Studies on dendritic cells and ‘leukemia derived dendritic cells’ in the treatment of acute myeloid leukemia and myelodysplastic syndrome

Kumulative Dissertation

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2020

Aus der Medizinischen Klinik und Poliklinik III

Klinikum der Ludwig-Maximilians-Universität München

Vorstand: Prof. Dr. rer. nat. Dr. med. Michael von Bergwelt

Studies on dendritic cells and 'leukemia derived dendritic cells' in the treatment of acute myeloid leukemia and myelodysplastic syndrome

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Christian Stephan Ansprenger

aus
München

Jahr
2021

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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Tag der mündlichen Prüfung: 10.06.2021

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1 Abbreviations

ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
AML	Acute Myeloid Leukemia
BCR/ABL	Breakpoint Cluster Region-Abelson Murine Leukemia Viral Oncogene Homolog 1-Fusion Gene
CAR-T	Chimeric Antigen Receptor-T-Cell-Therapy
CD	Cluster of Differentiation
CIK	Cytokine-Induced Killer Cells
CML	Chronic Myelogenous Leukemia,
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DC	Dendritic Cells
DCleu	Leukemia-Derived Dendritic Cells
DLI	Donor-Lymphocyte Infusion
FLT3	Fms Like Tyrosinekinase 3
FLT3-L	FLT3-Ligand
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice
GvHD	Graft Versus Host Disease
HLA	Human Leukocyte Antigen
hTERT	Human Telomerase Reverse Transcriptase
IFN-γ	Interferon-γ
IL	Interleukin
iNKT	Invariant Natural-Killer T-Cells
KIR-receptor	Killer Cell Immunoglobulin-Like Receptor
LAA	Leukemia-Associated Antigens
mHA	Minor Histocompatibility Antigen
MHC	Major Histocompatibility Complex
MLC	Mixed Lymphocyte Culture
MNC	Mononuclear Cells
mRNA	Messenger Ribonucleic Acid
MUC1	Mucin-1
NK	Natural-Killer Cells
NKG2D	Natural-Killer Group 2, Member D
NKT	Natural-Killer T-Cells

NPM1	Nucleophosmin 1
NY-ESO	New York Esophageal Squamous Cell Carcinoma-1
PD1	Programmed Cell Death Protein 1
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PIND	Paramunity Inducer
PR1	HLA-A2 Nonameric Peptide Derived From Neutrophil Elastase and Proteinase 3
PRAME	Preferentially Expressed Antigen in Melanoma
RNA	Ribonucleic Acid
SCT	Stem Cell Transplantation
TIM-3	T-Cell Immunoglobulin and Mucin Domain-Containing Protein 3
Tcm	Central memory T-Cells
TNF-α	Tumor Necrosis Factor-α
Tnaïve	Naïve T-Cells
UTY	Ubiquitously Transcribed Tetratricopeptide Repeat Containing, Y-Linked
WB	Whole Blood
WT1	Wilms Tumor Suppressor Gene1

2 Publications

2.1 Publications Included in this Thesis

The following publications were summarized for this cumulative medical thesis in accordance with the examination rules of the medical faculty of the LMU Munich:

Publication I:

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.
Anja Liepert, Christine Grabrucker, Andreas Kremser, Julia Dreyßig, **Christian Ansprenger**, Markus Freudenreich, Tanja Kroell, Roland Reibke, Johanna Tischer, Cornelia Schweiger, Christoph Schmid, Hans-Jochem Kolb, Helga Schmetzer
Cell Immunol. 2010;265:23–30. doi:10.1016/j.cellimm.2010.06.009

Publication II:

Profiles of Activation, Differentiation–Markers, or β-Integrins on T Cells Contribute to Predict T Cells' Antileukemic Responses After Stimulation With Leukemia-derived Dendritic Cells
Valentin Vogt, Julia Schick, **Christian Ansprenger**, Marion Braeu, Tanja Kroell, Doris Kraemer, Claus-Henning Köhne, Andreas Hausmann, Raymund Buhmann, Johanna Tischer, Helga Schmetzer
J Immunother. 2014;37:331–47. doi:10.1097/CJI.0000000000000037.

Publication III:

Paramunity-inducing Factors (PINDs) in dendritic cell (DC) cultures lead to impaired antileukemic functionality of DC-stimulated T-cells.
Christian Ansprenger, Valentin Vogt, Julia Schick, Annika Hirn-Lopez, Yvonne Vokac, Ihor Harabacz, Marion Braeu, Tanja Kroell, Axel Karenberg, Hans-Jochem Kolb, Helga Schmetzer
Cell Immunol 2018. doi:10.1016/j.cellimm.2018.03.005.

