune responses, including specific anti-tumor T cell responses is coordinated by dendritic cells (DC). Vaccination with DC has been recognized as a promising investigational therapy due to the uniquely powerful antigen (Ag) presenting capacity of DC and its potential to circumvent immunosuppressive features of leukemia [4]. The first steps down the road to DC vaccination in AML have been taken and results from small clinical trials have been reported [5]. The general lack of clinical responses evoked important questions concerning the optimal methodologies for DC vaccine preparation as well as the design of clinical vaccination protocols. Many

strategies are explored in the preparation of DC vaccines *ex vivo*; among these, autologous monocyte-derived DC (MoDC) loaded with leukemia-associated Ag (LAA) are promising [6]. Various sources of LAA and different methods of loading LAA onto DC have been explored in an attempt to optimize anti-tumor responses [7]. For AML, several relevant LAA have been identified including PRAME, RHAMM, WT1 and PR1. Unfortunately, overexpression of these LAA is common but not uniform in leukemia [8]. Moreover, HLA restriction of LAA-derived peptides limits application of such vaccines to patients with certain HLA profiles. These restrictions inherent to the use of defined LAA or LAA-derived peptides may be overcome by using whole AML cells as a source of LAA, for instance by generation of AML lysates or apoptotic leukemic cells. Among other whole AML cell derived antigen loading strategies that have been explored, is electroporation of DC with AML derived RNA [9]. Also vaccination with modified AML cells, such as AML derived DC or fusions between AML cells and DC has been investigated; further modification of DC with 4-1BB-L or CD40 might enhance efficacy of such vaccines [10-13]. It has long been assumed that apoptotic cell death is poorly immunogenic or even tolerogenic, whereas necrotic cell death is considered to be immunogenic. However, stress-induced heat shock protein (HSP)-peptide complexes (commonly induced during apoptosis) are more efficiently taken up via scavenger receptors and Toll-like receptors (TLR) on the DC surface and induce efficient cross-priming and skewing of the immune response towards a Th1-type profile [14;15], whereas necrosis has been associated with local immune suppression in solid tumors [16]. Furthermore, apoptosis induction after irradiation with UV light or by treatment with chemotherapeutic drugs results in upregulation of calreticulin, a scavenger receptor class-A ligand associated with immunogenic apoptosis as demonstrated for colon carcinoma cells [17;18]. For AML it is not yet clear whether cell lysates or apoptotic cells are preferable for Ag loading onto MoDC [9;19-25]. Next to enhancing immunogenicity of tumor Ag sources by, for instance, heat shock, addition of DC-maturing stimuli such as TLR-ligands (TLR-L) are explored; for example, electroporation of TLR3-L poly(I:C) into AML cells results in enhanced uptake of leukemic cells by DC and improves their subsequent maturation and cytokine production [26]. Furthermore, intracellular binding of TLR8 by its ligand R848 has been reported to result in enhanced cross-priming of exogenous Ag by MoDC [27]. Various whole AML cell preparations loaded onto MoDC in combination with DC maturation-inducing cocktails have been explored [9;21]; however the quantitative effects of TLR-L and cytokines on uptake of leukemic cells is still unclear.

In this study we compared uptake of allogeneic apoptotic leukemic cells with lysates derived from leukemic cells. We investigated the uptake of heat shock-induced apoptotic leukemic cells by MoDC and DC maturation by combining a standard cytokine cocktail (CC) with the clinically applicable TLR7/8-L R848.

Material and Methods

Patient samples

Peripheral blood or bone marrow mononuclear cells from AML patients were isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). All samples were drawn after obtaining the patients' informed consent at time of collection. Cells were cultured immediately or cryopreserved at a controlled rate in liquid nitrogen

using RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 20% heat-inactivated FCS (Greiner, Alphen a/d Rijn, The Netherlands) and 10% dimethylsulphoxide (Merck, Darmstadt, Germany). Before application of stored AML cells, cryopreserved material was rapidly thawed and washed twice in RPMI-1640 supplemented with 40% FCS. The cells were then resuspended in culture medium as described below.

Culture of monocyte-derived dendritic cells (MoDC)

Buffy coats were obtained from healthy volunteers according to institutional guidelines. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque. Subsequently, monocytes were obtained by magnetic bead isolation, using CD14-labeled Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). MoDC were cultured from $CD14^+$ monocytes in CellGro serum-free medium (CG) (CellGenix, Freiburg, Germany) supplemented with 100IU/ml penicillin and 100µg/ml streptomycin, GM-CSF (800IU/ml, specific activity $1x10^5$ IU/mg protein; Pepro Tech, Rocky Hill NJ) and IL-4 (500 IU/ml, specific activity $1x10^7$ U/mg protein, Pepro Tech). After five days immature MoDC were harvested. Purity and phenotype of immature DC were evaluated by flow cytometry for expression of CD14, CD40, CD34, CD80, CD86, HLA-DR, CD54 and CD83. For maturation induction the following TLR-L were used: R848 (3 µg/ml; Alexis Biochemicals, Axxora, Grünberg, Germany), LPS (100ng/ml; Sigma-Aldrich, Saint Louis, MO), Polyinosinic-polycyticylic acid potassium salt (Poly(I:C) 25µg/ml; Sigma-Aldrich), Flagellin (5µg/ml; Invivogen, San Diego CA) and Peptidoglycan (PGN; 5µg/ml; Sigma-Aldrich). Maturation with CC was induced using the following combination of cytokines and reagents: recombinant TNF-α (200IU/ml; Strathmann Biotec, Hannover, Germany), IL-1β (10 ng/ml; Strathmann Biotec), PGE_2 (1 µg/ml; Sigma-Aldrich) and IL-6 (10 ng/ml; R&D Systems, Abingdon, UK).

Loading of MoDC with allogeneic leukemic cell lysates and apoptotic leukemic cells

Leukemic cells were labeled with 5- (and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon). Cells were suspended in buffered saline (PBS) at $10x10^6$/ml and labeled for 10 min with 1 M CFSE at 37°C, washed three times with RPMI-1640, 10% FCS and incubated overnight in RPMI-1640, supplemented with 100IU/ml penicillin and 100µg/ml streptomycin and 10% FCS. Leukemic cells were harvested and taken up in PBS and apoptosis was induced by heat shock (two hours at 42ºC) or by incubation for two hours at 37ºC with ARA-C (10µg/ml; Mayne Pharma, Warwickshire, UK). The percentage of necrotic, apoptotic and viable cells was determined before and after apoptosis induction by incubation with Syto-62 (5 nM; Molecular Probes, Eugene OR), PSC833 (2µM; Novartis, Basel, Switzerland) and 7-amino-actinomycin D (7-AAD; ViaProbe, Pharmingen, San Diego CA) for 45 min at 37ºC and by flow cytometric analysis as described previously [28]. Syto-62 is a fluorescent nucleic acid stain that exhibits bright fluorescence upon binding to nucleic acids and is retained in viable cells. Together with the dead-cell dye 7-aminoactinomycine-D (7-AAD) discrimination between viable ($Syto^+/7AAD^-$), and early ($Syto^-/7AAD^-$) and late ($Syto^-/7AAD^+$) stages of apoptosis can be made. MoDC were labeled with CellVue Plum, (Polysciences, Washington, CO), or with PKH26 (Sigma, MO, USA) according to manufacturers' guidelines. After labeling, DC were washed three times and put in CG medium. MoDC were cultured in a 1:1 or 1:3 ratio with pretreated leukemic cells in CG medium combined with 100IU/ml penicillin and 100µg/ml streptomycin and 800IU/ml

GM-CSF and 500IU/ml IL-4 at 37ºC, 5% CO_2. Using different time schedules, cultures were supplemented with the indicated maturation stimuli.

Flow cytometric analysis of MoDC

Four-color flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose CA). Results were analyzed using CellQuest software (Becton Dickinson). Immature DC and DC after maturation were stained by appropriate dilutions or mouse isotype-matched control moAb of fluorescein isothiocyanate (FITC)-labeled CD14 (Becton Dickinson, San Jose CA), CD1a (Sanquin), CD54 (DAKO, Glostrup, Denmark). Phyco-erythrin (PE)-labeled monoclonal antibodies (moAb) were used against CD40 (Coulter Immunotech, Marseilles, France), CD80, CD86, CCR-7 (CD197, Becton Dickinson, San Jose CA), CLIP (cerCLIP.1; Santa Cruz Biotechnology, Santa Cruz CA), CD83 (Coulter Immunotech) and peridinin chlorophyll protein (PerCP)-labeled anti-HLA-DR and CD34 (Becton Dickinson). Isotype controls used were FITC-labeled IgG1 (DAKO) and IgG2b (Sanquin), PE-labeled IgG1 (Becton Dickinson) and IgG2b (DAKO) and PerCP-labeled IgG1 and IgG2a (Becton Dickinson). For HLA-DO and HLA-DM the following mouse moAb were used: HLA-DM (Becton Dickinson) and IgG1 isotype control (Becton Dickinson); FITC-labeled HLA-DO (Becton Dickinson) and IgG2b (Becton Dickinson). Cells were stained for DO and DM after fixation with paraformaldehyde followed by permeabilization with PBS-0.1% Saponine (Sigma-Aldrich). Results are presented as either the percentage of positive cells compared to the appropriate isotype control or the mean fluorescence index (MFI). MFI represents the mean fluorescence obtained through binding of the moAb of interest divided by the mean fluorescence of the appropriate isotype control. Relative CLIP expression was determined by calculating the ratio between CLIP and HLA-DR expression based on both the percentage and MFI of positive cells, as described previously [2].

DC migration assay

The migratory capacity of DC was evaluated in a transwell system (5 µm poresize, Corning Costar, Corning, NY, USA) under serum-free conditions. Cells were allowed to migrate 16h towards medium with or without chemoattractant CCL19 (MIP3β, 300ng/ml; R&D Systems, Abingdon, UK). The percentage of migrated cells was quantified by flow cytometry using a fixed amount of fluorescent beads as a reference (FlowCount Fluorospheres, Coulter, Miami, FL, USA).

CD40 ligation and cytokine release

DC were harvested, washed and seeded in a 96-well round-bottomed plate at 40 x 10^3 cells in 200 µl of medium containing 10% FCS per well. CD40L expressing cells (i.e. muCD154-transfected J558 cells) were used as stimulator cells at 40 x 10^3 per well. After twenty-four hour, supernatants were harvested and analyzed for IL-12 by ELISA, as described previously [29].

Mixed Leukocyte Reaction

The ability of the various AML-loaded DC to stimulate T cells was tested in an allogeneic mixed leukocyte reaction (MLR). After isolation of peripheral blood mononuclear cells (PBMC) from a buffy-coat, $CD8^+$ cells were obtained by magnetic cell sorting with anti-CD8-

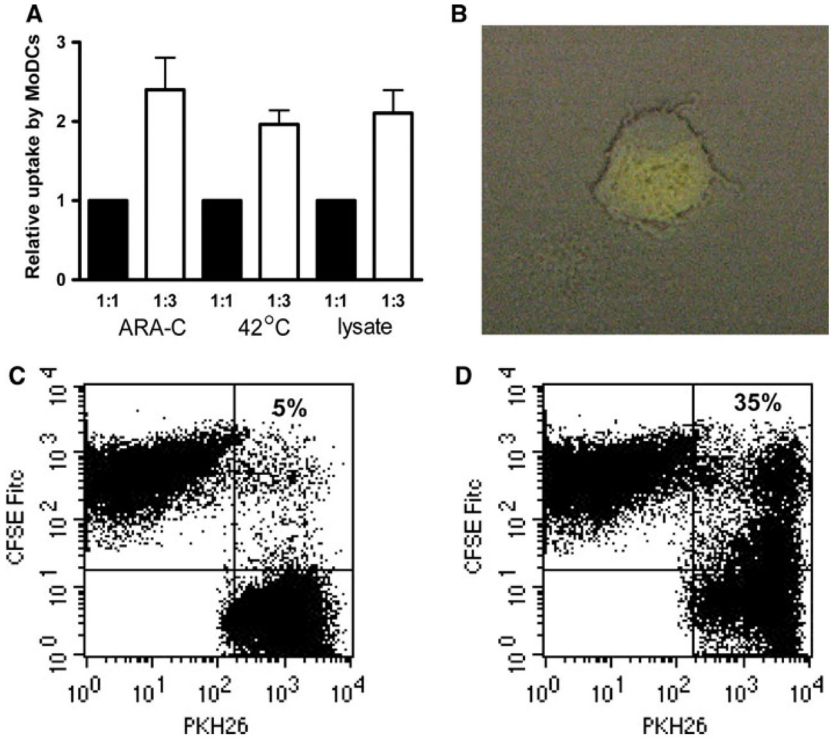


Figure 1. Uptake of leukemic cells by MoDC is a dose-dependent and active process. Blast cells were labeled with CFSE and subsequently apoptosis was induced by heat shock at 42ºC or incubation with ARA-C for 2 hours; lysates were generated by three freeze-thawing cycles. Immature MoDC were labeled with CellVue Plum (emitting in the FL4 channel) or with PKH-26 (emitting in the FL2 channel) and co-incubated with leukemic cells. (A) Uptake of leukemic cells by MoDC is depicted after 24h of co-incubation. Results of 1:3 blast:DC ratio (filled bars) are presented as relative uptake compared to uptake at blast:DC ratio of 1:1 (open bars) (±SEM) (n=14). (B) CFSE-positive MoDC after co-incubation of CFSE labeled lysate (400x magnification). (C) Dot plots representing uptake of CFSE-labeled blasts after 2h incubation at 0ºC or (D) 37ºC; insets represent percentage of PKH26-labeled MoDC positive for CFSE.

labeled magnetic Microbeads (Miltenyi Biotec GmbH Bergisch Gladbach, Germany) and used as responder cells. DC were irradiated at 30Gy and then added to round-bottomed 96-well tissue-culture plates (Costar, Corning NY) at various stimulator to responder ratios. All ratios were tested in triplicate; cells were cultured in RPMI containing 10% FCS. After 5 days Thymidine was added (0.4 µCi per well; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) for 16h, after which the cells were harvested onto fiberglass filters and 3H-Thymidine incorporation was determined using a flatbed scintillation counter (Wallac, Turku, Finland).

Statistical analysis

Statistical significance of differences between the various loading and maturation procedures was determined by the paired sample student's t-test (two-tailed). P-values of <0.05 were regarded as significant.

Results

Uptake of apoptotic leukemic cells or lysates by MoDC is a dose-dependent and active process

To investigate the use of whole leukemic cells as a source of LAA for loading onto DC, we co-incubated monocyte-derived DC from healthy donors (HD) with either apoptotic leukemic cells or lysates, each labeled with different fluorochromes. "Apoptotic samples contained less than 25% (range 25-0%) viable cells; the ratio early apoptotic/viable cells was 5 times higher after 2h incubation at 42ºC compared with the control situation (2h at 37ºC, data not

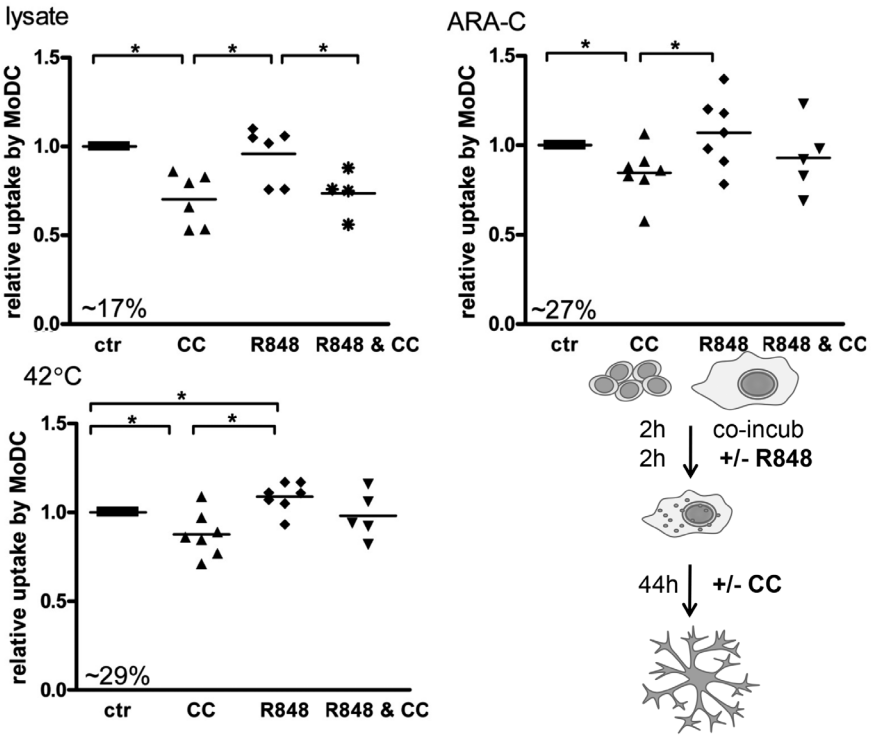


Figure 2. Differential effects of R848 and a cytokine cocktail on uptake of apoptotic AML cells or AML lysates. Apoptosis of AML cells was induced by heat shock at 42ºC or incubation in ARA-C for 2h. Leukemic cells and MoDC were incubated for 2h, R848 was added for another two hours for the R848 and R848 & cytokine cocktail (CC) conditions. Four hours after the start of co-culture CC was added for the CC and R848 & CC condition (as depicted in the schematic illustration). Uptake was measured by flow cytometry and indicated as the percentage of CFSE-positive MoDC, 48h after the start of co-culture. Relative uptake of blast products by MoDC is depicted, i.e. relative increase as compared to no addition of cytokines or R848. Mean percentage of CFSE-positive DC in the control group is listed in the lower left corner of each graph; horizontal lines represent means; * = $p<0.05$; n=6.

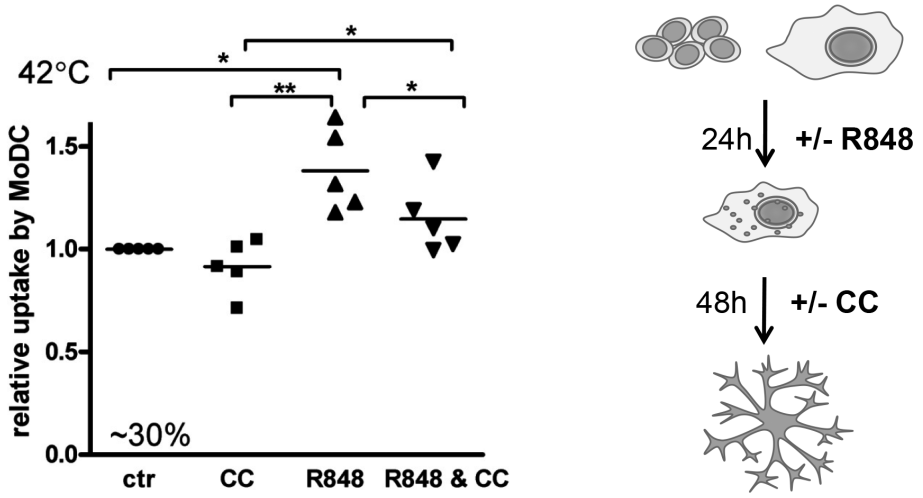


Figure 3. Differential effects of R848 and a cytokine cocktail on uptake of apoptotic AML. Uptake of blast preparations by MoDC is depicted. Apoptosis of AML cells was induced by heat shock for 2h at 42ºC. After 24h of co-incubation of apoptotic blasts and MoDC, either in presence or absence of R848, a cytokine cocktail (CC) was added. Uptake was measured by flow cytometry and indicated as the percentage of CFSE-positive MoDC, 72h after the start of co-culture. Relative increase to no addition of cytokines or R848 is depicted. Mean percentage of CFSE-positive MoDC from the control group is listed in the lower left corner of the graph; horizontal lines represent means; * = p<0.05; **= p<0.005, n=5.

shown). The percentages of apoptotic cells did not differ between the methods (incubation with Cytarabine (ARA-C) or heat shock (HS) at 42ºC) used to induce apoptosis. Uptake of apoptotic cells or apoptotic cell fragments and lysates by MoDC was dose-dependent (*figure 1A*). Moreover, uptake of HS-induced apoptotic AML cells was more efficient than AML lysates (p=0.03; paired t-test, n=6; tested in MoDC from three different HD). Fluorescence microscopic observations revealed actual uptake of AML preparations and not mere binding to DC cell surface (*figure 1B*). Co-incubation of CFSE-labeled leukemic cells with MoDC for 2h at 37ºC resulted in high levels of $CFSE^+$ MoDC (35%), whereas co-incubation for 2h at 0ºC resulted in only low percentages of $CFSE^+$ MoDC (5%), indicating that the observed uptake is an active endocytic process (*figure 1c* and *figure 1d*). The efficiency of uptake differed per patient; no correlation was observed regarding AML subtype or percentage of apoptotic cells. Since HS-induced apoptotic leukemic cells were more efficiently taken up than lysates further analysis was focused on uptake of HS-induced apoptotic leukemic cells.

Uptake of heath shock induced-apoptotic AML cells by MoDC is enhanced by R848 and diminished by a DC maturation-inducing cytokine cocktail

As TLR-mediated activation of DC has previously been associated with enhanced endocytosis [30], we tested the influence of the clinically applicable TLR-L Poly(I:C) and R848 and, as well as PGN, flagellin and LPS on AML cell uptake by MoDC. Administration of a maturation-inducing cytokine cocktail (CC) two hours after co-incubation of MoDC with lysed or apoptotic leukemic cells resulted in a significantly lower uptake by MoDC (*figure 2*). Of all the TLR-L tested (data of Poly(I:C), R848, LPS, PGN and flagellin not shown), only R848

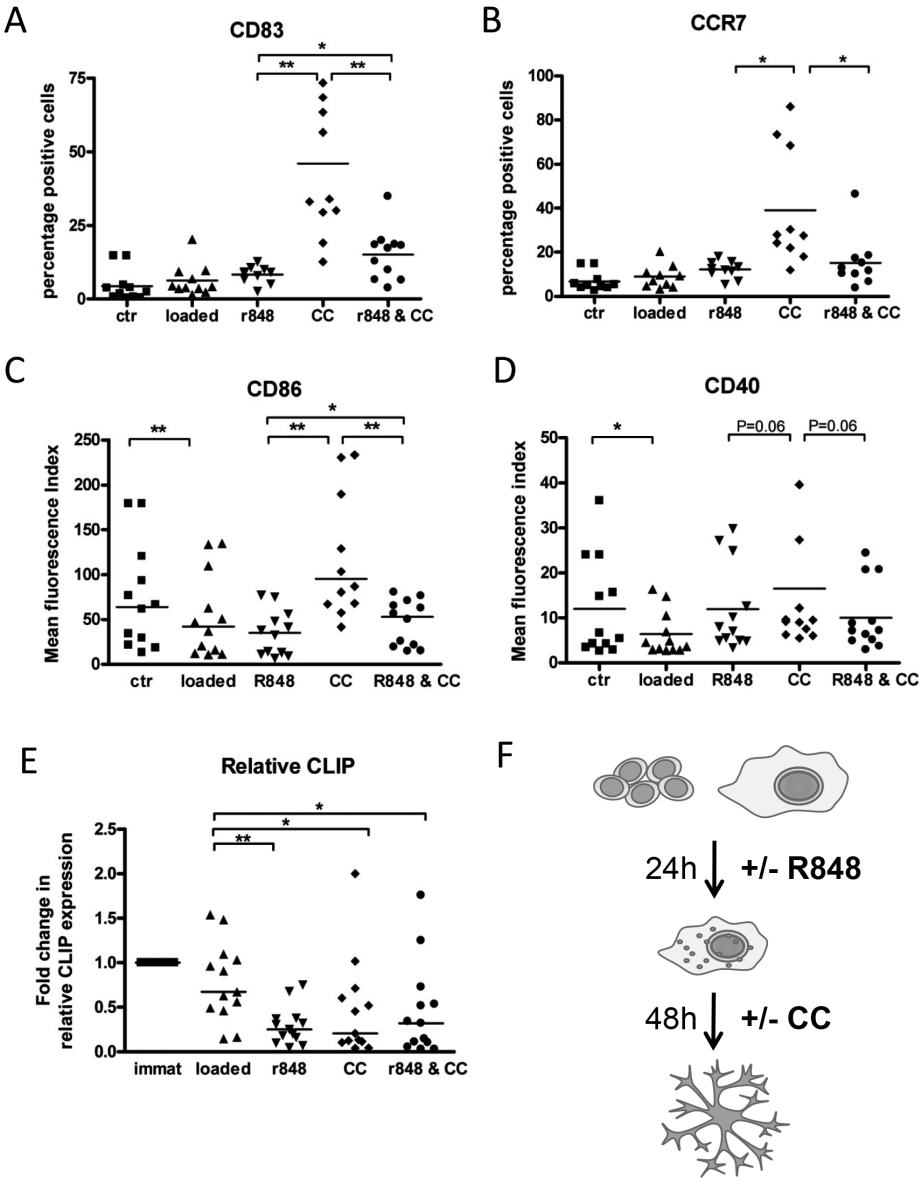


Figure 4 Immunophenotype of MoDC after co-incubation with apoptotic blast cells. Marker expression levels of (A) CD83, (B) CCR7, (C) CD86, (D) CD40 and (E) relative CLIP are shown after uptake of heat shock induced apoptotic blast cells by MoDC, either in presence or absence of R848 or CC; the time intervals used are depicted in the schematic illustration in panel F. For CD83 and CCR7, the percentage of positive cells are shown (panel A and B, respectively). For CD86 and CD40, mean fluorescence indices (MFI, as calculated in relation to the isotype control) are shown (panel C and D, respectively) (n=10). Fold change compared with immature MoDC (immat) in relative CLIP expression is depicted in panel E by calculating the ratio of CLIP and HLA-DR expression as indicated in the material and methods section (n=13). Horizontal lines represent mean, * $p<0.05$; ** $p<0.005$,

was able to enhance uptake of HS-induced apoptotic leukemic cells significantly. Of note, addition of R848 enhanced uptake of apoptotic but not of lysed AML cells by MoDC (*figure 2*). Remarkably, combined administration of R848 and CC abolished this positive effect. We hypothesized that prolonged incubation with R848 combined with delayed administration of CC might result in higher uptake. Therefore, we pre-incubated HS-induced apoptotic leukemic cells and MoDC with R848 for 24h, after which CC was added for 48h (*figure 3*). In this time frame, we found more efficient uptake of HS-induced apoptotic leukemic cells by R848-treated MoDC as compared to control and CC-treated MoDC. However, this enhanced uptake was counteracted by sequential incubation with CC.

Maturation induction of AML-loaded MoDC

Next, we investigated if incubation of MoDC from HD with AML cells with or without R848 and/or CC affected their maturation state. Uptake of HS-induced apoptotic cells by immature MoDC resulted in significant decrease of CD86 and CD40 expression (*figure 4*). Addition of CC 24h after loading resulted in MoDC maturation as shown by *de novo* expression of CD83 and chemokine receptor CCR7 (*figure 4*). Only partial maturation was induced after addition of R848 alone, as demonstrated by low-level CD83 expression and the absence of CCR7. Of note, pre-incubation with R848 blocked full maturation by CC as shown by significantly lower levels of CD83, CCR7, CD40 and CD86 (*figure 4*).

MHC class II antigen presentation requires the exchange of CLIP for an antigenic peptide in the peptide-binding groove, a process that is catalyzed by HLA-DM. HLA-DO, another nonclassical MHC class II molecule, is able to down-regulate the catalytic function of HLA-DM [2]. The intensity of HLA-DR varied between different maturation stimuli (data not shown). Relative to HLA-DR, moderate CLIP down-regulation was observed upon loading of immature DC with HS-induced apoptotic AML cells, but a more profound decrease in CLIP expression was seen upon maturation induction by R848 and/or CC (*figure 4E*). These data indicate efficient HLA-DR mediated presentation of endocytosed antigens after maturation. Both HLA-DM and -DO levels were in accordance with the observed relative CLIP levels (data not shown).

Function of heat shock-induced apoptotic AML-loaded versus unloaded MoDC

To further investigate effects of the applied loading strategies on HD-derived MoDC, we examined their migratory and T cell stimulating capacity. Upon CC-induced maturation, the lymph node homing receptor CCR7 was upregulated (*figure 4B*). In line with this, maturation with CC resulted in a high migration rate towards the CCR7 ligand CCL19; combined maturation with R848 resulted in lower migration efficiencies (*figure 5A*).

In response to overnight CD40 ligation, HS-induced apoptotic AML-loaded MoDC stimulated with R848 or CC released reduced levels of the Th1-skewing and CTL-activating cytokine IL-12p70 in comparison to non-stimulated unloaded MoDC (mean relative decrease (±SD) 0.65 (±0.27) and 0.52 (±0.16), respectively; median IL12 production unloaded non-stimulated MoDC: 520 (286-5199) pg/ml) (*figure 5B*). Of note, IL-12p70 release was almost completely abolished by the combined sequential use of R848 and CC (mean relative decrease 0.97 (±0.06)). However, incubation with R848 and CC increased the ability of MoDC to prime naive T cells in an allogeneic MLR in accordance with observations for phenotypic maturation: addition of CC after 24h pre-incubation with R848 enhanced T cell stimulating capacity but not to the same level as seen for CC alone (*figure 5C*).

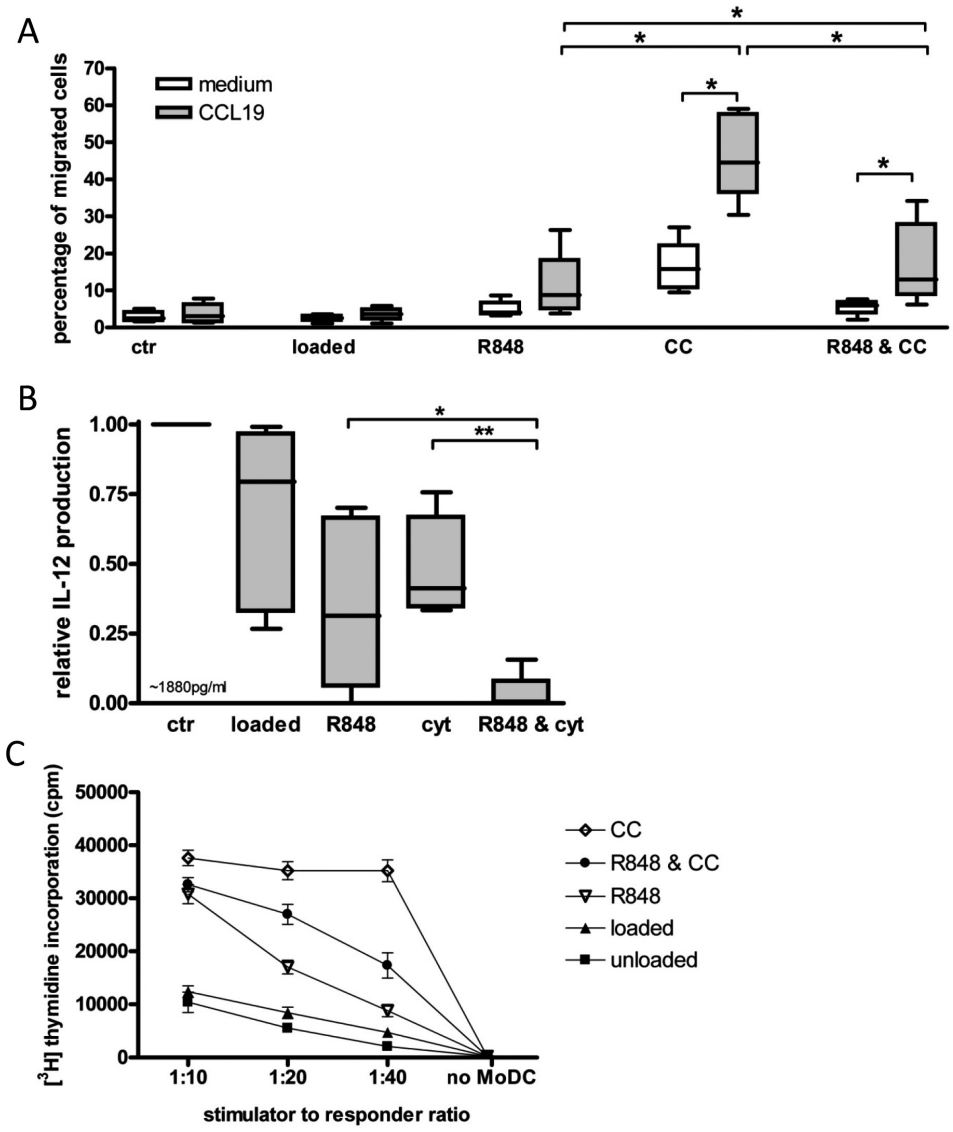


Figure 5 Function of unloaded and heat shock induced apoptotic blast loaded MoDC after incubation with or without R848 and/or cytokine cocktail. (A) Migration of DC towards CCL19 was tested in a 0.5µm-pore transwell system; percentages of MoDC that migrated towards CCL19 (grey bars) and medium (white bars) are depicted; * = $p<0.05$.(B) IL-12p70 production was measured after overnight CD40 ligation; relative IL-12p70 release compared with immature unloaded MoDC is depicted, mean IL-12p70 release in the control group is listed in the lower left corner of the graph; * = $p<0.05$; ** = $p<0.005$. (C) The ability of DC to induce proliferation in a mixed leukocyte reaction is shown after 5 days of co-incubation of MoDC and allogeneic $CD8^+$ T cells in various MoDC: T cell ratios. [3H] thymidine was added and incorporation was measured after 16h. Each ratio was tested in triplicate and means±SD are shown. One representative experiment out of six is shown.

Discussion

This study was undertaken to investigate various MoDC vaccine preparation strategies in order to create an optimized MoDC vaccine for treatment of MRD in AML. The ability of HD-derived MoDC to take up whole cell derived material in combination with TLR-L was investigated in order to enhance uptake of LAA. Furthermore, we explored the capability of MoDC to mature under influence of various TLR-L and a standard cytokine cocktail (CC) as positive control.

Immature DC are capable of taking up Ag, e.g. from infected cells or tumor cells, in the form of apoptotic bodies or necrotic cell particles [31;32]. Next to the Ag source, agents such as TLR-L or cytokines can affect the outcome AML of this uptake [33]. Upon encounter with for instance microbial agents or other inflammatory stimuli, DC mature from cells specialized in Ag uptake into cells specialized in T cell stimulation. Inflammatory cytokines are considered as strong maturation inducers but, as we demonstrated, CC inhibits further Ag uptake by DC. In contrast, TLR-L are known to enhance uptake of antigenic material and subsequently induce maturation [30]. In accordance with this we found enhanced uptake after co-incubation with TLR- L R848, whereas addition of CC after 24h of co-incubation with R848 halted further uptake.

The method by which leukemic cell death was induced, influenced the amount of cellular material taken up by MoDC: less uptake was achieved in DC after co-incubation with lysate as compared to apoptotic cells. In the process of dying, apoptotic cells are known to shed immunogenic particles, i.e. so-called blebs. Murine studies have demonstrated that these blebs are taken up more efficiently by DC than the remaining, larger, apoptotic bodies [34]. This may offer an explanation for our observation that apoptotic AML cells are more efficiently taken up by MoDC than AML lysates. Additional knowledge about the ability of DC to prime LAA-specific T cells is clearly warranted to further differentiate between both whole leukemic cell derived antigen sources.

One of the key players in the MHC class II presentation pathway is CLIP and its regulators HLA-DM and HLA-DO. TLR-L regulate processing of the Invariant chain (Ii) and influence the exchange of CLIP with antigenic peptides. TLR signaling is necessary to direct internalized antigens via the classical pathway for presentation by MHC II, whereas tolerogenic DC can upregulate CLIP expression and hamper T cell signaling [35-37]. Consistent with this, CLIP expression was decreased when DC and apoptotic AML cells were co-incubated in the presence of R848 and/or CC, indicative of a putative increase in LAA presentation by the loaded MoDC.

Maturation of DC is necessary to enable migration to lymph nodes and activation of T cells. Consistent with literature, CC induced upregulation of CD86, expression of CD83, CCR7 and hence enhanced migratory capacity, the latter most likely due to the presence of Prostaglandin-E2 (PGE_2) in the cocktail. R848 is able to induce phenotypic maturation, but far less prominently than CC [38;39]. In this setting our results indicate that R848 is rather an enhancer of antigen uptake than a straightforward maturation inducer, whereas CC only promotes maturation. We hypothesized that both high uptake and maturation might be achieved by combining prolonged R848 incubation (24h) followed by addition of CC. However, maturation was only slightly increased compared to R848 alone. These data indicate that TLR-L antagonize the maturation efficacy of CC. Recent studies showed inhibitory effects of TLR-L LPS and Poly(I:C) on DC by activation of so-called suppressors

of cytokine signaling (SOCS). It has been described that SOCS1, 2 and 3 are required for appropriate TLR signaling in maturing human DC via both the MyD88-dependent and -independent signaling pathway [40-43]. By first enhancing uptake with TLR-L the DC might become resistant to cytokines due to activation of SOCS1/2, resulting in disturbed maturation. Moreover, TLR-mediated induction of maturation-inhibitory cytokines (e.g. IL-10) might be responsible for this maturation inhibitory effect [44;45]. We did attempt inhibition of putatively involved down-stream signaling elements (a.o. p38 MAPK, PI3K, STAT3) to abolish this R848-mediated block in CC-induced DC maturation, but to no avail.
PGE_2, part of the cytokine cocktail, is known to impair production of IL-12p70 [46;47]. As a consequence of reduced IL-12p70 levels, immunosuppressive cells such as T regulatory cells might be recruited [48;49]. Various combinations of cytokines and other DC-maturing factors such as TLR-L direct MoDC maturation towards a migratory and/or Th1-activating cytokine release profile [38;50]. Recently, it was shown that simultaneous addition of PGE_2 and TLR 3/7/8 ligands resulted in higher levels of co-stimulatory molecules on DC as well as IL-12p70 production and migration capacity [38;51]. We hypothesized that by pre-incubating MoDC with R848, followed by maturation with CC (containing PGE_2), both effective IL-12 p70 production, migratory capacity as well as improved AML cell uptake might be established. Addition of CC after prolonged incubation with R848 enhanced the migratory capacity but not to the same levels as CC alone. Furthermore, IL-12p70 production was abolished as compared to CC stimulation alone. This might be explained by the longer incubation time and thereby exhaustion of MoDC before measuring the overnight IL-12p70 production. However, in a mixed leukocyte reaction we demonstrated that the $CD8^+$ T cell stimulatory capacity of R848 and/or CC matured MoDC was significantly higher than that of controls, indicating that irrespective of IL-12p70 production, R848 and/or CC matured DC are able to stimulate $CD8^+$ T cells. Since IL-12p70 is an important cytokine in Th1 and CTL activation, careful analysis of the ability to prime functional leukemia-specific T cells is needed to design optimal MoDC vaccine preparation strategies.
In conclusion, our results demonstrate that uptake of leukemic cells by MoDC and their maturation state is differentially affected by TLR-L R848 and CC. As compared to CC alone, the sequential use of R848 and CC interfered with effective MoDC maturation as indicated by diminished migration and T cell stimulatory capacity and abolished IL-12p70 production. These findings do not justify incorporation of TLR7/8-L in DC-maturing cocktails for clinical development of whole-cell AML-loaded MoDC vaccines.

Reference List

1. Provan D, Singer CRJ, Baglin T, Lilleyman J. Acute myeloblastic leukaemia (AML). In: Provan D, Singer CRJ, Baglin T, Lilleyman J, eds. Oxford handbook of clinical haematology. Oxford, UK: Oxford University Press; 2004:149-157.
2. Chamuleau ME, Souwer Y, Van Ham SM et al. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res. 2004;64:5546-5550.
3. van Luijn MM, Chamuleau ME, Thompson JA et al. Class II-associated invariant chain peptide down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased $CD4^+$ T-cell responses. Haematologica 2010;95:485-493.

4. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat.Rev. Immunol. 2005;5:296-306.
5. Ridgway D. The first 1000 dendritic cell vaccinees. Cancer Invest 2003;21:873-886.
6. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat.Med. 2004;10:909-915.
7. van den Ancker W., Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Back to basics: in search of the optimal dendritic cell for vaccination in AML. Leuk.Res. 2008;32:1641-1643.
8. Greiner J, Ringhoffer M, Taniguchi M et al. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. Int.J.Cancer 2004;108:704-711.
9. Galea-Lauri J, Darling D, Mufti G, Harrison P, Farzaneh F. Eliciting cytotoxic T lymphocytes against acute myeloid leukemia-derived antigens: evaluation of dendritic cell-leukemia cell hybrids and other antigen-loading strategies for dendritic cell-based vaccination. Cancer Immunol.Immunother. 2002;51:299-310.
10. Chan L, Hardwick NR, Guinn BA et al. An immune edited tumour versus a tumour edited immune system: Prospects for immune therapy of acute myeloid leukaemia. Cancer Immunol.Immunother. 2006;55:1017-1024.
11. Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA. Leukemia-derived dendritic cells: towards clinical vaccination protocols in acute myeloid leukemia. Haematologica 2006;91:348-355.
12. de Gruijl TD, Luykx-de Bakker SA, Tillman BW et al. Prolonged maturation and enhanced transduction of dendritic cells migrated from human skin explants after in situ delivery of CD40-targeted adenoviral vectors. J.Immunol. 2002;169:5322-5331.
13. Melero I, Shuford WW, Newby SA et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat.Med. 1997;3:682-685.
14. Castellino F, Boucher PE, Eichelberg K et al. Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J.Exp.Med. 2000;191:1957-1964.
15. Shi H, Cao T, Connolly JE et al. Hyperthermia enhances CTL cross-priming. J.Immunol. 2006;176:2134-2141.
16. Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. Nat.Rev.Immunol. 2004;4:641-648.
17. Obeid M, Tesniere A, Ghiringhelli F et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat.Med. 2007;13:54-61.
18. Berwin B, Hart JP, Rice S et al. Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. EMBO J. 2003;22:6127-6136.
19. Lee JJ, Choi BH, Kang HK et al. Monocyte-derived dendritic cells from HLA-matched allogeneic donors showed a greater ability to induce leukemic cell-specific T cells in comparison to leukemic cell-derived dendritic cells or monocyte-derived dendritic cells from AML patients. Leuk.Res. 2008;32:1653-1660.
20. Lee JJ, Park MS, Park JS et al. Induction of leukemic-cell-specific cytotoxic T lymphocytes by autologous monocyte-derived dendritic cells presenting leukemic cell antigens. J.Clin.Apher. 2006;21:188-194.
21. Decker WK, Xing D, Li S et al. Double loading of dendritic cell MHC class I and MHC class II with an AML antigen repertoire enhances correlates of T-cell immunity in vitro via amplification of T-cell help. Vaccine 2006;24:3203-3216.

22. Gong J, Koido S, Kato Y et al. Induction of anti-leukemic cytotoxic T lymphocytes by fusion of patient-derived dendritic cells with autologous myeloblasts. Leuk.Res. 2004;28:1303-1312.
23. Lee JJ, Nam CE, Nam JH et al. Generation of cytotoxic donor $CD8^+$ T cells against relapsing leukemic cells following allogeneic transplantation by stimulation with leukemic cell- or leukemic lysate pulsed donor cell-derived dendritic cells. Leuk.Res. 2004;28:517-524.
24. Spisek R, Chevallier P, Morineau N et al. Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. Cancer Res. 2002;62:2861-2868.
25. Klammer M, Waterfall M, Samuel K, Turner ML, Roddie PH. Fusion hybrids of dendritic cells and autologous myeloid blasts as a potential cellular vaccine for acute myeloid leukaemia. Br.J.Haematol. 2005;129:340-349.
26. Smits EL, Ponsaerts P, Van de Velde AL et al. Proinflammatory response of human leukemic cells to dsRNA transfection linked to activation of dendritic cells. Leukemia 2007;21:1691-1699.
27. Weck MM, Grunebach F, Werth D et al. TLR ligands differentially affect uptake and presentation of cellular antigens. Blood 2007;109:3890-3894.
28. Westers TM, Houtenbos I, Schuurhuis GJ, Ossenkoppele GJ, van de Loosdrecht AA. Quantification of T-cell-mediated apoptosis in heterogeneous leukemia populations using four-color multiparameter flow cytometry. Cytometry A 2005;66:71-77.
29. Snijders A, Hilkens CM, van der Pouw Kraan TC et al. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J.Immunol. 1996;156:1207-1212.
30. West MA, Wallin RP, Matthews SP et al. Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. Science 2004;305:1153-1157.
31. Cella M, Scheidegger D, Palmer-Lehmann K et al. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J.Exp.Med. 1996;184:747-752.
32. Caux C, Massacrier C, Vanbervliet B et al. $CD34^+$ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to $GM\text{-}CSF^+$TNF alpha. Adv.Exp.Med.Biol. 1997;417:21-25.
33. Niedergang F, Chavrier P. Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. Curr.Opin.Cell Biol. 2004;16:422-428.
34. Fransen JH, Hilbrands LB, Ruben J et al. Mouse dendritic cells matured by ingestion of apoptotic blebs induce T cells to produce interleukin-17. Arthritis Rheum. 2009;60:2304-2313.
35. Blander JM. Coupling Toll-like receptor signaling with phagocytosis: potentiation of antigen presentation. Trends Immunol. 2007;28:19-25.
36. Blander JM, Medzhitov R. On regulation of phagosome maturation and antigen presentation. Nat.Immunol. 2006;7:1029-1035.
37. Torres-Aguilar H, guilar-Ruiz SR, Gonzalez-Perez G et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory $CD4^+$ T cells. J.Immunol. 2010;184:1765-1775.
38. Boullart AC, Aarntzen EH, Verdijk P et al. Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E(2) results in high interleukin-12 production and cell migration. Cancer Immunol.Immunother. 2008;57:1589-97

39. Assier E, Marin-Esteban V, Haziot A et al. TLR7/8 agonists impair monocyte-derived dendritic cell differentiation and maturation. J.Leukoc.Biol. 2007;81:221-228.
40. Hu J, Winqvist O, Flores-Morales A, Wikstrom AC, Norstedt G. SOCS2 influences LPS induced human monocyte-derived dendritic cell maturation. PLoS.One. 2009;4:e7178.
41. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat.Rev.Immunol. 2007;7:454-465.
42. Bartz H, Avalos NM, Baetz A, Heeg K, Dalpke AH. Involvement of suppressors of cytokine signaling in toll-like receptor-mediated block of dendritic cell differentiation. Blood 2006;108:4102-4108.
43. Melillo JA, Song L, Bhagat G et al. Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function. J.Immunol. 2010;184:2638-2645.
44. Frick JS, Grunebach F, Autenrieth IB. Immunomodulation by semi-mature dendritic cells: a novel role of Toll-like receptors and interleukin-6. Int.J.Med.Microbiol. 2010;300:19-24.
45. Oliveira CJ, Carvalho WA, Garcia GR et al. Tick saliva induces regulatory dendritic cells: MAP-kinases and Toll-like receptor-2 expression as potential targets. Vet.Parasitol. 2010;167:288-297.
46. van Helden SF, Krooshoop DJ, Broers KC et al. A critical role for prostaglandin E2 in podosome dissolution and induction of high-speed migration during dendritic cell maturation. J.Immunol. 2006;177:1567-1574.
47. Lebre MC, Burwell T, Vieira PL et al. Differential expression of inflammatory chemokines by Th1- and Th2-cell promoting dendritic cells: a role for different mature dendritic cell populations in attracting appropriate effector cells to peripheral sites of inflammation. Immunol.Cell Biol. 2005;83:525-535.
48. Kalinski P, Vieira PL, Schuitemaker JH, de Jong EC, Kapsenberg ML. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. Blood 2001;97:3466-3469.
49. Luft T, Jefford M, Luetjens P et al. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. Blood 2002;100:1362-1372.
50. Dowling D, Hamilton CM, O'Neill SM. A comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with LPS compared with a panel of TLR ligands. Cytokine 2008;41:254-262.
51. Lehner M, Morhart P, Stilper A et al. Efficient chemokine-dependent migration and primary and secondary IL-12 secretion by human dendritic cells stimulated through Toll-like receptors. J.Immunother. 2007;30:312-322.