2.2 Other original publications

C. Ansprenger, D. C. Amberger, H. Schmetzer

Potential of immunotherapies in the mediation of antileukemic responses for patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) - with a focus on Dendritic cells of leukemic origin (DCleu)

Clin Immunol. 2020 May 26;217:108467. doi: 10.1016/j.clim.2020.108467

A. Hirn Lopez, D. Deen, Z. Fischer, A. Rabe, **C. Ansprenger**, K. Stein, V. Vogt, J. Schick, T. Kroell, D. Kraemer, H.J. Kolb, J. Tischer, C. Schmid, H. Schmetzer

Role of Interferon (IFN)α in “Cocktails” for the Generation of (Leukemia-derived) Dendritic Cells (DCleu) From Blasts in Blood From Patients (pts) With Acute Myeloid Leukemia (AML) and the Induction of Antileukemic Reactions
J. Immunotherapy, 42, 5, 143-161 (2019)

C. Ansprenger, E. Burri
Diagnose und Monitoring bei chronisch-entzündlicher Darmerkrankung
Ther Umsch. 2019 Jan;75(5):316-328. doi: 10.1024/0040-5930/a001002

C. Ansprenger, J. Alder, A. Bergerhoff, H. Kummer, F. Meyer zu Bentrup
Case-Report: Anilin-Intoxikation und Hypothermie, Kälteschutz, der wirkt
Swiss Medical Forum 2016; 24: 526-528

2.3 Poster Presentation

C. Ansprenger, J. Schick, V. Vogt, M. Zerwes, T. Kroell, H.J. Kolb, B. Mayr, A.,ayr, H.M. Schmetzer
Paramunity-inducing factors have the capacity to improve DC-maturity, proportions of DCleu and early T-cell proliferation, but impair ex-vivo antileukemic functionality and therefore require thorough analyses before application in man.
Bone Marrow Transplantation 47, suppl. 1, abstract 883, (2012)

3 Co-Authors’ confirmation

All co-authors signed a confirmation document, that Christian Ansprenger has the permission to use the publications for his medical thesis. Furthermore, they confirmed that the publications are not part of another doctoral theses.

The Documents were submitted separately with this thesis.

4 Introduction

4.1 Acute myeloid leukemia

Acute myeloid leukemia is the most common acute leukemia in adults. The term encompasses a group of heterogeneous aggressive leukemic cancers that develop from and are upheld by leukemic stem cells. Further mutational events in cells developed from leukemic stem cells and their clonal expansion lead to the genetic/molecular heterogenicity found in the blast-population at diagnosis. Malignantly transformed blasts are characterized by expression of myeloid and leukemia-associated antigens (e.g., WT1, PR1, PRAME). Accumulation of clonally expanded blasts replace physiological hematopoiesis and thereby cause the typical symptoms, e.g. anemia, bleeding, infections. Some cases present with extramedullary disease, such as affection of the central nervous system. Typically symptoms develop rapidly and may be fatal within weeks to months, if left untreated. Fast diagnosis and therapy are therefore imperative, especially if complications like disseminated intravascular coagulation or tumor lysis syndrome are present. Prognosis depends on clinical features (e.g. age, comorbidities, prior MDS) as well as certain cytogenetic and/or molecular features. The latter can be stratified according to the 2017 European Leukemia Net risk stratification as being favorable, intermediate or adverse [1–5].

4.2 Standard Treatment

If the Patient is deemed fit for intensive chemotherapy, complete remission is set as the initial goal in most cases. It is defined as no evidence of blasts in the peripheral blood and detection of less than 5% blasts in the bone marrow in combination with a recovery of peripheral blood parameters. Complete remission is associated with longer overall survival as well as improved quality of life. Induction chemotherapy is performed with an anthracycline plus cytarabine-based regimen, possibly augmented by an FLT3 inhibitor or gemtuzumab ozogamicin, where suitable (FLT3 mutation, $CD33^{+}$). Cytarabine and daunorubicin (CPX-351) is preferred in therapy-related AML or AML with MDS-related changes. Depending on individual patients' risk of relapse, risk of HSCT itself as well as personal preferences and availability of a suitable donor-source, an intermediate-dose chemotherapy or allogeneic HSCT is chosen as consolidation-therapy. Depending on prognostic subtypes, up to 80% of AML patients who have had successful chemotherapy relapse within the next two

years. Allogeneic SCT is the only treatment modality, that can achieve permanent cure. It also offers the best chance of cure for (most) patients, who fail to achieve primary induction and for those, who relapse after standard therapy [2, 3, 6].

Unfortunately, SCT is a very onerous treatment and therefore less suitable for older patients or patients with significant comorbidities and/or high morbidity. Relapses may occur even after SCT, the risk of which depends on leukemic risk profile, stage of disease or preceded therapies at time of transplantation and installation of remission after SCT. Moreover, graft versus host disease (GvHD) often occurs [7–9].

4.3 New AML Therapies

A large number of new treatment strategies with novel and diverse compounds are currently under development: antileukemic protein kinase inhibitors, therapies targeting specific oncogenic proteins, new cytotoxic agents, epigenetic modulators, mitochondrial inhibitors including apoptosis therapies, therapeutic and immune checkpoint antibodies and cellular immuno-therapies, therapies targeting the AML microenvironment or strategies based on dendritic cells (DC) presenting leukemic antigens [1, 10, 11]. In general, many of these strategies are plagued by various problems such as low tolerance and/or adverse events after drug administration, subpar efficiency or blast-clearance (of all known subtypes) in blood, bone marrow or extramedullary sites, and poor generation and/or targeting of functional immune-reactive cells and molecules [5, 8].

Some of the most promising strategies are aimed at various leukemia-specific associated antigens (LAA), which are coded for by mutational events in the leukemic clone, as ‘immunological targets’ to elicit humoral or cellular reactions against them. Ideal antigens for these approaches are expressed evenly, are of central functional significance, are highly immunogenic and are malignancy-specific in order to avoid toxicity. Several single LAAs are being studied (e.g. WT1, NPM1, PRAME). Moreover, several minor histocompatibility antigens (mHA) have been analyzed for their potential to initiate leukemia-specific responses [12–14]. Exemplarily, CAR (Chimeric Antigen Receptor)-T-cell-therapy utilizes T-cells, which are genetically engineered to express antigen-receptors against a specific protein (e.g. CD33, CD123, FLT3, NKG2D). Its efficacy - at least for some CAR-T-subtypes - was recently shown in AML:

promising leukemia-cytotoxicity was demonstrated against cell lines and myeloid blasts of refractory/relapsed patients before or after SCT, although with varying results. High costs, the risk of (life threatening) adverse events, e.g. cytokine release syndrome or toxicities against alternate targets, and the often unknown role of the addressed antigen in the pathogenesis of AML, are some of the major disadvantages [13].

Clinical data shows an association between improved outcome and an antitumor-response comprised of both, innate natural-killer (NK, iNKT)- and adaptive T-cells. Therefore, strategies additionally utilizing the innate immune system have come into focus. These employ non-specific CIK-, NK-, or ɣδ-T-cells, mediated by and involved in the mediation of cytokine-, antibody- (ADCC), granzyme-, perforin- or (in case of NK-cells) KIR-receptor-mediated effects [13]. Fortunately there is no evidence, that links human GvHD to NK cell infusions [8]. Advantages of innate-cell-based strategies include their potential migration to ‚tumor-sites‘ and mediation of fast and effective HLA unrestricted responses against AML. The main disadvantage lies in the unspecific approach, which hinders the ability to sufficiently differentiate non-specific from specific effects and/or side effects. Additionally these cells have to be produced and expanded on a large scale ex vivo.

Antibody-based approaches in AML have been initiated and are currently being tested. Strategies include checkpoint-inhibitors, that disconnect the binding of inhibitory receptors (e.g. PD1, CTLA-4,TIM-3) to their ligands ("taking off the brakes"). Other antibody-based approaches (conjugated or not conjugated with drugs) against AML-targets address certain lineage-restricted or aberrantly expressed antigens in AML-subsets (e.g. WT1, CD33, NY-ESO, MUC1, FLT3). Bispecific antibodies can facilitate an interconnection of leukemic and immunoreactive cells (e.g. CD3) [11–13, 15]. First results of ongoing treatments using checkpoint-inhibitors and (mono- and bispecific) antibodies against lineage-restricted antigens show promising tolerability and efficacy (stabilization of disease, hematological improvements and partial responses), although overall comparative results are still pending. Main disadvantages include, that the inhibitory role of antigen expressions (checkpoint-, lineage-, leukemia-associated markers) in the pathogenesis as well as the treatment outcome of single patients and their effects as monotherapies are unknown [5].

Additionally, antibody-therapies and transfers of immunoreactive cells are passive immunotherapies. They do not lead to the creation of an immunological memory.

Cancer vaccines have been developed to discontinue tumor cell induced immunoescape and to (specifically) activate and expand anti-tumor immunoreactive cells. In myeloid malignancies peptide-based vaccinations have been performed using leukemia-associated antigens (WT1, PR1 or BCR/ABL in CML). In general, responses against single LAAs are known to be weak, whereas responses against mHA (e.g. UTY) appear to be stronger [8, 16, 17]. These vaccinations appear to be well tolerated and they lead to the induction of LAA specific T-cells. Clinically, immune responses were variable, but prolonged remission was achieved in some cases [12].