Apoptotic blebs from leukemic cells as a preferred source of tumor-associated antigen for dendritic cell-based vaccines

15

JM Ruben
W van den Ancker
HJ Bontkes
TM Westers
E Hooijberg
GJ Ossenkoppele
TD de Gruijl
AA van de Loosdrecht

submitted.

Abstract

Since few leukemia-associated antigens (LAA) are characterized for acute myeloid leukemia (AML), apoptotic tumor cells constitute an attractive LAA source for DC-based vaccines. However, loading DC with apoptotic tumor cells may interfere with DC function. Previously, it was shown in mice that apoptotic blebs induce DC maturation, whereas apoptotic cell remnants (ACR) do not. Here, we analyzed human monocyte-derived DC (MoDC) functionality *in vitro,* after ingesting either allogeneic AML-derived ACR or blebs. We show that MoDC ingest blebs to a higher extent, and are superior in migrating towards CCL19, as compared to ACR-loaded MoDC. Although MoDC cytokine production was unaffected, co-culturing bleb-loaded MoDC with T-cells led to an increased T cell proliferation and IFNγ production. Moreover, antigen-specific $CD8^+$ T-cells frequencies increased to 0.63% by priming with bleb-loaded MoDC, compared to 0.16% when primed with ACR-loaded MoDC. Importantly, $CD8^+$ T cells primed by bleb-loaded MoDC recognized their specific epitope at one to two orders of magnitude lower concentrations compared to ACR-loaded MoDC. In conclusion, superior ingestion efficiency and migration, combined with favorable T cell cytokine release and $CD8^+$ T cell priming ability and avidity, point to blebs as the preferred component of apoptotic leukemic cells for LAA loading of DC for the immunotherapy of AML.

Introduction

As key regulators of immune responses, dendritic cells (DC) have the ability to ingest and process exogenous proteins and to present derived peptides thereof at the cell surface in complex with HLA class II molecules, or via cross-presentation in the context of HLA class I molecules. DC can potentiate antigen-specific T cell responses and are therefore of major interest as mediators of anti-tumor immunity in vaccination strategies [1,2]. In order to induce tumor-specific immune responses, DC are required to present tumor-associated antigen(s) (TAA). Next to the generation of DC from chronic- and acute myeloid leukemia (CML and AML, respectively) cells [3, 4], multiple strategies for loading DC with TAA have been investigated, such as peptide pulsing [5, 6], TAA-specific mRNA electroporation [7–9] and loading of necrotic or apoptotic tumor cells via phagocytosis [4,5,10,11]. Currently, targeting DC *in vivo*, using (antibody-based) conjugates targeting DC-specific receptors (e.g. DEC-205, DC-SIGN, CD40 and Clec-9a) gains more interest [12–15]. Although the field of DC vaccination is clearly moving forward, many challenges remain, e.g. increasing clinical responses by overcoming T cell/tumor cell regulatory mechanisms, broadening the array of TAA used for vaccination, and inducing (long-term) immune protection in cancer patients. In most studies, the focus has been on targeting solid tumors (mostly melanoma), but DC vaccination is a potential therapy for treating AML patients in a minimal residual disease setting as well, either as a mono- or combination therapy. Recently, a small-scale phase I clinical study demonstrated vaccination of elderly AML patients with autologous AML blast cell-loaded monocyte-derived DC (MoDC) to be safe, and to induce (transient) disease stabilization and specific $CD8^+$ T cells [16]. The use of leukemic blasts as a source of leukemia-associated antigen (LAA) for DC loading is particularly attractive, as they will contain both known and as yet unidentified LAA. Using tumor cells as a source of LAA could, moreover,

pave the way to generate a tumor- and patient-specific therapy in a minimal residual disease setting in AML and other tumor types. However, loading DC with apoptotic blasts could impair DC function, due to the immunosuppressive nature of apoptotic cells [17–22].
In recent years, more knowledge has been gained on the tightly regulated process of apoptosis, which is controlled by the balance between the pro- and anti-apoptotic members of the Bcl-2 family and is executed by initiator and effector caspases [23, 24]. Apoptotic cells undergo multiple macro- and microcellular rearrangements, e.g. cellular condensation, pyknosis, karyorrhexis, redistribution of specific organelles into peripheral membrane protrusions (blebbing) and the exposure of apoptosis-specific structures on the cell membrane e.g. phosphatidyl serine, calreticulin as well as alterations in the glycocalyx that act as “eat-me” signals for phagocytes [24–27].
The peripheral membrane protrusions (or blebs) are formed during the active blebbing phase, a process dependent on the activation of caspases, phosphorylation of the myosin light chain and death-associated protein kinase [26,28,29]. During the blebbing phase mainly the endoplasmic reticulum (ER), RNA and chromatin are enclosed in blebs, which become detached from the main apoptotic cell at later stages of apoptosis [26,30]. Although the exact functional relevance of blebbing during apoptosis remains elusive, it was shown that ingestion of blebs by mouse bone marrow-derived DC (BMDC) induced DC maturation, and subsequent T helper-17 cell activation when co-cultured with splenocytes in vitro, whereas the remaining apoptotic cell remnants (ACR) did not. Of note, there is some confusion in literature regarding the use of the term apoptotic bodies, which is at times used to describe both ACR and blebs [30,31]. Although multiple studies have analyzed the effect of loading apoptotic cells or apoptotic bodies (ACR) on DC, no studies have been conducted thusfar which analyzed the selective use of apoptotic blebs (microvesicles that require additional isolation steps) as a source of TAA. Since blebs have been shown to activate BMDC in mice [30] , and in view of a growing recognition of the need to utilize the unique mutanome of individual tumors for vaccination strategies, DC loaded with AML blast-derived blebs may constitute an effective vaccine. Here, we compared MoDC phenotype and function *in vitro,* after ingestion of either ACR or blebs derived from a human AML cell line.

Material and methods

Cell lines

The human HLA-A2 negative AML cell line HL60 (ATCC, Wesel, Germany) was cultured in RPMI containing 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (all Gibco, Paisley, UK) , further referred to as complete medium.

Generation of MoDC

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy volunteers by centrifugation on a Ficoll-Paque density gradient medium, after informed consent. Subsequently, monocytes were isolated from PBMCs by positive selection using magnetic activated cell sorting with CD14 microbeads (Miltenyi biotec, Utrecht, The Netherlands). MoDC were generated by culturing monocytes for 5 days at 37°C in complete medium, in the presence of 800 U/ml GM-CSF (10 * 10^6 U/mg, Peprotech, The Netherlands)

and 500 U/ml IL-4 (5 * 10^6 U/mg, Peprotech, The Netherlands). After 5 days of culture, MoDC were either used directly or stored in liquid nitrogen until further use. 1 hour after loading MoDC with ACR or blebs, maturation was induced by a cytokine cocktail consisting of IL-1β (10 ng/ml, 1 * 10^9U/mg, Sanquin, Amsterdam, The Netherlands), IL-6 (10 ng/ml, 2 * 10^6 U/mg, R&D systems, Abingdon, UK), TNFα (200 U/ml, 20 * 10^6 U/mg, Sanquin, Amsterdam, The Netherlands) and PGE_2 (10 ng/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands).

Preparation of apoptotic cell fractions

Apoptosis of HL60 cells was induced by adding 3 μM mitoxantrone dihydrochloride (mitoxantrone; Sigma-Aldrich, Zwijndrecht, The Netherlands) per 2 * 10^6 HL60 cells per ml of complete medium, for 48 hr at 37°C, 5% CO2. We were unable to perform priming experiments with MoDC loaded with either ACR or blebs from mitoxantrone-induced apoptotic HL60 cells, since T cells did not expand, or underwent apoptosis during co-culture (data not shown). This is likely to be the result of leaked mitoxantrone from the loaded MoDC. We therefore alternatively induced apoptosis of HL60 cells by heat shock, as described before [11]. In short, cells were washed with RPMI 1640 and resuspended at 5 * 10^6 cells/ml in RPMI 1640, and subsequently incubated for 2 hours at 42°C. Next, the cells were irradiated at 5000 rad, washed, resuspended at 2 * 10^6 per ml in full medium, and incubated for 48 to 72 hours at 37°C, 5% CO_2.

The percentage of apoptotic cells was determined by Syto16 labeling (Molecular Probes, Leiden, The Netherlands). In short, 50 * 10^3 cells were resuspended in 1 ml PBS, containing 10% FCS (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), 20 mM HEPES (Sigma-Aldrich), 1 ml/ml glucose anhydrous (Baker B.V. Deventer, The Netherlands), 4 mM L-glutamine (Invitrogen), MEM amino acids (Sigma-Aldrich). Syto16 (42 nM) and the PgP pump inhibitor PSC833 (5 μM; a kind gift from Novartis, Basel, Switzerland) were added, and the cells were incubated for 30 minutes at 37 °C, 5% CO_2 and washed with an excess of PBS. The subsequent caspase-dependent loss of syto16 labeling was analyzed on a BD FACSCanto™ II flow cytometer (BD Biosciences), to assess the level of apoptosis. In all cases, > 90% of the cells was apoptotic.

ACR and blebs were isolated by differential centrifugation. The centrifugal force for separating ACR and blebs was determined by assessing the size and appearance of the separated fractions by a Zeiss Axioskop 50 fluorescence microscope (Zeiss, The Netherlands), and a CM100 BioTwin electron microscope (Philips, The Netherlands). ACR were isolated by centrifugation at 600g at 4 ˚C for 10 minutes, after which the apoptotic blebs were isolated from the resulting supernatant by centrifugation at 4,000g at 4 ˚C for 10 minutes. Apoptotic fractions were washed twice with excess PBS prior to determining the protein concentration. Apoptotic cell material was lysed by 5 cycles of snap-freezing in liquid nitrogen, followed by thawing in a water bath at 37 °C. Subsequently, cellular material was lysed by 5 cycles of sonication (5 second pulse, followed by 5 seconds rest) at 18 micron (Sanyo MSE Soniprep 150, Amsterdam, The Netherlands) in the presence of a protease inhibitor cocktail, following manufacturers' protocol (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Almere, The Netherlands). Subsequently, the protein concentration was determined by analyzing the absorption at 280/260 nm from 50 μl of lysed samples, using a ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific, Breda, The Netherlands). ACR and blebs were either used directly or frozen in liquid nitrogen and thawed prior to usage.

Uptake and internalization of ACR and blebs by- and immunophenotyping of MoDC

Prior to inducing apoptosis, HL60 cells were labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Breda, The Netherlands) and Hoechst 33342 1 μg/ml (Molecular Probes, Eugene, OR, USA) for fluorescence microscopic imaging and MoDC were labeled with 1 μM CellVue Plum (eBioscience, Vienna, Austria). Analysis of the ingestion of apoptotic material by MoDC was analyzed by flow cytometry using a BD FACSCanto™ II flow cytometer and BD FACSDiva™ software.

Internalization of the apoptotic fractions was visualized using confocal microscopy. Thawed ACR and blebs were labeled with 1 μM PKH26 red fluorescent cell linker kit (Sigma-Aldrich), following the manufacturer's protocol. MoDC were labeled with 2 μM CFSE, and co-cultured with PKH26-labelled ACR or blebs for 8 hours. Loaded MoDC were harvested and washed, and transferred to 4 wells Lab-Tek™ Permanox Chamber Slides (Thermo Scientific, The Netherlands). After overnight adherence, the supernatant was carefully removed and the loaded MoDC were fixed using 1% paraformaldehyde solution for 15 minutes at 4 °C, washed with PBS and dried. Vectashield (Vector Laboratories, United Kingdom) was added on the slides, which were covered by a coverslip and analyzed using a Leica TCS SP2 confocal microscope (Leica Microsystems, The Netherlands).

MoDC phenotype was determined by flow cytometric analysis 48 hours after loading the apoptotic material, using fluorochrome-conjugated antibodies CD1a, CD54 (Dako Cytomation), CD14, CD80, CD86, CD209, (BD Biosciences) CD40, CD83 (Beckman Coulter) and the unlabeled CCR7 antibody (BD Biosciences) followed by PE-conjugated goat anti-mouse IgM (Beckman Coulter).

Transwell migration assay

Migratory capacity of MoDC after ingesting apoptotic material was analyzed using a transwell migration assay, using membranes with a 5 μM pore size (Corning). Specific versus non-specific migration of 1 * 10^5 MoDC (deposited in the upper compartment) was determined by performing the transwell migration assay in the presence or absence of 250 ng/ml MIP-3β (added to the lower compartment, CCL19, R&D systems, Abingdon, UK). After 5 hours, the number of migrated viable MoDC was quantified using FlowCount™ beads (Dako, Enschede, The Netherlands) on a BD FACSCanto™ II flow cytometer.

MoDC cytokine release

4 * 10^4 (loaded) MoDC were cultured overnight with 4 * 10^4 CD40-ligand expressing J558 cells (irradiated at 50 Gy) [32] in the presence of 1000 U/ml IFN-γ (Sanquin, Amsterdam, The Netherlands) in a 96-wells plate. Next, the plates were spun at 1000 x G and the supernatants were directly analyzed for the presence of cytokines using an inflammatory cytokine bead array (BD Biosciences, Breda, The Netherlands), following the manufacturer's protocol.

Mixed lymphocyte reaction and cytokine release

Peripheral blood lymphocytes (PBL) from healthy donors were labeled with 1 μM CFSE and subsequently co-cultured with allogeneic (loaded) MoDC for 6 days, after which the cells were harvested and labeled with CD3, CD4, and CD8 (BD biosciences), and the CFSE dilution over the daughter $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was measured using flow cytometry (BD

FACSCanto™ II) and analyzed using BD FACSDiva™ software. Moreover, supernatants were harvested for cytokine analysis using a Th1/Th2/Th17 cytokine bead array (BD Biosciences, Breda, The Netherlands), following the manufacturers protocol.

CD8$^+$ T cell priming and avidity analysis

Autologous in vitro T cell priming experiments were conducted as described previously [33]. CD14$^+$ monocytes from healthy HLA-A2 positive donors were isolated and differentiated into MoDC, as described above. CD8$^+$ cells were positively selected from CD14$^-$ PBMC using CD8 microbeads (Miltenyi biotec), frozen and stored in liquid nitrogen until further use. The remaining PBL were frozen and stored in liquid nitrogen. In order to analyze MART-1-specific CD8$^+$ T cell outgrowth, we generated a MART-1 expressing HL60 cell line (as well as a K562 cell line, referred to as K562-M, as a (HLA-A2) negative control for the analysis of cytotoxicity) by retroviral transduction of LZRS-MART-1-IRES-ΔNGFR, as reported previously [34]. After 5 days of culture, MoDC were isolated and loaded with 40 μg MART-1 positive ACR or blebs per 0.2 * 10^6 MoDC, and one hour after loading MoDC with ACR or blebs, maturation was induced by a cytokine cocktail consisting of IL-1β (10 ng/ml, 1 * 10^9 U/mg, Sanquin, Amsterdam, The Netherlands), IL-6 (10 ng/ml, 2 * 10^6 U/mg, R&D systems, Abingdon, UK), TNFα (200 U/ml, 20 * 10^6 U/mg, Sanquin, Amsterdam, The Netherlands) and PGE_2 (10 ng/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands). After overnight co-culture, loaded MoDC were isolated and seeded at 0.1 * 10^6 per 24-well (8 x 24-wells per condition) in IMDM (Gibco, Paisley, UK), containing 50 μM β2-Mercaptoethanol (Merck, The Netherlands), 100 IU/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), 5% Yssels supplement [33], and 1% pooled human AB serum (MP biomedicals, Amsterdam, The Netherlands), further referred to as Yssels medium. Next, isolated CD8$^+$ cells and CD14$^-$/CD8$^-$ PBL were thawed and washed twice with complete medium. CD8$^+$ cells were resuspended in Yssels medium and added to the (loaded) MoDC at 1 * 10^6 per 24-well. CD14$^+$ monocytes from healthy HLA-A2 positive donors were isolated and differentiated into MoDC, as described above. CD8$^+$ cells were positively selected from CD14$^-$ PBMC using CD8 microbeads (Miltenyi biotec), frozen and stored in liquid nitrogen until further use. The remaining PBL were frozen and stored in liquid nitrogen. In order to analyze MART-1-specific CD8$^+$ T cell outgrowth, we generated a MART-1 expressing HL60 cell line (as well as a K562 cell line, referred to as K562-M, as a (HLA-A2) negative control for the analysis of cytotoxicity) by retroviral transduction of LZRS-MART-1-IRES-ΔNGFR, as reported previously [34]. After 5 days of culture, MoDC were isolated and loaded with 40 μg MART-1 positive ACR or blebs per 0.2 * 10^6 MoDC, and one hour after loading MoDC with ACR or blebs, maturation was induced by a cytokine cocktail consisting of IL-1β (10 ng/ml, 1 * 10^9 U/mg, Sanquin, Amsterdam, The Netherlands), IL-6 (10 ng/ml, 2 * 10^6 U/mg, R&D systems, Abingdon, UK), TNFα (200 U/ml, 20 * 10^6 U/mg, Sanquin, Amsterdam, The Netherlands) and PGE_2 (10 ng/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands). After overnight co-culture, loaded MoDC were isolated and seeded at 0.1 * 10^6 per 24-well (8 x 24-wells per condition) in IMDM (Gibco, Paisley, UK), containing 50 μM β2-Mercaptoethanol (Merck, The Netherlands), 100 IU/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), 5% Yssels supplement, and 1% pooled human AB serum (MP biomedicals, Amsterdam, The Netherlands), further referred to as Yssels medium. Next, isolated CD8$^+$ cells and CD14-/CD8- PBL were thawed and washed twice with complete medium. CD8+ cells were resuspended in Yssels medium and added to the (loaded) MoDC at 1 * 10^6 per 24-well.

Cytotoxicity assay

Cytotoxicity was determined by co-culturing primed and sorted $CD8^+$ T cells (labeled with 50 nM CFSE (Invitrogen)), at effector to target ratios starting from 0:1 through 10:1, with 200 nM CellVue (eBiosciences) labeled melanoma Mel AKR cells (HLA-A2 positive & MART-1 positive; Netherlands Cancer Institute, Amsterdam, The Netherlands), JY cells (HLA-A2 positive, MART-1 negative), or K562-M (HLA-A2 negative & MART-1 positive). After an overnight co-culture, target cells were shortly (< 5 minutes) incubated with 50 µl 0.05% Trypsin-EDTA solution (Invitrogen), suspended by careful tapping and washed with 150 µl complete medium. Next, the cells were resuspended in a 2% BD Via-Probe/PBS/0.1%human serum albumin solution, incubated for 10 minutes and the percentage of BD Via-Probe-positive $CellVue^+/CFSE^-$ target cells was measured using flow cytometry (LSRFortessa™, BD Biosciences).

Statistical analysis

Statistical analysis was performed in GraphPad Prism version 5 for Windows (GraphPad Software Inc.), using a paired two-tailed Student T-test. P-values ≤ 0.05 were regarded as significant.

Results

ACR and blebs isolated from HL60 AML cells represent distinct cellular components

After isolation of ACR and blebs from mitoxantrone treated apoptotic HL60 cells, we analyzed their morphological appearance and size, using transmission electron microscopic imaging (*figure 1A-B*). ACR appeared as a heterogeneous population of large cellular bodies of approximately 2 to 10 µm in size with a ruffled plasma membrane that appeared to be locally disintegrated (*figure 1A*, arrows). Electron dense areas resided within the ACR (*figure 1A*, asterisk), that were enclosed by a membrane and represent the latest stages of chromatin translocation to the periphery of the cell, which was confirmed by fluorescence microscopy after the labeling of proteins (CFSE) and DNA (Hoechst 33342) in apoptotic ACR (*figure 1C*).

Apoptotic blebs are described as 100 nM to 1000 nM microvesicles, containing both chromatin and endoplasmic reticulum (ER). Both cellular components are actively translocated from the apoptotic cell into peripheral vesicles during apoptosis, and become enclosed by a sheet of ER membrane and a sheet of plasma membrane [26;36]. The isolated fraction contained a heterogeneous population of blebs 200 nM to 600 nM in diameter (*figure 1B*). They were indeed enclosed by a characteristic double membrane, as described previously [26], and as shown in *figure 1B*, open arrows. Moreover, characteristic structures resembling ER and ribosomes were also observed (*figure 1B*, closed arrows). Using electron microscopic imaging, we could not detect blebs containing electron dense areas (chromatin). Fluorescent imaging showed few blebs to stain positive for Hoechst 33342 (*figure 1D*).