Despite the plethora of promising approaches, outcomes for AML-patients remain unsatisfactory: more than 50% ultimately die from AML-related causes [3].

4.4 Dendritic Cell-Based Therapy

DCs function as a vital link between the innate and adaptive immune system. Since the turn of the millennium numerous treatment-strategies have been developed, that employ DCs' properties as professional antigen presenting cells as well as their capability as key regulators of antigen-specific immune responses, stimulating - amongst others - tumor cytotoxic T-cells. DC can be generated ex vivo from precursor cells and loaded with tumor-antigens. Common techniques are LAA- or telomerase reverse transcriptase (hTERT) protein-pulsing, mRNA transfection or fusion with AML-cells [11, 12]. Promising results were shown for patients (intradermally or subcutaneously) vaccinated with such manipulated DC: (leukemia-specific) immune responses could be induced and residual blasts were reduced [18]. Data from our group has shown antileukemically active $CD4^+$ T-cells after stimulation with LAA/mHA-presented through antigen-presenting cells. This finding highlights the central role of $CD4^+$ as well as $CD8^+$ cells in the mediation of antileukemic reactions [19]. The induction of an immunological memory can be postulated due to the 'DC-concept'. The disadvantage of this concept is, that although the ex vivo generation and creation of manipulated DCs is possible, the process is time-consuming and has to be performed under GMP(good manufacturing practice)-conditions. Additionally the resulting "products" must undergo strict quality-testing

(e.g. for infectious contaminations). Moreover, resulting cell products are limited in quantity.

4.5 DCleu Based Therapy

It has been demonstrated by others and us, that T-cells' immunological restraint in the presence of leukemic blasts can be (more elegantly) overcome by differentiating blasts to DC (DC of leukemic origin, DCleu). These antigen presenting cells thereby display the individual patient's entire leukemic antigen repertoire, independent of age, MHC-, mutational- or transplantation-status [5].

The superior response of myeloid compared to lymphoid leukemias to SCT and/or DLI led to the hypothesis of an spontaneous conversion of myeloid blasts to 'leukemia-derived DC' (DCleu) in vivo, thereby presenting the patients' individual LAA (e.g. WT1, PR1, PRAME, NPM1 or BCR/ABL translocation in CML) and/or other immunological targets such as minor histocompatibility antigens (e.g. UTY) together with MHC molecules in conjunction with (induced) co-stimulatory receptors (e.g. CD80,CD86), resulting in (specific) activation of effector T-cells [8, 17, 20, 21]. Additionally, our data has shown, that iNK-T-cells - members of the innate immune system - might be involved in the mediation of DC/DCleu-triggered antileukemic activity [22].

The advantage of a 'DCleu'-based concept is, that those DC present the whole antigenic repertoire of the individual patient. Moreover DCleu can not only (in analogy to DC) be generated ex vivo and be used to vaccinate patients, but possibly be induced/ produced from patients' blasts in vivo: combinations of substances stimulating hematopoietic cells as well as inducing DC differentiation and DC maturation, can be used to facilitate blasts' conversion to DCleu (ex vivo or in vivo). Subsequently, the activation of the immune system as well as the installation of an immunological memory in vivo are induced.

4.6 Publications Included in this Thesis and contributions

The central theme of the submitted publications is the ex-vivo generation of DC/DCleu with various methods and the analysis of the resulting functionality in mixed lymphocyte culture.

4.6.1 Publication I, Liepert et al. 2010

Title: *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.*

Authors: Liepert A, Grabrucker C, Kremser A, Dreyssig J, Ansprenger C, Freudenreich M, Kroell T, Reibke R, Tischer J, Schweiger C, Schmid C, Kolb HJ, Schmetzer H.

Journal: Cell Immunol. 2010;265(1):23-30. doi: 10.1016/j.cellimm.2010.06.009. Epub 2010 Jun 20.

In this project DC/DCleu were generated ex vivo with various standard methods: 1. ‘MCM-Mimic’ (GM-CSF, IL-4, TNFa, FL, IL-1ß, IL6, PGE2) is a cytokine based DC-differentiation method, PGE2 increases CCR7-expression and improves migration. 2. In ‘Picibanil’ (GM-CSF, IL-4, TNFa, lysat from Streptococcus pyogenes, PGE2) bacterial lysat and PGE2 stimulate DC differentiation. 3. ‘Ca-ionophore’ (IL-4, A23187) is a bypass of cytokine-driven DC differentiation. Generated DC/DCleu were co-cultured with patients’ thawn T-cells (autologous, allogenic or after SCT) in MLC. Then the T-cells’ composition after culture was analyzed by flow cytometry. In a final step a cytotoxicity assay was performed. It was shown, that DCs’ composition and quality after culture and T-cells’ after MLC is predictive for their leukemia cytotoxic potential ex- and in-vivo

Contribution: Christian Ansprenger contributed to data analysis as well as statistical work and its representation.