Blebs are ingested by a higher percentage of MoDC than ACR

We analyzed whether MoDC were able to internalize both mitoxantrone-induced ACR and blebs, by co-culturing CFSE-labeled MoDC with PKH26-labelled apoptotic material. Indeed, analysis using confocal microscopy demonstrated that both ACR (*figure 2A*, upper panel)

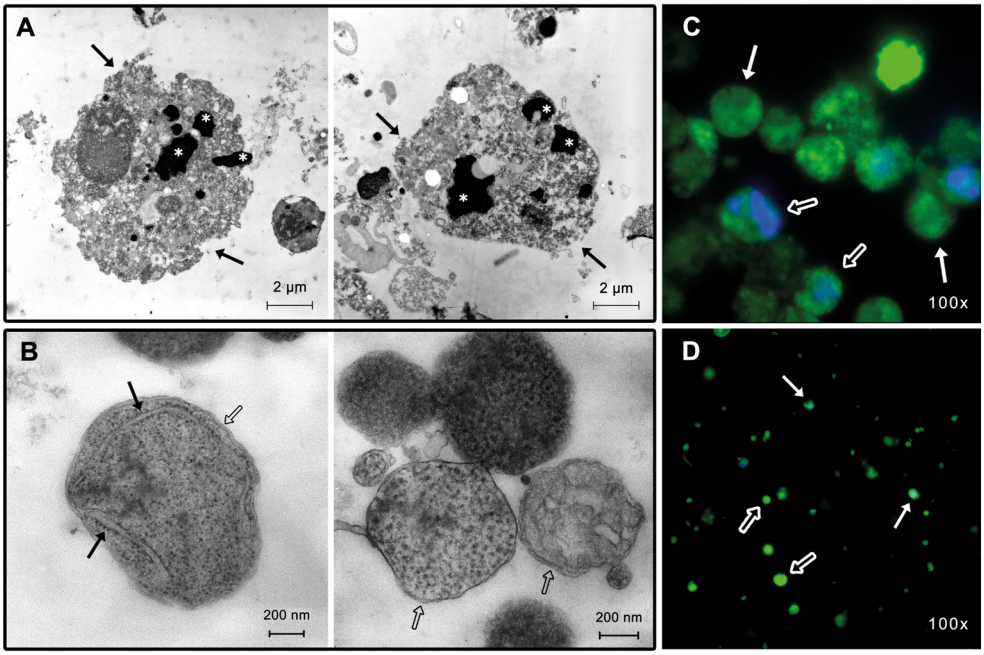


Figure 1: Analysis of isolated apoptotic fractions. (A-D) Transmission electron microscopic analysis of isolated ACR (A & B) and blebs (C & D). The isolated ACR fraction contained large cellular bodies of 4 – 10 µm in diameter, of which the membrane appeared to be locally disintegrated (A & B, arrows). Electron dense areas reside within the ACR (A & B, asterisks), that are enclosed by a membrane and represent the latest stages of chromatin translocation to the periphery of the cell. The bleb fraction contains an heterogeneous population of vesicles which are 0.2 µm to 0.6 µm in diameter and have a double membrane (C & D open arrows). Structures resembling ER and ribosomes (C, closed arrows) could be detected in some blebs.
(E-F) Fluorescence labelling with CFSE (green; protein) and Hoechst 33342 (blue; DNA) of ACR (E) and blebs (F) derived from apoptotic HL60 cells. 48 hours after inducing apoptosis using mitoxantrone, most ACR lost all (E; solid arrows) or most (E; open arrows) chromatin. The isolated bleb fraction contained mainly vesicles that did not stain positive for Hoechst 33342 (F; open arrows). Few blebs did enclose detectable amounts of chromatin (F; solid arrows).

and blebs (*figure 2A*, lower panel) could be internalized by MoDC. Next, we quantified the ingestion of ACR or blebs using flow cytometry (*figure 2B*), by co-culturing MoDC overnight with either CFSE-labeled ACR or blebs. During co-culture, MoDC did not receive any additional maturation stimuli (further referred to as immature DC), or received a maturation inducing cocktail one hour after initiation of the co-culture, consisting of the cytokine cocktail IL-1β, IL-6, PGE-2, and TNF-α (further referred to as maturing DC). Blebs were ingested by 70% (mean, range 41% - 95%) of the immature MoDC whereas ACR were ingested by 46% (mean, range 26% - 86%) (*figure 2C*, immature). These percentages decreased to a mean of 63% (range 13% - 92%) and 35% (range 6% - 80%), respectively, after inducing MoDC maturation (*figure 2C* maturing). Similar results were obtained when loading MoDC with ACR or blebs derived from heat shock (HS)-induced apoptosis (data not shown). Moreover, the uptake of blebs was significantly increased, whereas there was no significant difference in the uptake of ACR, compared to unseparated apoptotic cells (*figure 2D*). Therefore, subsequent experiments were performed using the seperated fractions.

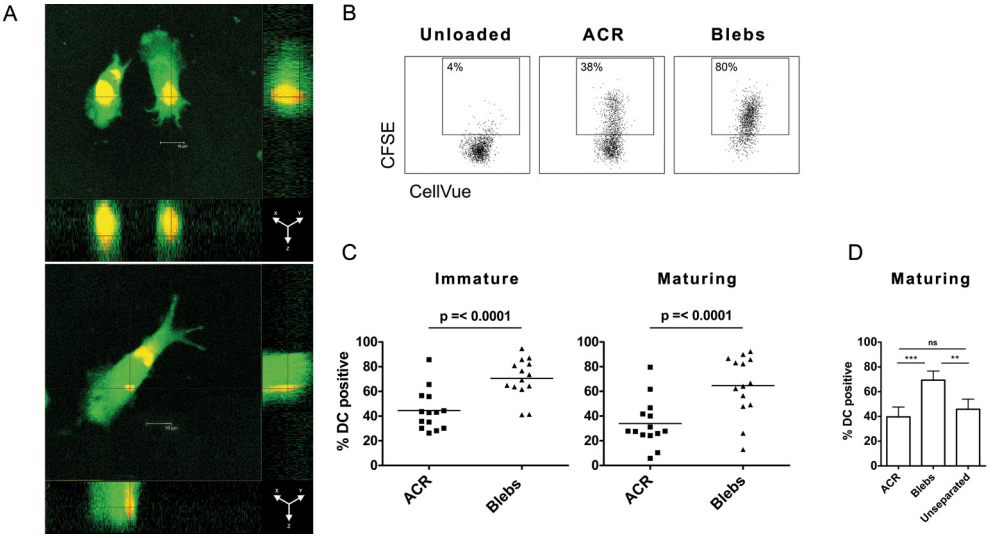


Figure 2: Uptake of apoptotic material by MoDC. (A) CFSE-labeled MoDC (green) were loaded with either PKH26-labelled ACR or blebs (red) and analyzed using confocal microscopy. Both ACR (upper panel) and blebs (lower panel) are internalized by MoDC (yellow), as visualized by the enclosure of both apoptotic fractions by MoDC in X, Y and Z directions. Optical magnification 42x and 3.5x digital zoom. (B) Representative flow cytometric analysis of the uptake quantification of the apoptotic fractions by MoDC. (C) Uptake by immature and maturing DC of blebs and ACR (n = 14). (D) The percentage ingestion of isolated ACR and blebs, as compared to the unseperated apoptotic cell preparation, by maturing MoDC (n = 5).

Increased CCR7 expression and migration towards CCL19 by MoDC which have ingested blebs.

Next, we determined whether ingestion of mitoxantrone-induced ACR or blebs altered MoDC phenotype. After 48 hours of co-culture, we could not detect differences in the expression levels of the tested markers (CD1a, CD14, CD40, CD54, CD80, CD83, and CD86, data not shown), between ACR- or bleb-loaded MoDC, except for the lymph node homing receptor CCR7. CCR7 expression was upregulated on mature MoDC that had ingested blebs, compared to unloaded or ACR-loaded MoDC (*figure 3A*). In line with the latter, an increase in the migration of bleb-loaded mature MoDC towards the lymph node homing chemokine CCL19 (Mip-3β) was observed; mean 14% (range 2% - 35%) of the MoDC that had ingested blebs were able to migrate towards CCL19, as compared to only 5% (range 1% - 16%) of MoDC that had ingested ACR (*figure 3B*).

Unaffected production of pro-inflammatory cytokines by MoDC after ingesting either ACR or blebs

To avoid interference of trace amounts of mitoxantrone with MoDC function (as described in the methods and materials section), we assessed whether ingestion of either HS-derived ACR or blebs induced an altered cytokine profile, as compared to unloaded MoDC. To this end, ACR- or bleb-loaded MoDC were activated by the CD40 ligand expressing cell line J558

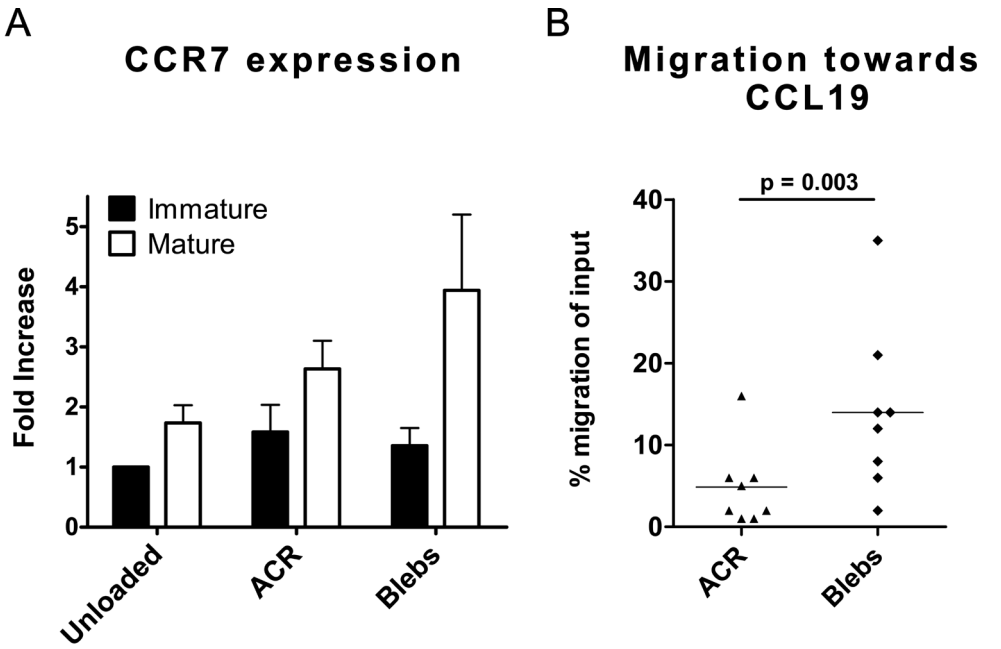


Figure 3: CCR7 expression on (loaded) MoDC, and subsequent migration towards CCL19. (A) Whereas no difference in expression of the lymph node homing chemokine receptor CCR7 could be detected in immature MoDC (closed bars), loading of mature MoDC (open bars) with either ACR or blebs led to an increased expression of CCR7. This increase was most pronounced when MoDC were loaded with blebs (n=3). (B) In concordance with an increased expression of CCR7, migration of ACR or bleb loaded mature MoDC revealed that MoDC which had ingested blebs, were also able to migrate towards CCL19 (Mip-3β) to a higher degree (14%; range 2% - 35%) in a transwell migration assay, compared to ACR (5%; range 1% - 16%) (p = 0.003) (n = 8).

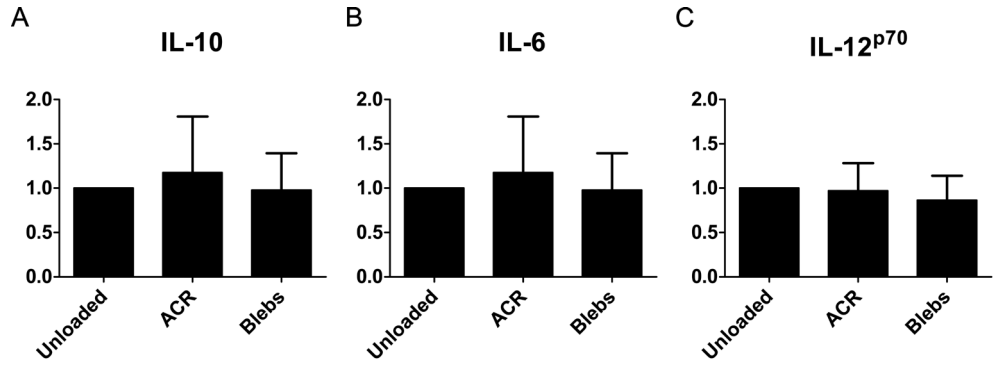


Figure 4: Cytokine production by (loaded) MoDC after CD40 ligation. (A-C) Cytokine production by unloaded, ACR-, or bleb-loaded immature MoDC after CD40 ligation. Supernatants were analyzed after an over-night incubation in the presence of 1000u/ml IFN-γ.
No differences could be detected in the production of the measured cytokines IL-10 (A), IL-6 (B) or IL-12p70 (C). The mean cytokine concentrations of unloaded MoDC are shown and set to one (n = 3).

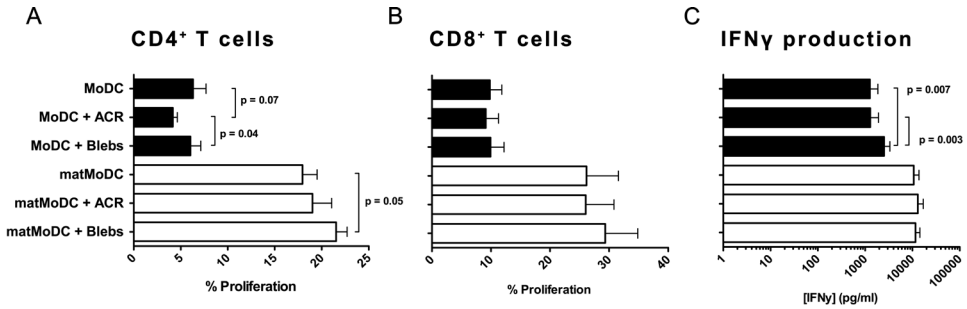


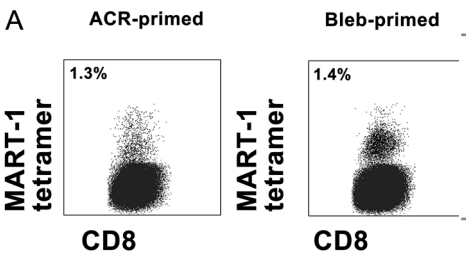
Figure 5: Allogeneic mixed lymphocyte reaction with (loaded) MoDC. (A) CFSE-labelled, CD14 negative, peripheral blood lymphocytes were cultured with MoDC, or MoDC loaded with either heat shock-induced ACR or blebs for 6 days, after which the CFSE dilution was analysed using flow cytometry, as a measure for T cell proliferation. The proliferation of $CD4^+$ T cells stimulated with ACR-loaded immature MoDC was diminished compared to unloaded- (p=0.07) and bleb-loaded immature MoDC (p=0.04). Moreover, Bleb-loaded mature MoDC induced a significant increase in proliferation of $CD4^+$ T cells, as compared to unloaded MoDC (p=0.05) (n=7). (B) No significant differences could be detected in $CD8^+$ T cell proliferation, between the different loading strategies (n = 7). (C) Peripheral blood lymphocytes were stimulated with (loaded) MoDC for 6 days, after which the cytokine production was determined in the supernatant. Of the cytokines tested (IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ and IL-17a), only IFNγ production was significantly altered. T cells stimulated with bleb-loaded MoDC produced approximately 2-fold higher levels of IFNγ, as compared to both unloaded and ACR-loaded MoDC (n = 4).

in the presence of 1000 U/ml IFN-γ. After overnight incubation, the concentration of the Th-skewing cytokines IL-6 (Th17), IL-10 (Th2) and IL-12p70 (Th1) were determined in the culture supernatants. No clear differences in the production of either IL-6 (*figure 4A*), IL-10 (*figure 4B*), or IL-12p70 (*figure 4C*) could be detected, when analyzing either unloaded, ACR-, or bleb-loaded MoDC.

Increased IFNγ production by allogeneic T cells after priming with bleb-loaded MoDC

Subsequently, we determined whether the loading of MoDC with either HS-derived ACR or blebs led to changes in T cell activation and/or proliferation, by priming with allogeneic T cells with differentially loaded MoDC. Loading of immature MoDC with ACR led to a reduction in $CD4^+$ T cell proliferation as compared to unloaded- and bleb-loaded MoDC (*figure 5A*). In contrast, mature MoDC loaded with blebs induced a slight, but significant increase in $CD4^+$ T cell proliferation as compared to unloaded- and ACR-loaded MoDC (*figure 5A*). No significant differences in $CD8^+$ T cell proliferation could be detected between the conditions (*figure 5B*).

Next, we determined whether we could detect differences in TH skewing by analyzing the cytokines produced by allogeneic T cells that were stimulated by differentially loaded MoDC. No differences could be detected in the production of IL-2, IL-4, IL-6, IL-10, IL-17a, or TNFα (data not shown). However, priming of T cells with bleb-loaded MoDC led to an increased release of the Th1 effector cytokine IFNγ as compared to unloaded or ACR-loaded MoDC (*figure 5C*).



B. Priming efficiency MART-1

	# Donors	Wells positive	Mean %	Range %	Range # Restim.
ACR	4	26/28	0.15	0.047%-1.009%	1-5
BLEBS	4	28/28	0.63	0.041%-11.662%	1-5

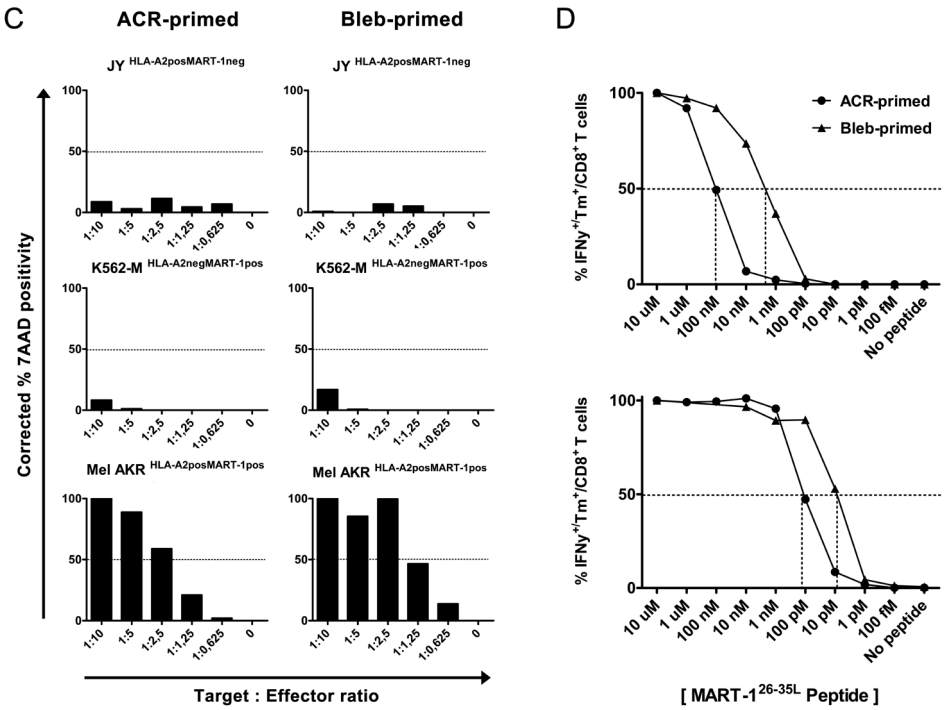


Figure 6: CD8⁺ T cell cross priming, cytotoxicity and avidity. (A) Priming of CD8⁺ T cells (gated on FCS/SSC, 7AAD⁻, CD3⁺, and CD8⁺) with heat shock-induced ACR- or bleb-loaded MoDC led to an expansion of MART-1$^{26\text{-}35L}$ specific CD8⁺ T cells, as measured by tetramer staining. (B) A total of 4 priming experiments with CD8⁺ T cells and autologous MoDC from healthy donors were performed, with a mean expansion of 0.16 % (range 0.05 % – 1.01 %) MART-1$^{26\text{-}35L}$ specific CD8⁺ T cells, when CD8⁺ T cells were primed with ACR-loaded MoDC. The mean expansion of MART-1$^{26\text{-}35L}$ specific CD8⁺ T cell, when primed with bleb-loaded MoDC, was 0.63 % (range 0.04 % - 11.66 %). Positive wells were defined as >0.03% and >15 Tm⁺ events, or >20 Tm⁺ events in two subsequent measurements. (C) Co-culture of sorted and expanded MART-1$^{26\text{-}35L}$ specific CD8⁺ T cells at increasing effector to target ratios with JY, or K562-M target cells, did not result in an increase in cytotoxicity, as measured by 7AAD positive target cells (C: top four panels; representative example of 2 cytotoxicity experiments). However, co-culture with Mel AKR target cells showed an increasing cytotoxicity with increasing number of effector cells, by MART-1$^{26\text{-}35L}$ specific CD8⁺ T cells primed with both ACR- and bleb- loaded MoDC (C: lower two panels). (D) T cell avidity analysis showed, that MART-1$^{26\text{-}35L}$ specific CD8⁺ T cells primed with bleb-loaded MoDC (triangles) could detect 12-fold to 107-fold lower MART-1$^{26\text{-}35}$ peptide concentrations, as compared to those primed with ACR-loaded MoDC (circles; avidity assays from 2 independent priming experiments).

Higher frequencies of antigen-specific CD8$^+$ T cells with a higher avidity are primed by bleb-loaded MoDC

Finally, we assessed the priming of antigen-specific CD8$^+$ T cells with MoDC loaded with HS-derived ACR or blebs. The apoptotic cell fractions were isolated from HLA-A2 negative HL60 AML cells, transduced with the MART-1 model tumor antigen, in order to prevent direct antigen-presentation of the apoptotic cell fractions to T cells. As such, outgrowth of antigen-specific T cells must be the result of cross-priming by loaded (HLA-A2-positive) MoDC. Priming CD8$^+$ T cells with differentially (i.e. with ACR or blebs) loaded MoDC, led to an expansion of MART-1$^{26\text{-}35L}$-specific CD8$^+$ T cells in both experimental conditions (*figure 6A*). However, the maximum percentage of MART-1$^{26\text{-}35L}$ positive CD8$^+$ T cells differed dramatically, with 1.01 % (mean 0.16 %, range 0.05 % – 1.01 %) vs.11.66 % (mean 0.63 %, range 0.04 % - 11.66 %) of CD8$^+$ T cells primed by ACR- or bleb-loaded MoDC respectively, as summarized in *figure 6B*. After sorting and polyclonal expansion, we analyzed the cytolytic capacity of the primed MART-1-specific CD8$^+$ effector T cells, by co-culturing them with Mel AKR (HLA-A2$^+$MART-1$^+$ positive target), or with HLA-A2$^+$MART$^-$ JY or HLA-A2$^-$MART-1$^+$ K562-M (negative control) target cells. MART-1$^{26\text{-}35L}$ CD8$^+$ T cells primed by either ACR- or bleb-loaded MoDC were capable of lysing Mel AKR at similar rates (*figure 6C*). Interestingly, analysis of functional T cell avidity by peptide titration followed by IFNγ release read-out, showed that MART-1$^{26\text{-}35L}$ CD8$^+$ T cells primed with bleb-loaded MoDC were able to recognize HLA class I:peptide complexes loaded with peptide concentrations two orders of magnitude lower as compared to CD8$^+$ T cells primed by ACR-loaded MoDC (i.e. 12-fold and 107-fold, see *figure 6D*).

Discussion

Broadening the repertoire of LAA presented by MoDC is likely to increase the efficacy of DC vaccination. However, only a limited number of LAA have been characterized to date and even fewer can be used in a patient-specific setting. Moreover, patient-specific frame-shift mutations are described to play a role in anti-tumor immunity [36], most of which are yet to be defined. The use of whole-tumor cell vaccines circumvents the need for LAA characterization and HLA matching, and furthermore broadens the array of presented LAA as well as facilitating personalized immunotherapy.

The finding that apoptotic blebs, in contrast to ACR, can induce maturation of BMDC in mice [30;37], led us to investigate the possibility of using blebs as source of LAA for DC loading in the context of a vaccination strategy. In the current study we show that blebs, as compared to ACR, derived from apoptotic AML cells, were ingested more efficiently by MoDC. It remains to be determined whether this increase was induced by 1) an enrichment of eat-me signals (e.g. phosphitadyl serine & calreticulin) on the surface of blebs, 2) a different mechanism of ingestion (phagocytosis vs. receptor-mediated endocytosis or fusion), or 3) whether the increased uptake was solely based on spatial considerations as a result of the approximately tenfold size difference between ACR and blebs.

One of the hurdles in effective DC vaccination, is the limited migration of (intradermally) injected DC from the site of injection towards the afferent lymph node [38]. The increased CCR7 expression we observed on MoDC that have taken up blebs and the subsequent CCL19 directed migration are clearly favorable in this regard. Apart from CCR7 expression, neither

ingestion of ACR nor of blebs resulted in clear alterations in phenotype, nor in cytokine production of MoDC. Contradictory data have been published on the effects of apoptotic cells on DC maturation. Presentation of self-antigens by DC under non-inflammatory conditions has been shown to induce self-tolerance [17]. Moreover, ingestion of apoptotic cells by DC inhibits NFκB activation, and induces the production of regulatory cytokines (e.g. IL-10, TGFβ), whereas the production of pro-inflammatory cytokines (e.g. IL-12 and TNFα) are hampered following Toll-like receptor ligation [18-22]. The expression of co-stimulatory molecules and T cell stimulatory capacity by apoptotic cell-loaded DC are diminished compared to unloaded DC [39-41]. In contrast, apoptotic cell-loaded DC have also been shown to induce T cell responses [42;43]. The contradictory data on apoptotic cell loading strategies for DC vaccination purposes could be a result of either the method of apoptosis induction, and/or the method of isolation after induction. Moreover, discrepancies observed after administration of apoptotic cells in murine models could be a result of the ingestion by different phagocytes (DC vs. macrophages, and non-professional phagocytes).

In contrast to dampening MoDC function and T cell activation, both ACR and bleb-loaded MoDC were able to stimulate T cells, although loading of MoDC with ACR, without the induction of DC maturation (using IL-1β, IL-6, PGE_2, and TNFα), did significantly diminish $CD4^+$ T cell proliferation compared to unloaded, or bleb-loaded MoDC. Importantly, co-culture of peripheral blood lymphocytes with bleb-loaded (immature) MoDC led to Th1 skewing, based on significantly higher levels of IFNγ production, compared to unloaded and ACR-loaded MoDC. Our data are in concurrence with the data obtained by Fransen *et al* [30], although we could not detect an increase in IL-17 production by PBL after stimulation with bleb-loaded MoDC (data not shown). This lack of IL-17 production in a MLR could be a result of 1) the species difference, 2) the origin of the DC used (MoDC vs. BMDC), or 3) the responder cells used in the MLR (PBL vs. splenocytes), and does not exclude the induction of IL-17 producing T cells *in vivo*.

We observed a higher percentage of MART-1^{26-35L} specific $CD8^+$ T cells when primed with bleb-loaded MoDC in the conducted priming experiments, which can be explained by multiple factors. For instance, the increase in uptake of blebs by MoDC could lead to an increased antigen-load per MoDC, by which these MoDC are able to present LAA in a greater number of HLA class I molecules, or for a prolonged period of time. Also, blebs could contain more readily processible sources of antigen, e.g. defective ribosomal products (DRIPs), rapidly degradable poly-peptides and translated proteins, due to the presence of the ER. These antigen sources have been described to be presented very efficiently in the context of HLA class I and are essential sources of presented antigen during viral infections [44;45]. Whether the presence of these peptides in blebs is elevated remains to be elucidated, but DRIPs within autophagosomes have been reported to be efficiently cross-presented [46]. Combined with the efficient uptake, this could render blebs a more effective source of LAA compared to ACR. In addition, membrane transfer or fusion can occur, by which HLA:peptide complexes (and other structures) are transferred onto MoDC, a process referred to as DC cross-dressing, or trogocytosis [47;48]. Whether cross-dressing occurs during the co-culture of blebs with MoDC remains to be determined. In this study we solely analyzed the effect of antigen uptake and the effects thereof on MoDC function, since we used HLA-A2 negative apoptotic material to prevent direct presentation of LAA by the apoptotic leukemic cell fractions, and thus it remains unclear whether, and to which degree, these mechanisms contribute to T cell priming. Our observation that bleb-loaded MoDC prime $CD8^+$ T cells of

higher avidity compared to ACR-loaded MoDC, is likely to be an effect of the ingested blebs on the functionality of the MoDC, rather than differences in processing and presentation efficiency of the antigen. As we found no differences in e.g. release of IL-12p70 or other T cell stimulatory cytokines or expression levels of co-stimulatory molecules, the underlying mechanism in this regard as yet remains obscure. Nevertheless, this impressive difference in functional avidity clearly indicates a preferred use of blebs over ACR for immunotherapeutic purposes.

In conclusion, we have shown that ingestion of human apoptotic blebs derived from the AML cell line HL60, can induce lymph node migration capacity in MoDC as well as the ability to prime IFNγ producing effector T cells and high-avidity tumor reactive CTL. Using bleb-loaded MoDC for the induction of tumor-directed cytotoxic T cell responses, could therefore be a potent novel strategy for treating AML patients.

Reference List

1. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat.Rev. Immunol. 2005;5:296-306.
2. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature 2007;449:419-426.
3. Westers TM, Stam AG, Scheper RJ et al. Rapid generation of antigen-presenting cells from leukaemic blasts in acute myeloid leukaemia. Cancer Immunol.Immunother. 2003;52:17-27.
4. Westers TM, van den AW, Bontkes HJ et al. Chronic myeloid leukemia lysate-loaded dendritic cells induce T-cell responses towards leukemia progenitor cells. Immunotherapy. 2011;3:569-576.
5. Thurner B, Haendle I, Roder C et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J.Exp.Med. 1999;190:1669-1678.
6. Nestle FO, Alijagic S, Gilliet M et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat.Med. 1998;4:328-332.
7. van Tendeloo VF, van de Velde A, van Driessche A, et a Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc.Natl.Acad.Sci.U.S.A 2010;107:13824-13829.
8. Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J.Exp.Med. 1996;184:465-472.
9. Bontkes HJ, Kramer D, Ruizendaal JJ et al. Dendritic cells transfected with interleukin-12 and tumor-associated antigen messenger RNA induce high avidity cytotoxic T cells. Gene Ther. 2007;14:366-375.
10. van den Ancker W, van Luijn MM, Ruben JM et al. Targeting Toll-like receptor 7/8 enhances uptake of apoptotic leukemic cells by monocyte-derived dendritic cells but interferes with subsequent cytokine-induced maturation. Cancer Immunol.Immunother. 2011;60:37-47.
11. van den Ancker W, van Luijn MM, Westers TM et al. Recent advances in antigen-loaded dendritic cell-based strategies for treatment of minimal residual disease in acute myeloid leukemia. Immunotherapy 2010;2:69-83.
12. de Gruijl TD, Luykx-de Bakker SA, Tillman BW et al. Prolonged maturation and enhanced transduction of dendritic cells migrated from human skin explants after in situ delivery of CD40-targeted adenoviral vectors. J.Immunol. 2002;169:5322-5331.

13. Bonifaz L, Bonnyay D, Mahnke K et al. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J.Exp.Med. 2002;196:1627-1638.
14. Bonifaz LC, Bonnyay DP, Charalambous A et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J.Exp.Med. 2004;199:815-824.
15. Tacken PJ, Figdor CG. Targeted antigen delivery and activation of dendritic cells in vivo: steps towards cost effective vaccines. Semin.Immunol. 2011;23:12-20.
16. Kitawaki T, Kadowaki N, Kondo T et al. Potential of dendritic cell immunotherapy for relapse after allogeneic hematopoietic stem cell transplantation, shown by WT1 peptide- and keyhole limpet hemocyanin-pulsed, donor-derived dendritic cell vaccine for acute myeloid leukemia. Am.J.Hematol. 2007;83:315-317.
17. Liu K, Iyoda T, Saternus M et al. Immune tolerance after delivery of dying cells to dendritic cells in situ. J.Exp.Med. 2002;196:1091-1097.
18. Morelli AE. The immune regulatory effect of apoptotic cells and exosomes on dendritic cells: its impact on transplantation. Am.J.Transplant. 2006;6:254-261.
19. Municio C, Hugo E, Alvarez Y et al. Apoptotic cells enhance IL-10 and reduce IL-23 production in human dendritic cells treated with zymosan. Mol.Immunol. 2011;49:97-106.
20. Ren G, Su J, Zhao X et al. Apoptotic cells induce immunosuppression through dendritic cells: critical roles of IFN-gamma and nitric oxide. J.Immunol. 2008;181:3277-3284.
21. Sen P, Wallet MA, Yi Z et al. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. Blood 2007;109:653-660.
22. Stuart LM, Lucas M, Simpson C et al. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. J.Immunol. 2002;168:1627-1635.
23. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N.Engl.J.Med. 2009;361:1570-1583.
24. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. Oncogene 2008;27 Suppl 1:S2-19.
25. Franz S, Herrmann K, Furnrohr BG et al. After shrinkage apoptotic cells expose internal membrane-derived epitopes on their plasma membranes. Cell Death.Differ. 2007;14:733-742.
26. Lane JD, Allan VJ, Woodman PG. Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells. J.Cell Sci. 2005;118:4059-4071.
27. Meesmann HM, Fehr EM, Kierschke S et al. Decrease of sialic acid residues as an eat-me signal on the surface of apoptotic lymphocytes. J.Cell Sci. 2010;123:3347-3356.
28. Bovellan M, Fritzsche M, Stevens C, Charras G. Death-associated protein kinase (DAPK) and signal transduction: blebbing in programmed cell death. FEBS J. 2010;277:58-65.
29. Mills JC, Stone NL, Erhardt J, Pittman RN. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J.Cell Biol. 1998;140:627-636.
30. Fransen JH, Hilbrands LB, Ruben J et al. Mouse dendritic cells matured by ingestion of apoptotic blebs induce T cells to produce interleukin-17. Arthritis Rheum. 2009;60:2304-2313.
31. Suber T, Rosen A. Apoptotic cell blebs: repositories of autoantigens and contributors to immune context. Arthritis and rheumatism 2009;60:2216–9

32. Liu Y, Qureshi M, Xiang J Antitumor immune responses derived from transgenic expression of CD40 ligand in myeloma cells. Cancer biotherapy & radiopharmaceuticals 2002;17:11–8.
33. Schreurs MW, Scholten KB, Kueter EW et al. In vitro generation and life span extension of human papillomavirus type 16-specific, healthy donor-derived CTL clones. J.Immunol. 2003;171:2912-2921.
34. Hooijberg E, Ruizendaal JJ, Snijders PJ et al. Immortalization of human CD8+ T cell clones by ectopic expression of telomerase reverse transcriptase. J.Immunol. 2000;165:4239-4245.
35. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J.Proteomics. 2010;73:1907-1920.
36. Fischer E, Kobold S, Kleber S et al. Cryptic epitopes induce high-titer humoral immune response in patients with cancer. J.Immunol. 2010;185:3095-3102.
37. Fransen JH, Hilbrands LB, Jacobs CW, et al. Both early and late apoptotic blebs are taken up by DC and induce IL-6 production. Autoimmunity 2009;42:325–327.
38. Verdijk P, Aarntzen EH, Lesterhuis WJ et al. Limited amounts of dendritic cells migrate into the T-cell area of lymph nodes but have high immune activating potential in melanoma patients. Clin.Cancer Res. 2009;15:2531-2540.
39. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nature medicine. 1999;5:1249–55.
40. Herr W, Ranieri E, Olson W et al. Mature dendritic cells pulsed with freeze-thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4(+) and CD8(+) T lymphocyte responses. Blood 2000;96:1857-1864.
41. Schnurr M, Galambos P, Scholz C et al. Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines. Cancer Res. 2001;61:6445-6450.
42. Kokhaei P, Rezvany MR, Virving L et al. Dendritic cells loaded with apoptotic tumour cells induce a stronger T-cell response than dendritic cell-tumour hybrids in B-CLL. Leukemia 2003;17:894-899.
43. Wieckowski E, Chatta GS, Mailliard RM et al. Type-1 polarized dendritic cells loaded with apoptotic prostate cancer cells are potent inducers of CD8(+) T cells against prostate cancer cells and defined prostate cancer-specific epitopes. Prostate 2011;71:125-133.
44. Dolan BP, Li L, Takeda K, Bennink JR, Yewdell JW. Defective ribosomal products are the major source of antigenic peptides endogenously generated from influenza A virus neuraminidase. J.Immunol. 2010;184:1419-1424.
45. Yewdell JW, Nicchitta CV. The DRiP hypothesis decennial: support, controversy, refinement and extension. Trends Immunol. 2006;27:368-373.
46. Li Y, Wang LX, Pang P et al. Tumor-derived autophagosome vaccine: mechanism of cross-presentation and therapeutic efficacy. Clin.Cancer Res. 2011;17:7047-7057.
47. Wakim LM, Bevan MJ. Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. Nature 2011;471:629-632.
48. Yewdell JW, Dolan BP. Immunology: Cross-dressers turn on T cells. Nature 2011;471:581-582.

Genereal discussion and future perspectives

16

W van den Ancker
TM Westers
AA van de Loosdrecht

Introduction

In 1811 William Cullen described a case of 'splenitis acutus' in which the serum of the blood had the appearance of milk; with our current knowledge Cullen is most likely the first to describe a case of chronic leukemia. In 1827 a more detailed case report on a 63 year old patient, Mr Vernis, was published by Alfred Velpeau. Mr Vernis's illness started at the age of 54 and was followed by a stable phase for three years. This phase was followed by a period of recurrent fevers and difficulty urinating. Eventually, the patient was admitted to hospital at the age of 63 with tumors in the abdomen and died the next morning. An autopsy revealed an enlarged liver and spleen and pus-filled blood. Then, Alfred Donné discriminated in his morphological atlas (published in 1845) between accumulation of pus in the blood as seen in infections or abscesses and of so called "mucous globules" (leucocytes) as he found in a case of a 44-year old woman with splenomegaly. John Bennett stated in 1845 that in case of leucocytosis this should be considered as a primary systemic blood disorder. In 1847 Rudolf Virchow was the first pathologist to describe this clinical and pathological condition as leukemia based on the Greek words leukos (λευκός), and aima (αίμα), meaning white and blood respectively. With further development of microscopy of blood smears Virchow was able to differentiate between splenic and lymphatic leukemia in 1856; it was not until 1869, when Neumann discovered that the origin of leukaemia should be connected to the bonemarrow. Further development of stained blood smears designated the beginning of a new era in which more details of leukemia were unravelled but yet many more complicated questions raised [1].

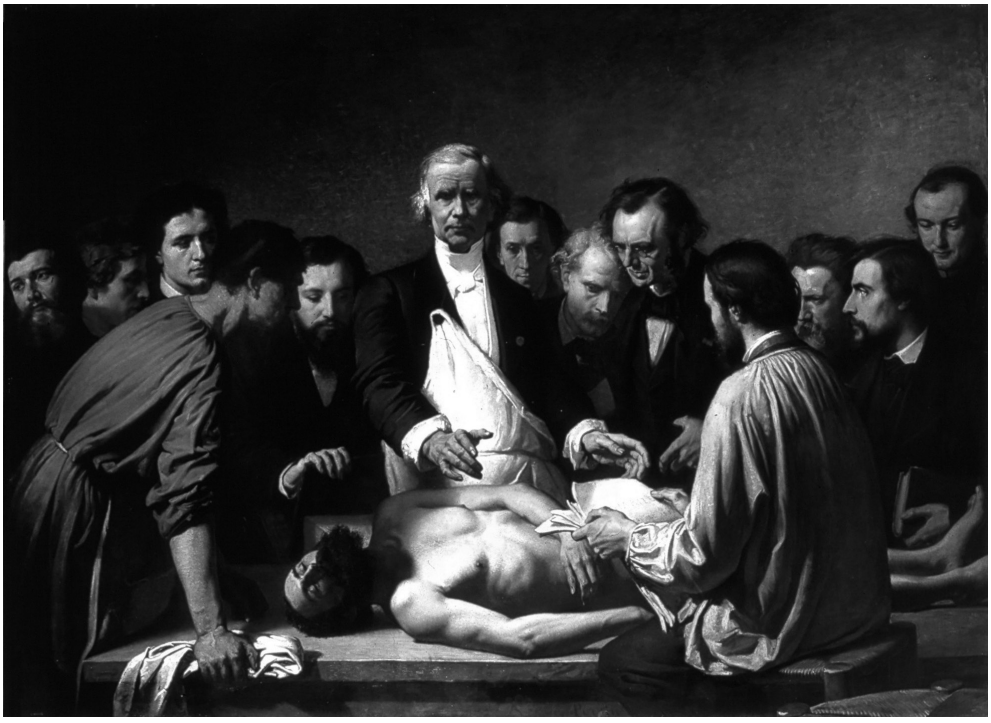


Figure 1. Anatomy lessons of Dr Velpeau. 1864, by Francois Nicolas Augustin Teyen-Perrin.

Models for the development of leukemia

Over the last decades, more and more is revealed about the development of cancer. Simplified models have been proposed, however, none of these are simple. For instance, massive exposure to radioactive substances will lead to breaks in double stranded DNA and thereby translation into proteins may stop which finally leads to death of hematopoietic stem cells: acute radiation syndrome. When one is exposed to lower amounts of radiation, this will lead to multiple DNA breaks which can be repaired by the cell itself. When this mechanism fails, the improperly repaired DNA might encode for proteins which promote unlimited growth of hematopoietic stem cells and in this case in months to years leukemia might develop. Especially mutations in DNA repair mechanisms or other tumor suppressor genes are well described. For instance, the P53 gene plays a decisive role in DNA repair and it can induce cell cycle arrest to provide time for DNA repair and when repair is not possible P53 can induce apoptosis. Mutation in P53 genes and abolishment of its function can promote development of cancer [2]. However, no model can yet explain why one hematopoietic progenitor cell becomes malignant and another progenitor cell will not. Moreover, the development of models answering these questions is complicated by the heterogeneity of leukemic cells between patients and even within one patient. Nevertheless, different models are developed trying to explain the heterogeneity of a tumor: the stochastic model points out that tumors are inherently biologically homogenous and heterogeneity is caused by random intrinsic or extrinsic influences that alter the behavior of individual cells in the tumor. In contrast, the deterministic model states that heterogeneity of tumors is caused by known influences and relationships between other cells and the environment, a role for random phenomena is excluded [3]. In some cases, a pre-leukemic phase of ineffective production and dysplasia of the myeloid cell lineage, the myeolodysplastic syndrome (MDS), might precede evolution to AML. In these cases the steps to malignant evolution into AML can be researched more thoroughly: Walter et al. proposed a model for the development of secondary AML from MDS based on single nucleotide variant analysis performed at various time points during evolution of MDS to AML (summarized in figure 2)[4]. All of these hypotheses have provided the basis for better understanding cancer and consequently

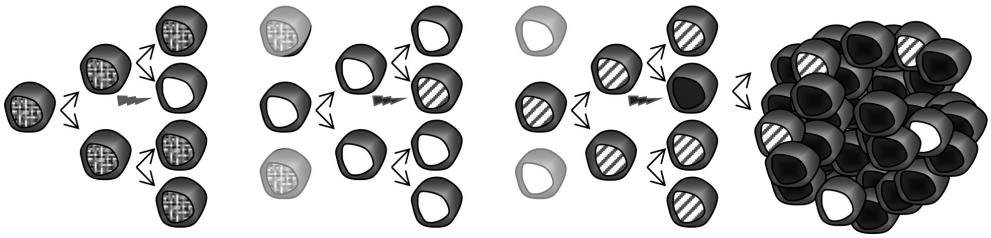


Figure 2. A model explaining clonal evolution (adapted from Walter et al. [4]). Based on Single nucleotide variant analysis of MDS (green cells) and secondary AML (purple) the following model was proposed for the evolution of MDS into AML. First (1) a single clone (green) is present. Cells in clone 2 (red) are derived from a single cell from clone 1 since newly acquired variants are found in addition to the same single nucleotide variants found in nearly all AML cells. Finally, AML has developed showing various subclones evolved through serial (2,3) acquisition of single nucleotide variants.

added to the development of new therapeutics and as a result, survival rates in leukemia have increased over the last decades

Nowadays, after induction and consolidation regimens complete remission can be established in 70-80% of adults with acute myeloid leukemia (AML) and even better results for acute lymphoid leukemia (ALL). Unfortunately, 5 year survival rates are significantly lower, for AML this is 30-40% and for ALL 40-50%.

The classification of acute (myeloid) leukemia

Despite heterogeneity as described above, in clinical studies AML patients have been regarded as one homogenous group, randomized in two cohorts: one for the standard and one for the new (chemo)therapeutics. With our current knowledge, treatment regimens may differ for patients with a (very) good and poor risk profile (see www.hovon.nl for Dutch treatment guidelines). These risk stratifications are primarily based on molecular and genetic aberrancies. Thus, as a start, there should be consensus on how to classify acute leukemias in order to provide a standardized platform for testing different treatment options for distinct subgroups of AML. A struggle on one hand is the heterogeneity of acute leukemias and potentially numerous subgroups and on the other hand the need for large (homogenous) groups of patients for testing new (chemo)therapeutic regimens.

In order to have a worldwide consensus on classification the WHO2008 provides guidelines for diagnosing different subgroups based on a combination of clinical, morphologic, and immunophenotypic features, and results obtained by molecular analysis and cytogenetics. Differentiation between AML and acute lymphoid leukemia (ALL) is important since ALL necessitates a different therapeutic regimen than leukemias from myeloid origin; for example, a longer maintenance treatment and intrathecal prophylaxis is needed for lymphoid leukemias.