4.6.2 Publication II, Vogt et al. 2014

Title: *Profiles of activation-, differentiation markers or ß-integrines on T-cells contribute to predict T-cells’ antileukemic responses after stimulation with leukemia-derived dendritic cells*

Authors: Vogt V, Schick J, Ansprenger C, Braeu M, Kroell T, Kraemer D, Köhne CH, Hausmann A, Buhmann R, Tischer J, Schmetzer H.

Journal: J Immunother. 2014 Jul-Aug;37(6):331-47. doi: 10.1097/CJI.0000000000000037.

In this project DC/DCleu were generated ex vivo with various standard methods, the generated DC/DCleu were co-cultured with patients' thawn T-cells (autologous, allogenic or after SCT) in MLC in analogy to publication I. The T-cells' composition was analyzed by flow cytometry at various time points during and after culture. A cytotoxicity assay was performed in order to assess antileukemic functionality. We were able to show, that T-cell stimulation with DC/DCleu leads to a shift in T-cell composition from naïve to non-naïve T-cells, DC contact antigens are up-regulated and ß-integrin expressing T-cells are induced. The increase of the latter correlated with an increase in antileukemic functionality. This could potentially help to predict the reactivity of T-cells during stimulation.

Contribution: Christian Ansprenger performed parts of the experiments, contributed to data acquisition and analysis as well as statistical work.

4.6.3 Publication III, Ansprenger et al. 2018

Title: *Paramunity-inducing Factors (PINDs) in dendritic cell (DC) cultures lead to impaired antileukemic functionality of DC-stimulated T-cells.*

Authors: Ansprenger C, Vogt V, Schick J, Hirn-Lopez A, Vokac Y, Harabacz I, Braeu M, Kroell T, Karenberg A, Kolb HJ, Schmetzer H.

Journal: Cell Immunol. 2018 Jun;328:33-48. doi: 10.1016/j.cellimm.2018.03.005. Epub 2018 Mar 16.

In this project DC/DCleu were generated ex vivo with various standard methods with and without the addition of different PINDs. Stimulator cells (DC/DCleu generated with or without PINDs as well as MNC) were co-cultured with effector cells (T-cells or MNC) in MLC with and without the (repeated) addition of PINDs (Zylexis). T-cell-composition was analyzed by flow cytometry and a cytotoxicity assay was performed. We were able to demonstrate, that addition of PINDS to DC culture and MLC changes the effects of DC/DCleu on effector cells in MLC: PIND-DC/DCleu lead to negative effects on T-cell composition, cytotoxicity and cytokine profile. Consequently, antileukemic trials involving PINDs cannot be recommended for the treatment of AML-patients.

Contribution: Christian Ansprenger performed the majority of experiments, data acquisition, interpretation and analysis including all statistical work and drafted the manuscript together with Prof. Schmetzer.

4.6.4 Conclusion

We were able to prove that DC/DCleu can be generated ex-vivo from AML-patients' mononuclear cells with at least one of various methods in any given case. 'Stimulator fractions' containing these DC/DCleu are able to (LAA-specifically) activate T-cells – as well as possibly cells of the innate immune-system - against leukemic cells ex vivo. We were able to demonstrate a (favorable) shift in T-cell composition and antileukemic functionality. However, this is variable. Our experiments with the addition of PINDs showed altered T-cell and DC composition as well as an altered microenvironment, resulting in a "blast protective capacity". This confirms the need for and the importance of a thorough analysis before therapeutic use: preliminary ex vivo testing is indispensable to assess which kind cells and which kind of reaction are induced.

5 Summary/Zusammenfassung

5.1 Summary

The publications included in this thesis are part of the development of a DC/DCleu-based treatment strategy for patients with (myeloid) leukemia that is applicable independent of age, MHC-, mutational- or transplantational status, that addresses every (residual) blast in a culture dish or in the body. It is based on the conversion of blasts to leukemia-derived DC, which retain the whole (leukemic) antigenic repertoire of the individual patient.

Our group was able to show, that suitable DCleu could be generated from at least one DC/DCleu-generating method (containing combinations of immune-modulatory/ danger-signaling factors: GM-CSF, IL-4, TNF-α, FLT3-L, IL-1β, IL-6, PGE2, PGE1, bacterial lysate of streptococcus pyogenes (PICIBANIL), or calcium ionophore) in every given patient. The generated DC/DCleu (or other cells, depending on the experiment) are then incubated as stimulator cells with effector cells (T-cells or MNC) in MLC and subsequently tested for antileukemic functionality (functional cytotoxicity assay) [23–25].

In publications 1 and 2 [26, 27] we further elucidated the composition and behavior of several subsets of T-cells in the context of stimulation by DC/DCleu. T-cells are central mediators of anti-leukemic functionality, for which $CD4^+$ as well as cytotoxic $CD8^+$ T-cells are key players. Antitumor T-cell anergy can be regularly reverted to anti-leukemic activity by stimulation with DC/DCleu [8, 19].

Activated T-cells and their clones can be selected and expanded [25, 28]. The Quality of anti-leukemic reactivity depends on and is predicted by DCs' and T-cells' composition and quality after MLC. Especially $CD4^+$ and $CD45RO^+$ T-cells were predictive of anti-leukemic activity after DC-stimulation. We were able to define cut-off-values predictive for successful ex vivo T-cell activity as well as for response to an immunotherapy. Successful blast lysis correlated with higher frequencies of (mature, leukemia derived) DC and higher proportions of proliferating, $CD4^+$ or $CD8^+$, non-naïve cells after MLC. Antileukemic activity increased especially after stimulation with DC and correlated with higher proportions of Tcm and Tnaive before stimulation, and with significantly higher proportions of activated and β-integrin expressing T-cells [27].

Publication 3: In an attempt to optimize the process and augment DC/DCleu antigen-presenting capabilities, to enhance the composition and function of DC-stimulated antileukemic T-cells and enable functioning of cells of the innate immune system, we investigated Paramunity-inducing-Factors (PINDs) as co-stimulants. They “consist of attenuated/inactivated viruses of various poxvirus-genera and are used in veterinary medicine as non-antigen-specific, non-immunizing stimulators of the innate immune system against infectious and malignant diseases” [23]. Their efficacy is hypothesized to rely on danger-signaling-interactions to activate and regulate the “paraspecific”, i.e. innate/ unspecific immune system against external (e.g. bacteria) or internal (tumorous cells) noxa. Addition of PINDs to DC-cultures resulted in an (positive) increase of mature DC and DCleu. However, it reduced the fractions of viable as well as $TLR4^+$ and $TLR9^+$ DCs. We observed increased early ($CD8^+$) T-cell activation ($CD69^+$), tough proportions of effector-T-cells after MLC were reduced. T-cells' and innate cells' antileukemic functionality was diminished in the presence of PINDs in DC- and MLC-cultures. Fittingly, PIND-containing DC- and MLC-culture-supernatants resembled an inhibitory microenvironment (decreased IFN-γ, TNF-α and IL-2, increased IL10). This led us to believe, PINDs might induce some kind of “blast-protective effect”. Therefore we concluded, that PINDs had no place in antileukemic trials [23].

In conclusion we were able to show, that DC/DCleu can (leukemia antigen-specifically) activate T-cells and potentially cells of the innate immune-system and thereby induce antileukemic functionality ex vivo. However, not all DC-generating methods result in such an activation. Therefore a thorough analysis before therapeutic use is of the utmost importance. Even more so, as the future focus will lie on the adaption of this ex vivo strategy to an in vivo application: antileukemic potential/functionality has to be evaluated and tolerogenic DC or other harmful mechanisms have to be excluded before clinical adoption.

Compared to an adoptive transfer strategy, it would be easier and more elegant to circumvent complicated, expensive and logistically demanding ex vivo procedures, by activating anti-leukemic immunoreactive cells through drugs that initiate blast-differentiation to DCleu in patients directly. In order to achieve this, we selected certain drug combinations (“Kits”), containing GM-CSF (as myelopoesis-inducing factor) in combination with one or two DC-maturing and

danger-signalling factors (TNFα, IFNα, PGE1, PGE2, Picibanil, Calciumionophore), that produce DC/DCleu without inducing blast proliferation (Helga Schmetzer, German Patent: 10 2014 014 993, US and Europ. Patent Application). The next step is to test these kits in whole blood in order to simulate in vivo conditions.

5.2 Zusammenfassung

Die in dieser Dissertation enthaltenen Publikationen sind Teil der Entwicklung einer DC/DCleu-basierten Therapie für Patienten mit (myeloischer) Leukämie, unabhängig von Alter, MHC-, Mutations- oder Transplantations-status, basierend auf der Umwandlung von Blasten zu DC leukämischen Ursprungs, die das gesamte (leukämische) Antigen-Repertoire des individuellen Patienten aufweisen. Sie richtet sich gegen alle (residuellen) Blasten - ex-vivo und in-vivo.