In *PART I* of this thesis we evaluated the role of the WHO2008 guidelines in differentiating classification of myeloid and lymphoid leukemias by flow cytometry. In *chapter 3* we describe two case reports highlighting the importance of immunophenotyping. The first report is a lymphoid leukemia at diagnosis which relapses as an immunophenotypic myeloid leukemia with the same complex cytogenetic aberrancies as at diagnosis. This subclone with a myeloid phenotype was already present at diagnosis and after chemotherapy following the ALL protocol. The other acute leukemia case describes a paradoxical appearance: morphology revealed a clear monocytoid picture with prominent vacuolization and erythrophagocytosis, whereas flow cytometry revealed a clear lymphoid phenotype. Besides the lymphoid markers, also MPO was found positive, thus classifying this case as a mixed phenotype acute leukaemia following the WHO2008 guidelines. The patient was treated with an AML chemotherapy regimen combined with intrathecal methotrexate prophylaxis, resulting in complete remission. Unfortunately the patient experienced a relapse (with the same phenotype) after 11 months, and further chemotherapy was not given. In about 2-4% of acute leukemias flow cytometry cannot unravel whether an acute leukemia should be assigned to the myeloid or lymphoid lineage. When expression of both myeloid and lymphoid markers is found, the acute leukemias are classified as mixed phenotype acute leukemias (MPAL), it is unclear whether MPAL benefit from an ALL or AML based treatment protocol. Moreover, acute leukemias with both lymphoid and myeloid

features have a poor prognosis and therefore may benefit from an intensified treatment scheme. International guidelines implementing these intensified schemes are lacking. For comparison of these different chemotherapy schemes a uniform classification has to be used. However, the last decades various guidelines have been used for classification of acute leukemias of ambiguous lineage. In *chapter 4* we discussed the implications of the various guidelines and compared the latest WHO2008 guidelines with the former WHO2001 guidelines in classification of MPAL. Noteworthy, according to the WHO2001 criteria 5.8% of all acute leukemias referred to our institute were classified as acute leukemias with both lymphoid and myeloid features (biphenotypic acute leukemia (WHO2001)), whereas according to the WHO2008 guidelines only 1.3% would have been classified as MPAL. In addition, clearly defined cut offs for positivity and negativity of certain immunophenotypic diagnostic markers are lacking in WHO2008; e.g. no cut off for cytoplasmic myeloperoxidase (cMPO), the hallmark of the myeloid lineage is defined. In *chapter 5*, we demonstrated that a 10% cut off is a secure lower limit for MPO expression by flow cytometry and can be used independently from the cytomorphology based Sudan Black analysis. Compared with a regular cut off of 20% (according to the former WHO2001 guidelines), this proposed cut off of 10% for cMPO expression would have changed the diagnosis into MPAL in only two cases in our cohort (1%). Nowadays, treatment schemes for MPAL are based on a lymphoid or myeloid based treatment scheme, the latter often combined with intrathecal methotrexate prophylaxis. Accordingly, it is necessary to distinguish between a myeloid or lymphoid lineage predominance within an acute leukemia with both characteristics. Classification of MPAL might be complemented by the use of other diagnostic tools, such as gene or microRNA expression profiling. In *chapter 6* we demonstrated by microRNA arrays that MPAL did not segregate as a separate entity but showed microRNA expression profiles similar to that of either AML, B-ALL or T-ALL. This implies that MPAL might not be a unique clinical entity but can be traced back to their original genotypic lineage using microRNA expression. We hypothesize that microRNA classification of those leukemias with both lymphoid and myeloid characteristics could provide a more accurate classification as compared to immunophenotyping and is instrumental for therapy decision making. However, prospective studies are needed for implementation of microRNA arrays in clinical practice.

In addition to microRNA arrays, the development of other mass data analysis on a gene or protein level (such as gene arrays, proteomics and mass cytometry [5]) will help to define more accurate signatures for acute leukemias and leukemic cells and thus more accurate definitions of subgroups of acute leukemias. In the near future these diagnostic tools might help defining (sub)groups for comparing different therapeutic schedules. Ideally, a personalized leukemic signature might lead to a personalized treatment: for instance a personalized immunotherapy which is discussed in *PART III*.

The development of AML: immunesurveillance

Most models describing the role of the immune system in cancer are deduced from the innate and adaptive immune response to foreign micro-organisms. One should be careful to extrapolate these models to acute leukemia. For instance, the process of a pre-malignant cell evolving into leukemia might take years such as is the case for MDS, whereas micro-

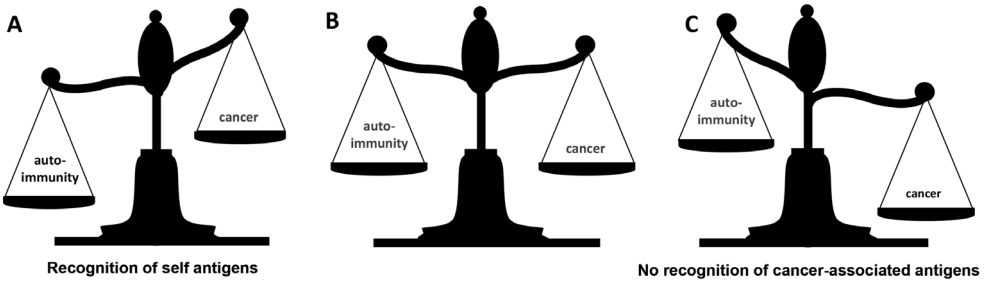


Figure 3. A simple (A) and complex (D) model explaining immunologic processes. In (A) the immune system is represented as a balance. In A an over-reactive immune system will result in auto-immune diseases and an unresponsive (C) immune system will result in the development of cancer. When the immune system is balanced (B) no auto-immune diseases will develop and the immune system is capable of eliminating cancer. I

organisms can cause fulminant infections within days. Furthermore, in contrast to micro-organisms, leukemic cells are derived from previous non-malignant cells and thus express more self than non-self-antigens. Nonetheless, these models provide insights in the role of the immune system in the development of cancer and thus might provide a tool for the development of therapies. One interesting model trying to explain the development of leukemia is discussed in this thesis and is based on escape of the immune system. In this model the immune system is depicted as a balance (*figure 3*). A balanced immune system reacts in a balanced manner to micro-organisms and malignant cells (*figure 3b*). An overacting immune system (*figure 3a*) results in hyper inflammation and even worse, an immune response against normal cells and subsequently auto-immune diseases. In contrast, in case of hypo-inflammation (*figure 3c*), the immune system leaves (pre)leukemic cells untouched resulting in outgrowth of leukemia. In other words, in order for leukemia to develop the leukemic cells must acquire escape mechanisms to prevent recognition by the immune system, this is researched in *PART II* of this thesis. However, this model can not be generally applied: for instance, chronic myeloid leukemia (CML) cells are able to activate BCR-ABL-specific T cells, however, these T cells were not able to proliferate [6]. Furthermore, it is evidenced that persistent antigen exposure can result in loss of high affinity leukemia reactive T cells or T cell tolerance [7-10]. Besides, many other immunosuppressive mechanisms are described involving the tumor environment such as regulatory and suppressor T cells and molecules expressed by the tumor cell itself such as indoleamine 2,3-dioxygenase (IDO) and Class II associated invariant chain peptide (CLIP) [11;12]. In *PART II* we have investigated the role of class II associated Invariant Chain peptide (CLIP) in antigen presentation via HLA class II by leukemic cells as a possible immune escape mechanism. Previously, we have shown that patients with a high CLIP expression on leukemic cells have a worse prognosis [12]. In this thesis we showed that CLIP expression on minimal residual disease can predict relapse more accurately (*Chapter 8*). The model explaining this difference in survival assumes that CLIP is preventing presentation of leukemia associated antigen (LAA) derived peptides by HLA-class II. However, CLIP expression is regulated by HLA-DM and HLA-DO; as such, CLIP expression might be the result of alterations in other pathways and molecules. CLIP expression implies that exogenous proteins are not efficiently presented by the leukemic cell onto HLA class II molecules. This assumption is substantiated by the finding that leukemic cells positive for CLIP have reduced $CD4^+$ T cell activating capacity compared with the CLIP negative cells

within the same patients in an autologous setting (*Chapter 9*). These results have led to the assumption that analysis of CLIP can be used to predict which patient might be likely to experience a relapse. High CLIP expression results in a poor prognosis and warrants more intensive chemotherapy, or more preferably therapy directly targeting CLIP. The latter may entail potentiation of the antigen processing machinery, such as the proteasome and TAP. For instance, clinically applicable HDAC inhibitors enhance the expression of TAP subunits in tumor cells. By incubation of leukemic cells with HDAC inhibitors *in vitro* we found strong down modulation of CLIP (unpublished data). Another option might be to directly inhibit CLIP expression via modulation of HLA-DM/DO, for instance using PKC inhibitors [13]: in activated B cells PKC inhibition affects DO expression potentially leading to a decline in CLIP. In *chapter 15* we investigate the role of R848 on monocyte derived dendritic cells (MoDC) and show down-modulation of CLIP. The role of TLR-ligands in affecting CLIP expression on leukemic cells should be further investigated; preliminary experiments showed that by incubation of leukemic cells with TLR-ligands CLIP was down regulated. However, due to presence of CLIP on non-malignant cells, such as B-cells one should be very careful in application of CLIP modulation in clinical practice in order to prevent auto-immunity.

Models for development of immunotherapeutic strategies

The immune-escape model provides a model for the development of cancer but can also provide a starting point for new treatment modalities such as immunotherapy (vaccination). After initial chemotherapy the bulk of leukemic cells are lysed, this is followed by consolidation therapy to attack remaining residual cells. In addition to consolidation therapy, immunotherapy can add to the recognition and lysis of residual leukemic cells and thereby might prevent relapse of AML. Immunotherapy aims at the induction of leukemic antigen specific T cells. In *chapter 11* we show that PRAME-specific T cells efficiently recognize and lyse leukemic cells and thus are an attractive tool for development of additional immunotherapeutic strategies, such as adoptive T cell transfer or active immunotherapy aiming at the induction of PRAME or other LAA-specific T cells

The critical role of dendritic cells (DC) in induction, regulation and maintenance of primary immune responses, including specific antitumor responses, has been demonstrated. This has led to development of DC-based vaccination strategies as a way to actively induce antitumor immunity. Thus far, results are promising: T cell responses have been elicited by DC vaccines and DC vaccines have proved to have only few side effects [14;15]. Unfortunately, major clinical responses have only been reported in a minority of cancer patients. Partly this can be explained by the fact that DC-based vaccines have been administered to cancer patients who obtained a second complete remission (CR) after relapse and thus have a very poor prognosis; these results highlight the need for improvement of DC-based immunotherapeutic strategies. For the latter, lessons can be learnt from stem cell transplantations (SCT): whereas the patients' immune system fails to control leukemic cells, donor immune cells can induce long term CR due to activation of donor T cells. These donor-derived T cells recognize either alloantigens that are expressed on normal and malignant cells or leukemia-specific antigens. However, SCT is not suitable for all patients, for instance those patient with high morbidity

or older patients, and has a high treatment-related mortality and morbidity (graft versus host disease). Passive immunotherapy might be effective in the short term; it does not offer long term immunity. In contrast, active DC-based immunotherapy aims at the induction of the LAA-specific T cells including memory T cells. The leukemic cell contains already known and not yet discovered LAA and thus provides an attractive source for loading onto DC and for development of personalized treatment regimens. Another option is to use DC cultured from leukemic cells: by culturing leukemic cells with a cytokine cocktail consisting of GM-CSF and IL-4 differentiation into leukemic cell-derived DC is potentiated; this technique is exploited in chapter 12. In favor of this concept is that leukemic cells and leukemic cell-derived DC processes and presents the same leukemic antigens by MHC-molecules. However, the leukemic cell-derived DC might harbor immune suppressive features and the antigen presenting machinery is less efficient as compared to normal non-leukemia derived DC. [16]. Another option is the use of MoDC loaded with leukemia-associated antigen sources. There are various ways to supply leukemic cells to the dendritic cells. For instance by repetitive freeze (liquid nitrogen)/ thaw (42ºC) cycles cellular integrity is lost and intracellular proteins and peptides can be provided to the DC for uptake and further processing. In *chapter 13* we show that CML lysate-loaded DC are able to activate CML-specific T cells. Another option is the induction of apoptosis by for instance, heat-shock, UV-irradiation or chemotherapeutics to render leukemic cells suitable for loading onto MoDC. Beside the numerous antigen sources (e.g. lysate, blebs, RNA, peptides) even more options for adjuvants potentially enhancing the loading efficacy are becoming known. In *chapter 14* we have compared different DC-loading strategies for optimal immune responses. To shorten the list of numerous DC loading strategies to a researchable amount we focused on whole cell products: lysate and apoptotic cells. In innate immunity toll like receptors (TLR) are necessary to induce an optimal innate immune response and help the adaptive immune response. In contrast to lysate, by loading apoptotic cells in presence of TLR ligand more antigen is taken up by the DC (*chapter 14*). The addition of TLR ligands during loading of MoDC enhances the uptake of leukemic cell lysate and apoptotic cells. However, the sequential use of R848 and a conventional cytokine cocktail is counter indicated due to its adverse effects on MoDC maturation.

During apoptosis of non-malignant cells highly immunogenic blebs are shed; the remaining apoptotic body is considered less immunogenic. We explored the immunogenicity of apoptotic blebs in leukemic cells in *chapter 15* and show that bleb-loaded DC are more efficient in T cell activation than the apoptotic cell remnants. Further systematic studies comparing leukemic cell derived DC and MoDC loaded with leukemic cell-derived antigens sources are needed to evaluate the best DC source for inducing LAA specific T cells.

Future directions for development of immunotherapy

Only a few studies are available comparing different DC loading strategies [17-20]. These comparitive studies revealed that different AML patients might benefit from different vaccine preparations. Furthermore, comparison of various studies with each other is complicated by different MoDC culturing and subsequent loading procedures.

In this thesis, we show that LAA-specific T cells can efficiently recognize and lyse leukemic cells. Furthermore, for the development of active specific immunotherapy, DC-based

vaccines offer an interesting option. We show that leukemic cells can be differentiated into DC, whether or not after preceding expansion of leukemic cells. Furthermore, loading of MoDC loaded with blebs is favorable over loading with apoptotic cell remnants. And when loading the DC, one should be careful with the use of adjuvants such as TLR-L. These recommendations, which we provide in this thesis, offer a starting point for further research: these studies should focus on comparison of variations in DC culturing and loading possibilities. For example, AML-derived DC have not yet been compared side-by-side with MoDC loaded with whole cell derived antigens.
Only a few LAA are known and these are not generally expressed in AML and thus hamper a LAA-specific *in vitro* read-out system [21]. In these cases results obtained from research using CML-cell loaded MoDC might be helpful: BCR-ABL is generally expressed in CML and phenotype and genotype are more homogenous compared with AML
When taking the stem cell model into account another challenge is faced in DC loading strategies: DC primed LAA specific T cells should ideally target leukemic cells and leukemia initiating stem cells. Therefore, *in vitro* research should focus on the development of leukemic stem cell loading strategies onto DC, for instance by loading of amplified RNA isolated from the leukemic stem cell.

Summary

In summary, in *PART I* we described the struggle with on one hand the heterogeneity of acute leukemias and potentially numerous subgroups and on the other hand the need for large (homogenous) groups of patients for testing new therapeutic regimens. Immunophenotyping plays an important role in definition of various subgroups. In future, results obtained from e.g. gene or microRNA array or mass cytometry might add to deciphering the genetic and protein signature of a leukemic cell and more accurate definition of subgroups. In *PARTII* we showed that leukemic cells exploit immune escape mechanisms such as expression of CLIP. These immune escape mechanisms provide a starting point for developing of immunotherapeutic interventions as described in *PART III* of this thesis. In this part we evaluated different DC-based loading strategies and made a start in finding the most optimal vaccine preparation and compare different adjuvants potentially enhancing DC-preparation and function. Diagnostic tools have to be developed that can direct personalized treatment schemes, such as immunotherapeutic vaccination strategies. Several steps have to be taken for preparation of dendritic cell based vaccines: ongoing research is needed in order to achieve consensus about which antigen loading strategy for MoDC combined with administration of optimal adjuvants is most suitable. This includes optimization of LAA processing and presentation pathways in order to achieve the best-suited tailor-made vaccine preparation in each individual patient.

Reference List

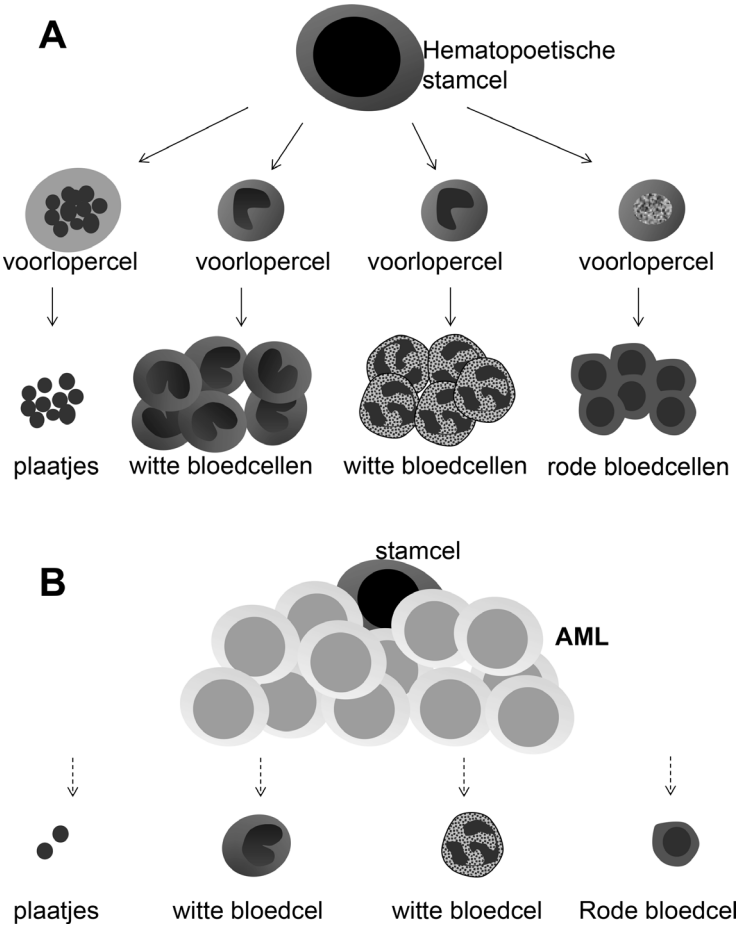
1. Kampen KR. The discovery and early understanding of leukemia. Leuk.Res. 2012;36:6-13.
2. Harutyunyan A, Klampfl T, Cazzola M, Kralovics R. p53 lesions in leukemic transformation. N.Engl.J.Med. 2011;364:488-490.

3. Becker MW, Jordan CT. Leukemia stem cells in 2010: current understanding and future directions. Blood Rev. 2011;25:75-81.
4. Walter MJ, Shen D, Ding L et al. Clonal architecture of secondary acute myeloid leukemia. N.Engl.J.Med. 2012;366:1090-1098.
5. Bendall SC, Simonds EF, Qiu P et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science 2011;332:687-696.
6. Rusakiewicz S, Madrigal A, Travers P, Dodi AI. BCR/ABL-specific CD8+ T cells can be detected from CML patients, but are only expanded from healthy donors. Cancer Immunol. Immunother. 2009;58:1449-1457.
7. Molldrem JJ, Lee PP, Kant S et al. Chronic myelogenous leukemia shapes host immunity by selective deletion of high-avidity leukemia-specific T cells. J.Clin.Invest 2003;111:639-647.
8. Fujii S, Shimizu K, Koji F, Kawano F. Malignant counterpart of myeloid dendritic cell (DC) belonging to acute myelogenous leukemia (AML) exhibits a dichotomous immunoregulatory potential. J.Leukoc.Biol. 2003;73:82-90.
9. Zhou G, Lu Z, McCadden JD, Levitsky HI, Marson AL. Reciprocal changes in tumor antigenicity and antigen-specific T cell function during tumor progression. J.Exp.Med. 2004;200:1581-1592.
10. Jonuleit H, Schmitt E. The regulatory T cell family: distinct subsets and their interrelations. J.Immunol. 2003;171:6323-6327.
11. Chamuleau ME, van de Loosdrecht AA, Hess CJ et al. High INDO (indoleamine 2,3-dioxygenase) mRNA level in blasts of acute myeloid leukemic patients predicts poor clinical outcome. Haematologica 2008;93:1894-1898.
12. Chamuleau ME, Souwer Y, Van Ham SM et al. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res. 2004;64:5546-5550.
13. Roucard C, Thomas C, Pasquier MA et al. In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. J.Immunol. 2001;167:6849-6858.
14. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat.Med. 2004;10:909-915.
15. Anguille S, Willemen Y, Lion E, Smits EL, Berneman ZN. Dendritic cell vaccination in acute myeloid leukemia. Cytotherapy. 2012;14:647-656.
16. Rasaiyaah J, Noursadeghi M, Kellam P, Chain B. Transcriptional and functional defects of dendritic cells derived from the MUTZ-3 leukaemia line. Immunology 2009;127:429-441.
17. Klammer M, Waterfall M, Samuel K, Turner ML, Roddie PH. Fusion hybrids of dendritic cells and autologous myeloid blasts as a potential cellular vaccine for acute myeloid leukaemia. Br.J.Haematol. 2005;129:340-349.
18. Weigel BJ, Panoskaltsis-Mortari A, Diers M et al. Dendritic cells pulsed or fused with AML cellular antigen provide comparable in vivo antitumor protective responses. Exp.Hematol. 2006;34:1403-1412.
19. Kotera Y, Shimizu K, Mule JJ. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. Cancer Res. 2001;61:8105-8109.
20. Larmonier N, Merino D, Nicolas A et al. Apoptotic, necrotic, or fused tumor cells: an equivalent source of antigen for dendritic cell loading. Apoptosis. 2006;11:1513-1524.
21. Greiner J, Schmitt M, Li L et al. Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. Blood 2006;108:4109-4117.