Unserer Arbeitsgruppe ist es gelungen zu zeigen, dass bei jedem einzelnen Patienten geeignete DCleu aus mindestens einer der DC/DCleu-Generierungsmethoden (Kombinationen aus Immunmodulatorischen/ “Danger-signaling“ Faktoren: GM-CSF, IL-4, TNF-α, FLT3-L, IL-1β, IL-6, PGE2, PGE1, bakterielles Lysat von Streptococcus pyogenes (PICIBANIL), oder Kalziumionophor) gewonnen werden konnten. Die generierten DC/DCleu (oder, je nach Experiment auch andere Zellen) werden im Anschluss als Stimulator-Zellen mit Effektorzellen (T-zellen oder MNC) in einer gemischten Lymphozyten Kultur (MLC) inkubiert und schließlich mittels funktioneller Zytotoxizitätsprüfung auf ihre antileukämischen Eigenschaften getestet [23–25].
In den Publikationen 1 und 2 [26, 27] haben wir die Zusammensetzung und die Veränderungen in verschiedenen T-Zell-Subgruppen nach Stimulation durch DC/DCleu analysiert. T-Zellen sind die Hauptmediatoren antileukämischer Reaktionen - $CD4^+$ und zytotoxische $CD8^+$ T-Zellen nehmen dabei Schlüsselpositionen ein [8, 19]. T-Zell Anergie kann regelhaft durch Stimulation mit DC/DCleu in antileukämische Aktivität umgewandelt werden: aktivierte T-Zellen und ihre Klone können selektiert und gezielt vermehrt werden [25, 28]. Die Qualität der antileukämischen Reaktion hängt von der Komposition und Qualität von DC und T-Zellen nach der MLC ab und lässt sich anhand dieser auch prognostizieren. Vor allem $CD4^+$ und $CD45RO^+$ T-Zellen waren bezüglich einer antileukämischen Funktionalität nach DC-Stimulation prädiktiv. Wir konnten prädiktive Grenzwerte für antileukämische Funktionalität ex vivo definieren,

welche auch prognostisch bezüglich des Ansprechens auf Immunotherapie waren. Eine erfolgreiche Blastenlyse korrelierte mit höheren Anteilen von (reifen, leukämie-stämmigen) DC und höheren Anteilen proliferierender, $CD4^{+}$oder $DC8^{+}$, nicht-naiver Zellen nach MLC. Gesteigerte antileukämische Aktivität war vor allem nach DC-Stimulation zu beobachten und korrelierte mit höheren Anteilen von Tcm und Tnaive vor Stimulation, sowie mit signifikant höheren Anteilen aktivierter und β-integrin exprimierender T Zellen [27].

Publikation 3: Um die DC-Generierung und das DC/DCleu Antigen-Präsentationspotential zu optimieren, die Komposition und Funktion der DC-stimulierten antileukämsichen T-Zellen zu verbessern und um Zellen des unspezifischen Immunsystemes zu aktivieren haben wir Paramunitäts-induzierende Faktoren (PINDs) als Ko-Stimulanz untersucht (Publikation 3, [23]). PINDs bestehen aus attenuierten/inaktivierten Vieren verschiedener Poxvirus-Genera und werden in der Veterinärmedizin als nicht-Antigen-spezifische, nicht-immunisierende Stimulatoren des unspezifischen/ angeborenen Immunsystems in der Behandlung infektiöser und maligner Krankheiten eingesetzt. Man vermutet, dass die Wirkung auf Dangersignaling-Interaktionen beruhende Aktivation und Regulation des paraspezifischen, d.h. des angeborenen und unspezifischen Immunsystems in Bezug auf externe (z.B. Bakterien) und interne (z.B. maligne Zellen) Noxen zurückzuführen ist. Ihre Zugabe zum DC-Kulturmedium führte zu einem (erwünschten) Anstieg reifer DC und DCleu, reduzierte aber die absoluten Zahlen und den Anteil lebender sowie TLR4- und TLR9-exprimierender Zellen. Die Zugabe führte zu vermehrter früher ($CD8^{+}$)T-Zell Aktivierung ($CD69^{+}$), reduzierte aber den Anteil an Effektor-T-Zellen nach MLC. PINDs in DC- und MLC-Kulturmedien reduzierten die antileukämischen Eigenschaften von T-Zellen und Effektoren des angeborenen Immunsystems. Passend hierzu wiesen die Überstände der Kulturmedien ein inhibitorisches Mikroenvironment auf (reduziertes IFN-γ, TNF-α and IL-2, vermehrtes IL10). Dies ließ uns eine „Blasten-Protektive“ Eigenschaft der PINDs vermuten. Daher schlossen wir, dass PINDs im Rahmen antileukämischer Therapien /Experimente gemieden werden sollten [23].