Nederlandse samenvatting

Introductie

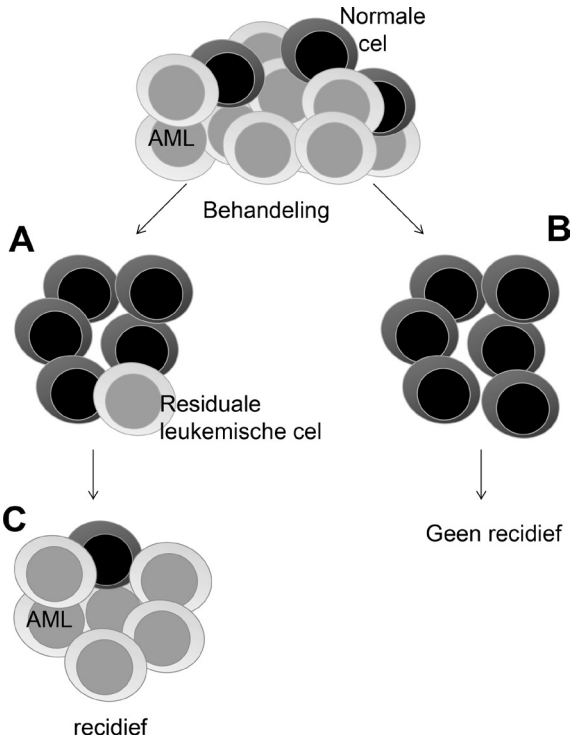
Aan het begin van een nieuw leven is één stamcel verantwoordelijk voor het ontstaan van alle weefsels. Later in het leven dragen weefselspecifieke stamcellen zorg voor het in stand houden van de huid, bloed en inwendige organen. De twee belangrijkste eigenschappen van een stamcel zijn dat zij in staat zijn uit te rijpen naar verschillende gespecialiseerde celtypen en dat zij zichzelf kunnen vernieuwen. Bloedvormende of te wel hematopoëtische stamcellen zijn te vinden in het beenmerg en zorgen ervoor dat er voldoende bloedcellen worden gemaakt. De hematopoëtische stamcel is de voorloper cel voor rode bloedcellen (erytrocyten), de witte bloedcellen (leukocyten, verantwoordelijk voor de afweer) en de



Figuur 1. Schematische weergave van de uitrijping van witte bloedcellen in gezonde toestand (A) en tijdens acute myeloïde leukemie (AML) (B). De hematopoëtische stamcel differentieert tot voorlopercellen die daarna verder uitrijpen tot bloedplaatjes (stolling), witte bloedcellen (afweer) en rode bloedcellen (A). Als er sprake is van acute leukemie dan verdringen de leukemische cellen de normale uitrijping en ontstaat er een tekort aan bloedplaatjes, witte bloedcellen en rode bloedcellen (B).

bloedplaatjes (trombocyten, verantwoordelijk voor de bloedstolling). De witte bloedcellen zijn te verdelen in myeloïde en lymfatische cellen. Een belangrijk verschil tussen de myeloïde en lymfatische afweercellen is dat de cellen van het myeloïde systeem een aspecifieke afweerreactie bewerkstelligen. De myeloïde cellen zijn het grove geschut: zij vallen bacteriën en virusgeïnfecteerde cellen op een aspecifieke manier aan. De afweercellen van de lymfatische reeks zijn verantwoordelijk voor de precieze en specifieke afweer en het ontstaan van een "afweer"geheugen, het principe waarop inenting of te wel vaccinatie is gebaseerd.

Acute myeloïde leukemie (AML) is een maligniteit van de witte bloedcellen waarin een myeloïde voorlopercel transformeert naar een leukemische cel. Deze leukemische cel deelt ongecontroleerd en rijpt niet meer uit naar functionele witte bloedcellen. De leukemische cellen hopen zich op in het beenmerg en belemmeren daarmee uitgroei van gezonde bloedcellen (*figuur 1*). Dit heeft tot gevolg dat er weinig rode bloedcellen (bloedarmoede: anemie) en myeloïde bloedcellen (neutropenie) zullen worden gevormd. Bij neutropenie ontstaat er een grote kans op een infectie. Daarnaast zal een tekort aan bloedplaatjes (trombopenie) leiden tot een verslechterde bloedstelping, zich uitend in blauwe plekken bij gering trauma, gemakkelijk bloedend tandvlees en/of bloedneuzen.



Figuur 2. Recidief en residuale ziekte na behandeling. Residuale leukemische cellen (A) kunnen na behandeling uitgroeien en een recidief van de leukemie veroorzaken (C). Indien alle leukemische cellen worden geëlimineerd na behandeling is een patiënt genezen (B)

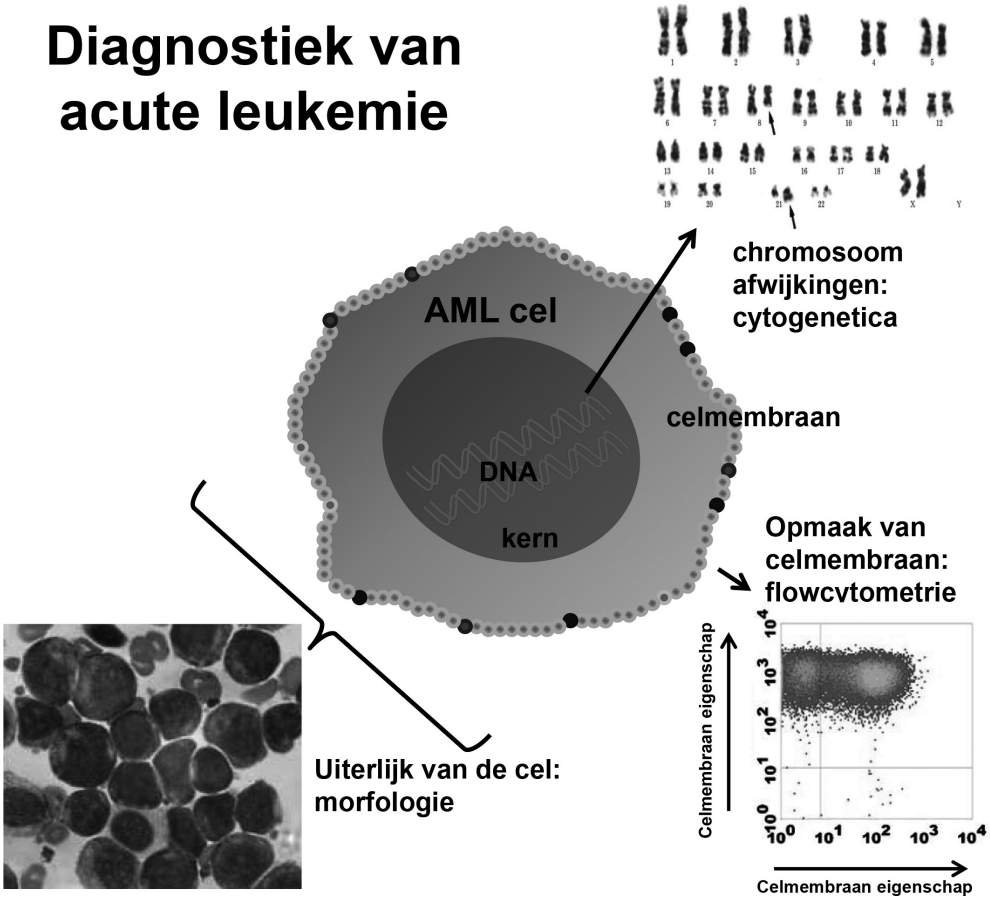
AML is een relatief zeldzame ziekte (2-3 gevallen per 100.000 inwoners per jaar in Nederland). Omdat mensen steeds ouder worden en de ziekte vooral voorkomt op hogere leeftijd, neemt het aantal gevallen van AML per jaar toe. Onbehandeld leidt AML tot de dood in enkele weken tot maanden. Omdat het een agressieve ziekte is, is intensieve behandeling met chemotherapie noodzakelijk. Als na therapie geen cellen meer worden gevonden in het beenmerg (complete remissie) betekent dit niet dat alle leukemiecellen zijn verdwenen. Een zeer kleine hoeveelheid leukemiecellen kan aan de therapie zijn ontsnapt: rest- of ook wel residuale ziekte genoemd. Om te voorkomen dat de kleine hoeveelheid achtergebleven leukemiecellen weer uitgroeit ("recidief", *figuur 2*) is het noodzakelijk dat er een tweede chemotherapiekuur volgt. Zonodig kan dit gevolgd worden door een stamceltransplantatie van een familielid of een niet-verwante donor met (bijna) hetzelfde type witte bloedcellen. Complete remissie wordt bereikt bij 70-80% van de patiënten. Helaas ervaart 30-40% van de patiënten een recidief, ondanks intensieve chemotherapie en stamceltransplantatie.

Met behulp van een betere classificatie en een beter begrip van de leukemische cel kunnen nieuwe therapieën worden ontwikkeld en dit wordt beschreven in dit proefschrift: Immunodiagnostiek en immuunsurveillance in een tijdperk van evoluerende AML behandelingen.
Deel I van dit proefschrift focust op de diagnostiek en classificatie van acute leukemie. *Deel 2* onderzoekt mechanismen welke de leukemische cel gebruikt om het afweersysteem te ontwijken. In *deel 3* worden tot slot nieuwe op de afweer gerichte (immunotherapeutische) behandelingsstrategieën bediscussieerd.

DEEL I: Immuundiagnostiek van AML.

Transformatie van een normale gezonde myeloïde voorloper cel naar een maligne leukemische cel kan gebeuren op elk moment gedurende differentiatie in de myeloïde lijn. Door te kijken naar de cellen onder de microscoop (morfologie) kan worden gezien op welk punt in de uitrijping (differentiatie) naar een rijpe cel de myeloïde cel is getransformeerd naar een leukemische cel. Zo bestaat er bijvoorbeeld een "minimaal gedifferentieerde acute leukemie"; bij deze leukemie is er nog geen kenmerk van uitrijping zichtbaar onder de microscoop. Andere voorbeelden zijn de "acute myeloide leukemie met kenmerken van differentiatie" of de "megakaryoblastaire acute leukemie" waarbij de leukemische cellen kenmerken vertonen van differentiatie of van voorlopercellen van bloedplaatjes. Bepaalde afwijkingen in de chromosomen zijn specifiek voor bepaalde typen leukemie, deze kunnen in kaart worden gebracht door middel van cytogenetisch onderzoek.
Daarnaast kunnen met behulp van flowcytometrie de verschillende eiwitten in de celmembraan worden geanalyseerd, deze techniek wordt veelvuldig toegepast in de diagnostiek van acute leukemie zoals in dit proefschrift is beschreven.

Heterogeniteit van acute leukemie
Er zijn zeer veel verschillende acute leukemiën beschreven die zich onderscheiden op basis van hun morfologische, cytogenetische, moleculaire en flowcytometrische kenmerken;



Figuur 3. Diagnostiek van acute leukemie. Bij het bepalen van het type acute leukemie wordt gekeken naar het uiterlijk van de cel onder de microscoop (morfologie), naar veranderingen in de chromosomen (cytogenetica) en afwijkingen in de opmaak van de celmembraan van de leukemiecel (flowcytometrie).

geen enkele leukemie is daardoor hetzelfde. Voor de verschillende typen acute leukemie zijn verschillende behandelingen noodzakelijk. Een acute leukemie met bepaalde slecht prognostische afwijkingen moet bij voorkeur een intensievere behandeling krijgen inclusief een stamceltransplantatie. Waarschijnlijk zal in de toekomst de behandeling meer worden toegespitst op de individuele patiënt.

Gemengd fenotype

Myeloïde en lymfatische leukemiecellen onderscheiden zich onder andere wat betreft verschillende eiwitten aan de buitenkant (celmembraan) en in de cel (cytoplasma). Deze verschillen kunnen in kaart worden gebracht met behulp van flowcytometrie. Het

onderscheid tussen een myeloïde of een lymfatische leukemiecel is zeer belangrijk omdat het de behandeling en de keuze van chemotherapie bepaalt. Op een acute myeloïde leukemie wordt regelmatig expressie van een lymfatisch membraaneiwit gevonden. In sommige gevallen heeft de leukemische cel evenveel myeloïde als lymfatische kenmerken; het is dan onduidelijk of de leukemie een myeloïde of lymfatische oorsprong kent/heeft; in die gevallen wordt er gesproken van een acute leukemie met een gemengd fenotype.

Beschrijving deel I van dit proefschrift

In *deel I* van dit proefschrift wordt de diagnostiek van leukemische cellen met een gemengd fenotype beschreven. *Hoofdstuk 1* en *2* vormen een inleiding op het gehele proefschrift en op *deel I* van dit proefschrift. Met behulp van een flowcytometer kan van elke afzonderlijk cel worden bekeken welke eiwitten zich op het celmembraan bevinden; dit heet het fenotype van de cel. Daarnaast worden de eiwitten vergeleken die op een leukemische cel zitten met de genetische opmaak van de cel.

In *hoofdstuk 3* worden de casus van twee patiënten beschreven met een bijzondere fenotypische opmaak van de leukemische cellen. De eerste patiënt presenteerde zich in eerste instantie met een acute lymfatische leukemie. Na chemotherapie ontstond er binnen 2 jaar een recidief. De cytogenetica liet dezelfde complexe afwijkingen zien als bij diagnose. Het fenotype was echter totaal veranderd; er was nu sprake van een myeloïde leukemie. De tweede casus laat een volgens de morfologie typisch myeloïde leukemie zien, echter bij flowcytometrische analyse werd een lymfatische opmaak gevonden. Beide casus laten de belangrijke rol van flowcytometrie zien bij de diagnostiek van acute leukemiën.

In *hoofdstuk 4* zijn twee verschillende classificatiesystemen (de WHO2001 en de WHO2008) voor acute leukemiën met een gemengd fenotype met elkaar vergeleken. De aanpassing van het scoringssysteem heeft tot gevolg dat de diagnose van de leukemie (lymfatische, myeloide of gemengd fenotype) zou veranderen bij 30 van de 517 patiënten.

In *hoofdstuk 5* worden de resultaten vergeleken van de morfologische kleuring Sudan black B met de flowcytometrische kleuring voor myeloperoxidase in 198 patiënten. Beide kleuringen tonen de aan- of afwezigheid aan van het eiwit myeloperoxidase in de cel, de resultaten van de kleuring kunnen echter verschillen. Myeloperoxidase is de gouden standaard voor het aantonen van de myeloïde herkomst van de cel en daarom zeer belangrijk in de diagnostiek. De morfologie en de flowcytometrie gebruiken een andere drempel om een leukemie positief te noemen voor dit eiwit. Indien bij 10% van de leukemische cellen MPO positiviteit wordt gevonden met flowcytometrie, dan kan de acute leukemie als myeloperoxidase positief worden beschouwd.

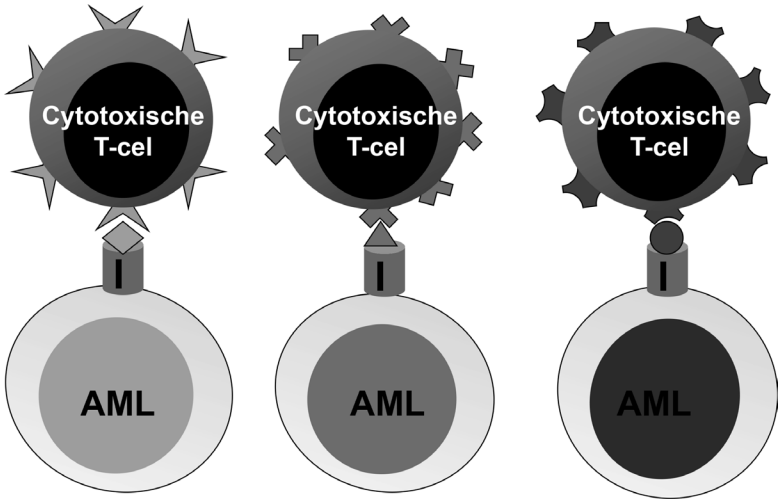
In *hoofdstuk 6* wordt het genetische profiel (door middel van micro-RNA analyse) van 17 patiënten met een acute leukemie met een gemengd fenotype onderzocht. Dit hoofdstuk laat zien dat er ongeacht de fenotypisch gemengde opmaak van de leukemische cellen een duidelijk lymfatische of myeloïde genetische opmaak bestaat. Onderzoek van de genetische opmaak van een acute leukemie met een gemengd fenotype kan op deze manier bijdragen om te bepalen of er sprake is van een myeloïde of lymfatische leukemie. De genetische analyse kan op deze manier helpen in het bepalen van de keuze van het juiste chemotherapieprotocol.

DEEL II: Het immuunsysteem en AML

Het afweersysteem in een notendop

De cellen van het afweersysteem beschermen de mens tegen bacteriën, virussen, parasieten en maligne cellen. Om goed te functioneren moeten de afweercellen onderscheid kunnen maken tussen “lichaamseigen” en “lichaamsvreemd”. Evenals bacteriën en virussen zijn de cellen in het lichaam opgebouwd uit (onder andere) eiwitten; het DNA in de cel bepaalt hoe de eiwitten van de cel eruit zien. De eiwitten waaruit virussen en bacteriën zijn opgebouwd zijn anders (lichaamsvreemd) dan die van de menselijke cel (lichaamseigen). Ook een leukemiecel heeft, door veranderingen in het DNA, eiwitten die in gezonde cellen niet worden gevonden. Deze lichaamsvreemde eiwitcombinaties worden antigenen genoemd en het afweersysteem is in staat om een afweerreactie tegen deze antigenen te initiëren en bacteriën en virussen of maligne cellen op te ruimen.

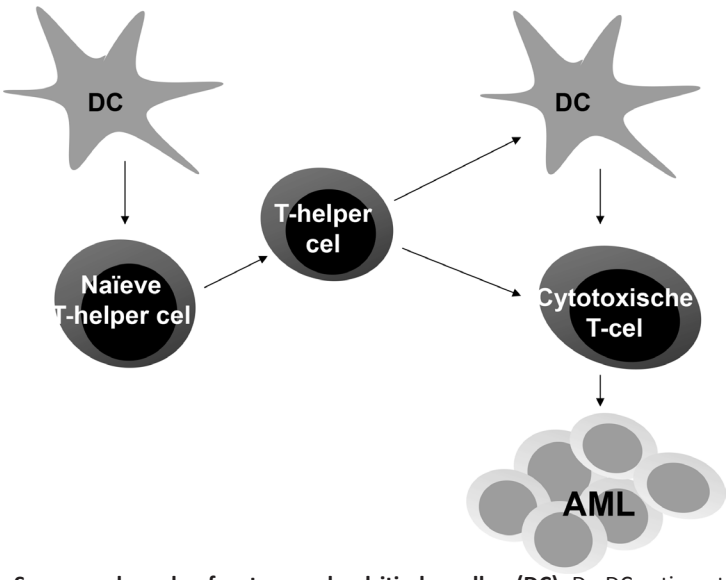
De cellen die verantwoordelijk zijn voor de aangeboren afweer vormen het grove geschut en herkennen “lichaamsvreemde ziekteverwekkers” op een aspecifieke manier. Myeloïde cellen die hiertoe behoren zijn de neutrofiele granulocyten en macrofagen, deze cellen herkennen ziektemakers zoals virussen en bacteriën via speciale receptoren. Deze receptoren herkennen



Figuur 4. Antigeenspecifieke herkenning van AML cellen door leukemie-specifieke cytotoxische T-cellen. AML cellen presenteren verschillende eiwitten in het HLA-klasse I molecuul. Deze gepresenteerde eiwitten vormen een afspiegeling van de eiwitten die in de cel worden gevonden. Een AML cel zal zo leukemie-specifieke eiwitten presenteren die niet worden gevonden in normale cellen. De Cytotoxische T-cel herkent leukemie-specifieke eiwitten, ook wel antigenen genoemd (◊, Δen ●). De antigenen worden gepresenteerd in het HLA-klasse I molecuul (I). De thymus zorgt ervoor dat er alleen T-cellen bestaan die lichaamsvreemde eiwitten herkennen, T-cellen die lichaamseigen eiwitten herkennen worden in de thymus verwijderd.

onderdelen van de virussen of bacteriën die veel voorkomen en zorgen er vervolgens voor dat deze worden opgeruimd.
De cellen van de verworven afweer herkennen "lichaamsvreemd materiaal" op een specifieke manier en zijn verantwoordelijk voor een precisie-aanval. Daarnaast zijn zij verantwoordelijk voor het ontstaan van "geheugen": op het moment dat een patiënt opnieuw te maken krijgt met dezelfde ziekteverwekker zal er direct een efficiënte immuunrespons ontstaan en wordt voorkomen dat een patiënt een tweede keer ziek wordt. Deze afweercellen heten lymfocyten: B-, T- en Natural Killer lymfocyten.
Er zijn meerdere typen T-cellen te onderscheiden waaronder cytotoxische T-cellen en T-helper cellen. Cytotoxische T-cellen zijn in staat om geïnfecteerde cellen en leukemiecellen direct op te ruimen. Hierbij worden ze ondersteund en geactiveerd door T-helper cellen.

De afweerreactie door antigeen-specifieke T-cellen
Voor efficiënte herkenning door de T-cellen zorgen alle cellen in het menselijk lichaam ervoor dat zij een afspiegeling van alle eiwitten in de cel presenteren op de celmembraan aan de T-cellen. Hiertoe worden kleine stukjes van de eiwitten in de cel gepresenteerd op de celmembraan in zogenaamde HLA-klasse I moleculen. Een virusgeïnfecteerde cel zal zo bijvoorbeeld stukjes viruseiwit (antigenen) presenteren op het HLA-klasse I molecuul. Ook leukemiecellen presenteren stukjes leukemie-specifieke antigenen in het HLA-molecuul. T-cellen die dit stukje virus- of leukemie-eiwit herkennen zullen vervolgens de geïnfecteerde cel opruimen.
T-cellen ontstaan uit de oorspronkelijke hematopoëtische stamcel in het beenmerg. Vervolgens verhuizen de jonge, voorloper T-cellen naar de thymus (zwezerik) waar alleen

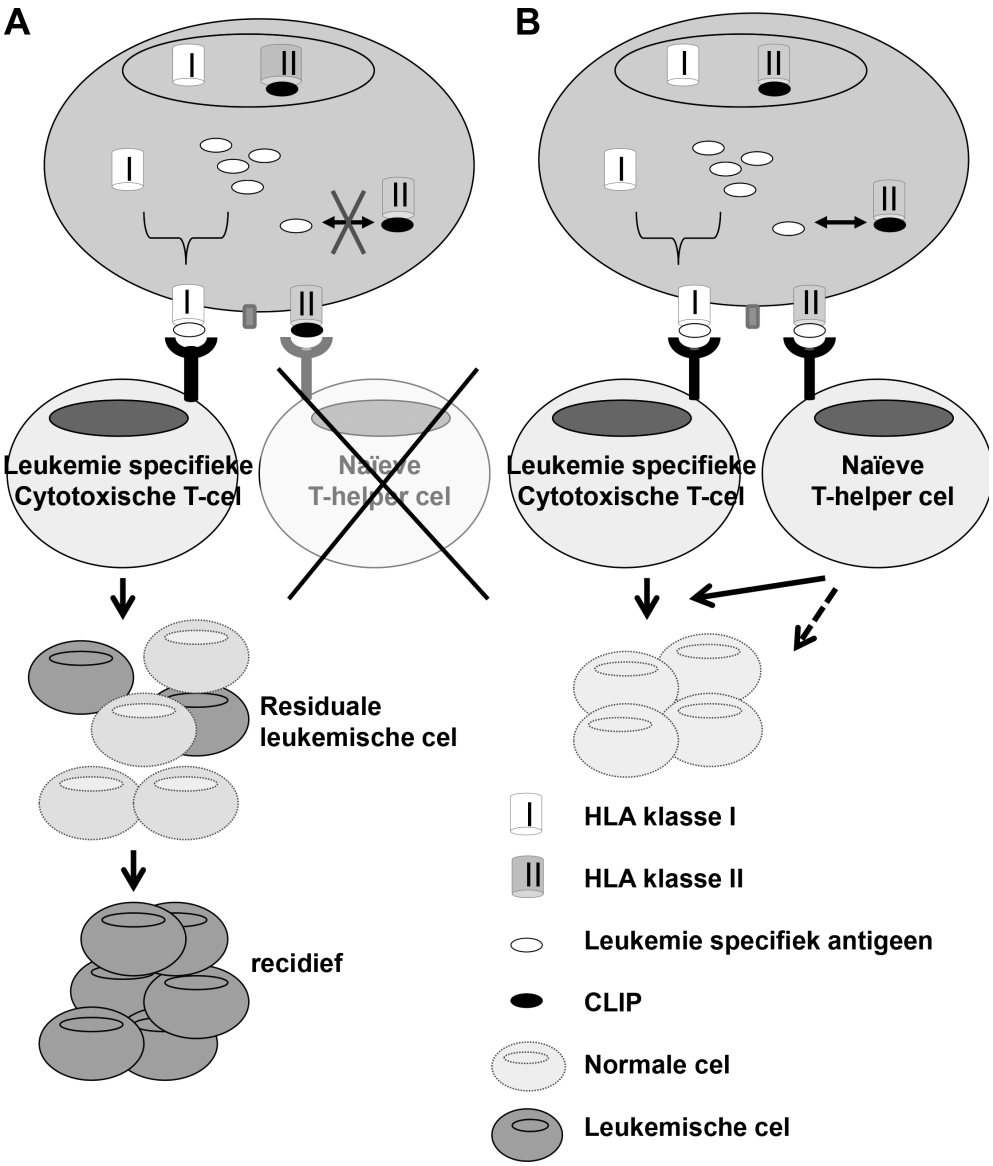


Figuur 5. Samenspel van lymfocyten en dendritische cellen (DC). De DC activeert de T-helper cel en de cytotoxische T-cel. De naïeve T-helper cel groeit uit tot een effector T-helper cel. De T-helper cel activeert vervolgens de DC en helpt bij activatie van de cytotoxische T-cel.

die T-cellen worden geselecteerd die een lichaamsvreemd eiwit herkennen. T-cellen die lichaamseigen eiwitten herkennen worden in de thymus verwijderd en op deze manier wordt er een afweerreactie tegen lichaamseigen eiwitten voorkomen. Elke T-cel die de thymus passeert kan maar één specifiek lichaamsvreemd eiwit herkennen, er zijn miljoenen verschillende T-cellen die elk specifiek zijn voor een ander eiwit (zie *figuur 4*).
Er zijn verschillende leukemie-specifieke eiwitten (leukemie-geassocieerde antigenen, LAA) bekend. Deze stukjes eiwit worden voornamelijk gevonden in leukemische cellen en niet in gezonde cellen.

Activatie van T-cellen door antigeenpresenterende cellen
Bovenstaande suggereert dat ons lichaam in staat is om leukemiecellen te herkennen en op te ruimen. Tumorcellen of geïnfecteerde cellen zijn echter niet goed uitgerust om T-cellen te activeren, hiervoor zijn professionele antigeen presenterende cellen noodzakelijk. Een voorbeeld van een antigeen presenterende cel is de dendritische cel (DC). De DC herkent bacteriën, geïnfecteerde of maligne cellen en neemt vervolgens de hele cel (of stukjes hiervan) in zich op en presenteert de eiwitten in HLA-klasse I moleculen aan T -cellen. Als bijvoorbeeld een leukemie-specifieke T-cel het gepresenteerde antigeen herkent zal deze worden geactiveerd en gaat de T-cel zich delen. Op deze manier ontstaat er een leger aan geactiveerde T-cellen dat maligne cellen herkent en op kan ruimen. Ook worden na activatie geheugen-T-cellen gevormd, deze memory T-cellen zijn in staat om bij een volgend contact met hetzelfde antigeen een efficiënte immuunrespons te bewerkstelligen en zo te voorkomen dat een patiënt opnieuw ziek wordt. Het samenspel tussen de DC en de verschillende T-cellen is weergegeven in *figuur 5.*

Ontsnappen aan het immuunsysteem: CLIP expressie op AML cellen.
Presentatie van antigenen op HLA moleculen is een complex proces waarin vele verschillende moleculen en processen in verschillende compartimenten van de cel zijn betrokken, in *figuur 6* staat dit proces vereenvoudigd weergegeven. Een belangrijk verschil tussen T-helper en cytotoxische T-cellen is dat T-helper cellen antigeen herkennen gepresenteerd in HLA klasse II en cytotoxische T-cellen antigenen gepresenteerd in HLA klasse I. Leukemische cellen proberen op verschillende wijze de antigeenpresentatie op HLA klasse I en II minder optimaal te laten verlopen en daarmee te ontsnappen aan herkenning door T-cellen: “immuunescape”. Een voorbeeld van een dergelijk immuunescape-mechanisme is de expressie van CLIP op leukemische cellen. In het proces van antigeenpresentatie op HLA klasse II wordt normaal gesproken het eiwit CLIP uitgewisseld voor een te presenteren antigeen. Gebeurt dit niet dan zal CLIP aanwezig blijven in het HLA molecuul; dit voorkomt dat de cel wordt herkend door de T-helper cel (*figuur 6A*).
Op gezonde cellen wordt ook CLIP expressie gevonden, hiermee wordt voorkomen dat het immuunsysteem geoveractiveerd wordt en zorgt CLIP expressie voor een evenwichtige immuunrespons. In het geval van leukemie gebruikt de leukemische cel CLIP om te ontsnappen aan het immuunsysteem, er wordt veel CLIP gepresenteerd in plaats van leukemie-specifieke antigenen. Het gevolg is dat T-helper cellen niet worden geactiveerd en er een suboptimale antileukemische immuunrespons plaatsvindt (*figuur 6A*). Indien CLIP wel wordt uitgewisseld voor een antigeen zal de T-helpercel worden geactiveerd en zal er een antileukemische immuunrespons plaats kunnen vinden (*figuur 6B*)



Figuur 6 De rol van CLIP in antigeenpresentatie door antigeenpresenterende cellen. In het proces van antigeenpresentatie op HLA klasse II moet het molecuul CLIP uitgewisseld worden met een antigeen (een eiwit in de cel specifiek voor de leukemische cel, B). Gebeurt dit niet, dan kan de T-helpercel (rood) cel niet worden geactiveerd en kan de cytotoxische T-cel niet optimaal zijn functie uitvoeren (A). Hierdoor zullen AML cellen (groen) niet goed worden herkend.

Beschrijving deel II van dit proefschrift
Presentatie van CLIP door leukemische cellen wordt bediscussieerd in *deel 2* van dit proefschrift. De aanwezigheid van CLIP op een leukemische cel kan bestudeerd worden met behulp van flowcytometrie. *Hoofdstuk 8* laat zien dat de aanwezigheid van CLIP op residuale leukemische cellen een minder gunstige prognose oplevert. In de meeste patiënten is maar een deel van de cellen positief voor CLIP, het andere deel brengt CLIP niet tot expressie; in *hoofdstuk 9* wordt beschreven dat de $CLIP^+$ fractie van leukemische cellen niet in staat is om T-helper cellen te activeren, terwijl $CLIP^-$ cellen van dezelfde patiënt dit wel goed kunnen.

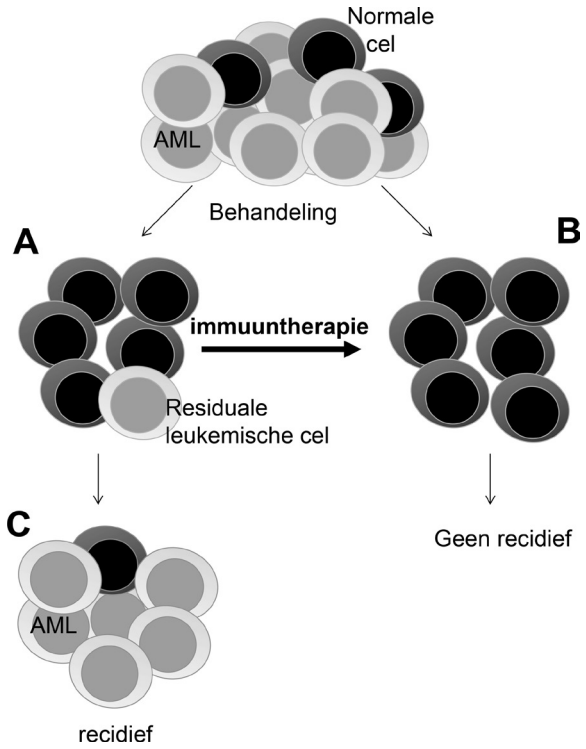
DEEL III: immuuntherapie

Immuuntherapie richt zich op het induceren van een afweerreactie tegen residuale tumorcellen welke ontsnapt zijn aan chemotherapie en later kunnen zorgen voor een recidief van de leukemie (*figuur 7*). *Hoofdstuk 10* vormt de inleiding van *deel III* van het proefschrift.

Immuuntherapie met leukemie-specifieke T-cellen
Door de acute leukemie wordt de uitrijping van gezonde normale bloedcellen verdrongen en is het immuunsysteem minder functioneel. Daarnaast heeft chemotherapie een negatief effect op de gezonde cellen van het immuunsysteem. Immuuntherapie kan bestaan uit het toedienen van leukemie-specifieke T-cellen. De behandeling met leukemie-specifieke T-cellen heeft effect zolang de levensduur van de cellen duurt. Het voordeel is dat de T-cellen hun functie kunnen uitoefenen zonder hulp van andere immuuncellen. *Hoofdstuk 11* laat zien dat leukemie-specifieke T-cellen AML cellen kunnen herkennen en opruimen. Cytotoxische T-cellen die een leukemie-specifiek antigeen herkennen kunnen worden geïsoleerd bij leukemiepatiënten of gekweekt in het laboratorium uit naïeve T-cellen. Een T-celkloon is een verzameling van identieke T-cellen die allen hetzelfde antigeen herkennen in context van het HLA molecuul. Voor het isoleren van de leukemie-specifieke T-cellen wordt in het laboratorium gebruik gemaakt van een flowcytometer. Daarna worden de leukemie-specifieke T-cellen geëxpandeerd in het laboratorium. Deze techniek wordt toegepast in *hoofdstuk 11* van dit proefschrift.

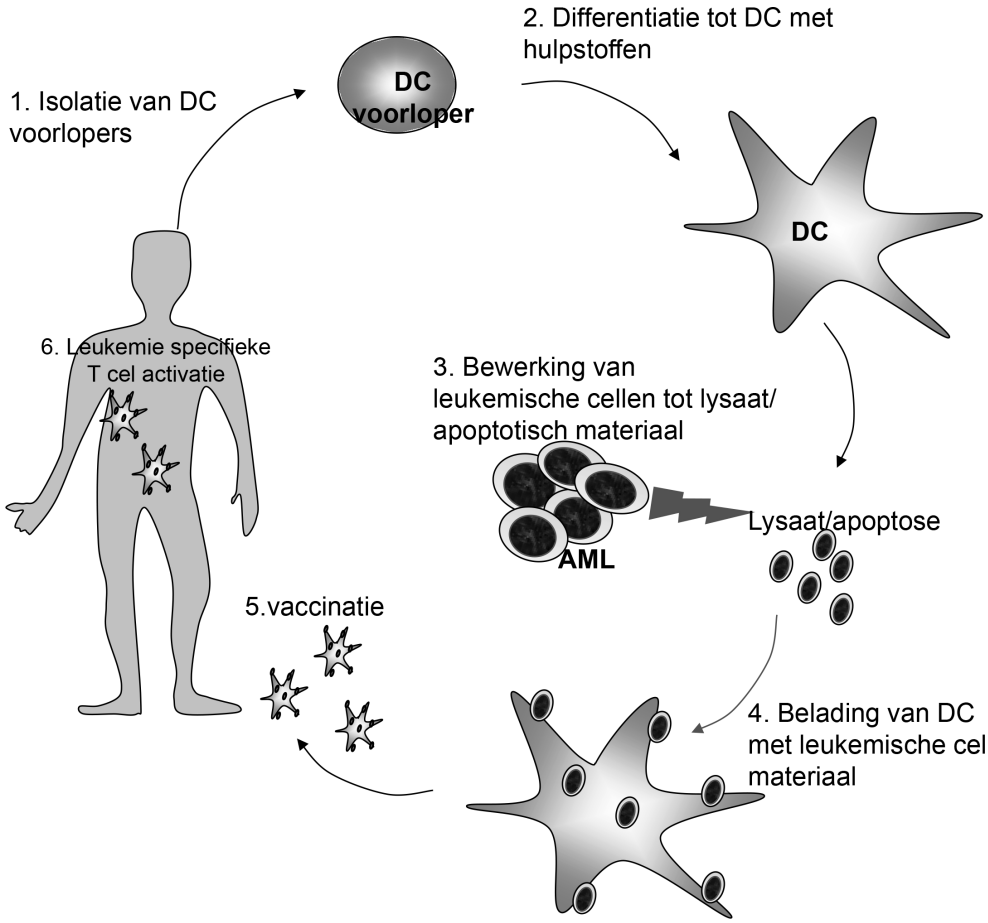
Immuuntherapie met DC vaccins.
Een andere vorm van immuuntherapie is vaccinatie; hierbij wordt geprobeerd om een cytotoxische T-cel- en memory T-celreactie te induceren. Voor vaccinatie is het belangrijk dat alle immuuncellen hun werk goed kunnen doen en dat er een goede T-cel respons kan worden opgewekt in het lichaam van de patiënt. Vaccinatie kan daarom pas worden toegepast als de patiënt in complete remissie is en helemaal is hersteld van de chemotherapie.
Een vorm van vaccinatie bij leukemie zijn DC-vaccins. Deze DC kunnen worden verkregen uit gezonde DC-voorlopers uit het bloed van de patiënt. Een andere optie is om de leukemiecel in het laboratorium te kweken tot een AML-afgeleide DC (AML-DC) met behulp van een cocktail van verschillende hulpstoffen. Een probleem bij het huidige protocol om leukemische DC te maken is dat er vaak te weinig leukemische DC worden verkregen. *Hoofdstuk 12* laat zien dat met een combinatie van verschillende hulpstoffen eerst het aantal leukemische cellen kan worden vergroot en dat de geëxpandeerde cellen vervolgens kunnen worden gekweekt tot een antigeen presenterende cel.

Bij het gebruik van gezonde DC als vaccin moeten de DC worden beladen met leukemie-specifieke antigenen (*figuur 8*). Dit kan door de DC verschillende preparaten aan te bieden met daarin leukemie-specifiek antigeen. Een voorbeeld van een dergelijke werkwijze is het lyseren van leukemische cellen. Lyseren gebeurt door cellen afwisselend op 42˚C en -80˚C te brengen. De cel valt zo uiteen en er blijven kleine fragmenten van de cel over. Hierin zitten normale eiwitten maar ook eiwitten specifiek voor de leukemische cel. In het laboratorium worden de DC en het lysaat samengevoegd en zal de DC het lysaat opnemen en verwerken. Hierna zal de DC de antigenen in het HLA-molecuul kunnen presenteren aan de T-cellen. Het principe van dendritische celvaccinatie staat weergegeven in *figuur 8*.
Het maken van een DC vaccin wordt beschreven in *hoofdstuk 13, 14* en *15* van dit proefschrift. Er worden verschillende strategieën met elkaar vergeleken en deze hoofdstukken laten zien dat lysaten maar ook cellen die in het proces van "dood gaan" zitten, zogenaamde apoptotische cellen, geschikt zijn als antigeenbron voor belading op een DC. In het geheel van apoptotische celmateriaal kunnen ook weer verschillende fracties worden onderscheiden. Hier wordt verder op in gegaan in *hoofdstuk 15*. Daarnaast kunnen er allerlei hulpstoffen worden gebruikt om de belading efficiënter te laten verlopen, in *hoofdstuk 14* worden verschillende van deze cocktails onderzocht. Er zijn zeer veel variaties mogelijk in de bereiding van DC vaccins. In de differentiatie van monocyten



Figuur 7. Recidief en residuale ziekte na behandeling. Residuale leukemische cellen (A) kunnen na behandeling uitgroeien en een recidief van de leukemie veroorzaken (C). Indien alle leukemische cellen worden geëlimineerd na behandeling is een patiënt genezen (B). Immuuntherapie richt zich op eliminatie van residuale leukemische cellen om zo een recidief te voorkomen.

tot DC kunnen verschillende cocktails van hulpstoffen worden gebruikt. En ook in het bereiden van leukemische cellen tot antigeenbron voor belading op DC kunnen zeer veel variaties worden aangebracht. Daarnaast kan tijdens de belading van DC gebruik worden gemaakt van weer andere hulpstoffen om de belading efficiënter te kunnen laten verlopen. In alle wetenschappelijke artikelen die tot nu toe hierover zijn gepubliceerd worden veel verschillende bereidingsstrategieën gebruikt; dit maakt het vergelijken van deze verschillende studies zeer ingewikkeld. Er zijn daarom grotere studies nodig waar deze verschillende strategieën worden vergeleken.



Figuur 8. Het principe van dendritische cel (DC) vaccinatie. Voorloper cellen van de DC worden geïsoleerd uit het bloed. Vervolgens worden deze gekweekt in het laboratorium tot DC. De DC worden beladen met antigeen. Dit kan worden verkregen uit leukemische cellen door het induceren van celdood gereguleerd door de cel zelf (apoptose) of het direct kapot maken van de cel (lyseren). Dit materiaal wordt daarna aangeboden aan de DC, de DC neemt het materiaal op en verwerkt het en presenteert de antigenen op de MHC klasse I en II moleculen. Deze DC kunnen vervolgens teruggegeven worden aan de patiënt: vaccinatie. Nadat het vaccin is toegediend migreren de DC naar de lymfklieren waar zij naïeve T-cellen kunnen activeren.

Conclusie

Bij acute leukemiën is het belangrijk of deze een myeloïde of lymfatische behandeling moeten krijgen; *deel I* van het proefschrift laat zien dat onderzoek naar genetische kenmerken kan helpen in het onderscheiden van de myeloïde of lymfatische oorsprong van een acute leukemie

Naast verbetering van diagnostiek en het in kaart brengen van de verschillende eigenschappen van de cel is het belangrijk om de verschillende eigenschappen van de cel te begrijpen. Het beter begrijpen van verschillende immuunescape mechanismen van de leukemische cellen kan helpen bij het ontwikkelen van nieuwe immuuntherapeutische strategieën. In *deel II* van dit proefschrift wordt beschreven dat leukemische cellen het CLIP-eiwit gebruiken om te ontsnappen aan herkenning door T-cellen. CLIP expressie kan in het laboratorium worden beïnvloed door allerlei hulpstoffen en medicijnen. In *deel III* worden verschillende immuuntherapeutische behandelingsstrategieën besproken. Een probleem bij het ontwikkelen van nieuwe (immuun-) therapeutische strategieën is de heterogeniteit van leukemie, elke leukemie is anders en zelfs binnen één patiënt worden veel variaties gevonden in de leukemische cellen. Verschillende leukemiën hebben daarom verschillende behandelingen nodig. Aan de andere kant zijn er grote homogene patiënten groepen nodig om het effect van verschillende behandelingen met elkaar te kunnen vergelijken. Dit contrast maakt het ontwikkelen van nieuwe therapeutische strategieën ingewikkeld. In de toekomst zal mogelijk met een op maat gemaakt vaccin worden voorkomen dat residuale leukemische cellen na behandeling uitgroeien en een recidief veroorzaken.

Samenvatting

DEEL I: diagnostiek van leukemie

- Leukemie is een ziekte van de witte bloedcellen. Acute myeloïde leukemie (AML) ontstaat uit de myeloïde voorlopercel, acute lymfatische leukemie uit de lymfatische voorlopercel.
- Door leukemie wordt de uitrijping van gezonde cellen verdrongen en ontstaat er een tekort aan witte en rode bloedcellen en bloedplaatjes (*figuur 1*).
- Diagnostiek van AML gebeurt door naar uiterlijke kenmerken te kijken onder de microscoop (morfologie), genetische afwijkingen en fenotypische afwijkingen in de eiwitten in de celmembraan en in de cel (flowcytometrie) (*figuur 3*).
- Met behulp van flowcytometrie kunnen de verschillende eiwitten in en op de cel van elke afzonderlijke cel in kaart worden gebracht. Met behulp van flowcytometrie kan er worden gedifferentieerd tussen myeloïde leukemie en lymfatische leukemie.
- In sommige gevallen heeft de leukemische cel evenveel lymfatische als myeloïde kenmerken, in dat geval spreekt men van een acute leukemie met een gemengd fenotype.
- Elke leukemie is anders en ook binnen één patiënt hebben leukemische cellen verschillende eigenschappen: dit wordt heterogeniteit van acute leukemie genoemd.

DEEL II: Het immuunsysteem en AML

- De cellen van het immuunsysteem beschermen de mens tegen bacteriën, virussen, parasieten en maligne cellen.
- De cellen van het immuunsysteem worden zodanig gevormd dat zij onderscheid kunnen maken tussen lichaamsvreemd (zoals bacteriën, virusgeïnfecteerde en maligne cellen) en lichaamseigen (gezonde cellen) materiaal.
- De immuuncellen kunnen worden verdeeld in de myeloïde en de lymfatische cellen.
- De myeloïde cellen zijn verantwoordelijk voor de aangeboren afweer en herkennen "lichaamsvreemde ziekteverwekkers" op een aspecifieke manier, hiertoe behoren onder andere de macrofagen.
- De lymfatische cellen zijn verantwoordelijk voor de verworven afweer en het herkennen van "lichaamsvreemd materiaal" op een specifieke manier, hiertoe behoren de T-cellen.
- Cytotoxische T-cellen herkennen lichaamsvreemde eiwitten (antigenen) met hun T-cel receptor als stukjes hiervan worden gepresenteerd door een geïnfecteerde cel of tumorcel in het HLA molecuul. Elke cel van het menselijk lichaam laat een afspiegeling zien van alle eiwitten in de cel in het HLA-molecuul op het celmembraan (*figuur 4*).
- T-helper cellen zijn noodzakelijk voor een goede functie van cytotoxische T-cellen (*figuur 5*).
- Leukemie cellen zijn slecht in staat om T-cellen te activeren. Dendritische cellen (DC) daarentegen kunnen leukemiecellen herkennen en de leukemische antigenen efficiënt presenteren aan de T-cellen.
- Expressie van CLIP door leukemische cellen zorgt ervoor dat T-helper cellen niet meer kunnen worden geactiveerd en dat vervolgens de cytotoxische T-celrespons suboptimaal verloopt. Dit is een immuun-escape mechanisme van de leukemische cel (*figuur 6*).

DEEL III: immuuntherapie bij AML.

- Immuuntherapie richt zich op het induceren van een immuunrespons tegen residuale leukemiecellen welke ontsnapt zijn aan chemotherapie en later kunnen zorgen voor een recidief van de leukemie (*figuur 7*)
- Bij actieve immuuntherapie probeert men een T-cel respons te induceren tegen de leukemie cellen, dit kan met behulp van DC vaccinatie.
- Gezonde voorlopers van DC kunnen worden verkregen uit het bloed van een patiënt of donor. Deze cellen kunnen worden gekweekt tot DC in het laboratorium. Vervolgens wordt het vaccin teruggeven aan de patiënt (*figuur 8*)
- De DC moeten daarvoor worden beladen met antigeen: de DC neemt het antigeen op, verwerkt het en presenteert dit op zijn HLA molecuul. Als er een exacte match is met de T-cel receptor van een naïeve T-cel zal de T-cel worden geactiveerd en zal de T-cel uitgroeien tot een leukemie-specifieke T-cel kloon.
- Uiteindelijk kan er zo een persoonlijk vaccin ontwikkeld worden voor de individuele patiënt.