Zusammenfassend konnten wir zeigen, dass DC/DCleu (Leukämie-Antigen-spezifisch) T-Zellen (und möglicherweise auch Zellen des angeborenen Immunsystems) aktivieren und dadurch ex vivo eine antileukämische

Funktionalität induzieren können. Allerdings führen nicht alle DC-generierenden Methoden zu einer solchen Aktivierung. Daher ist eine gründliche Analyse vor dem therapeutischen Einsatz von größter Bedeutung. Dies vor allem mit Blick auf den zukünftigen Fokus, der die Übertragung dieser ex vivo Strategie auf die in vivo Anwendung sein wird: Das antileukämische Potential/die antileukämische Funktionalität muss evaluiert und tolerogene DC oder andere inhibitorische Mechanismen müssen vor der klinischen Einführung ausgeschlossen werden.

Im Vergleich zu einer adoptiven Transferstrategie wäre es einfacher und eleganter, komplizierte, logistisch aufwendige und teure ex-vivo-Verfahren zu umgehen, indem antileukämische immunreaktive Zellen direkt in vivo durch die Verabreichung von Medikamenten aktiviert werden, die die Umwandlung von Blasten in DCleu einleiten. Um dies zu erreichen, haben wir bestimmte Arzneimittelkombinationen ("Kits") ausgewählt, die GM-CSF (als myelopoese-induzierenden Faktor) in Kombination mit 1-2 Faktoren, die ein ‚Danger Signaling' sowie DC-Reifung induzieren (TNFα, IFNα, PGE1, PGE2, Picibanil, Calciumionophor) enthalten. Diese „produzierten" DC/DCleu produzieren, ohne die Blastenproliferation zu induzieren (Helga Schmetzer, Deutsches Patent: 10 2014 014 993, US und Europäische Patentanmeldung). Der nächste Schritt besteht darin, diese Kits in Vollblut zu testen, um in vivo-Bedingungen zu simulieren und schließlich Patienten mit diesen Kits zu behandeln.

6 Publication I, Liepert et al. 2010

Cellular Immunology 265 (2010) 23–30

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

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

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ARTICLE INFO

Article history:
Received 17 March 2010
Accepted 16 June 2010
Available online 20 June 2010

Keywords:
Dendritic cells
Acute myeloid leukemia
Serum-free culture
T-cells
Immunotherapy
Stem cell transplantation

ABSTRACT

Myeloid leukemic cells can differentiate into leukemia-derived dendritic cells (DC_{leu}), presenting known/unknown leukemic-antigens. Induced anti-leukemic T-cell-responses are variable. To further elicit DC/DC_{leu}-induced T-cell-response-patterns we performed (functional)flow-cytometry/fluorolysis-assays before/after mixed lymphocyte cultures (MLC) of matched (allogeneic) donor-T-cells ($n = 6$), T-cells prepared at relapse after stem cell transplantation ($n = 4$) or (autologous) patients'-T-cells ($n = 7$) with blast-containing-mononuclear-cells ('MNC') or DC_{leu}-containing DC ('DC'). Compared to 'MNC' 'DC' were better mediators of anti-leukaemic T-cell-activity, although not in every case effective. We could define cut-off proportions of mature DC, DC_{leu}, proliferating, $CD4^+$, $CD8^+$ and non-naive T-cells after 'MNC'- or 'DC'-stimulation, that were predictive for an anti-leukemic-activity of stimulated T-cells as well as a response to immunotherapy. Interestingly especially ratios >1 of CD4:CD8 or CD45RO:CD45RA T-cells were predictive for anti-leukemic function after DC-stimulation.

In summary the composition and quality of DC and T-cells after a MLC-stimulating-phase is predictive for a successful *ex-vivo* and *in-vivo* anti-leukemic response, especially with respect to proportions of proliferating, $CD4^+$ and $CD45RO^+$ T-cells. Successful cytotoxicity and the development of a T-cell-memory after 'DC'-stimulation could be predictive for the clinical course of the disease and may pave the way to develop adoptive immunotherapy, especially for patients at relapse after SCT.

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1. Introduction

AML as well as MDS are clonal disorders of hematopoietic stem cells, characterized by an impaired normal cell differentiation [1]. About 70% of successfully chemotherapeutically treated AML-patients relapse soon [2]. There is a need for less intensive (post remission) immunotherapy in order to maintain stable remissions. Allogeneic stem cell transplantation (SCT) is the only curative treatment option [3]. Donor-T-cells are the most important mediators of anti-leukemic reactions [4,5], although relapses after SCT occur. We could already show, that remissions in relapsed patients after SCT can be restored by a transfusion of donor-T-cells (DLI) after a low dose Arabinosid chemotherapy—again proving the central role of T-cells [6]. However, not all relapsed patients respond to a DLI-based therapy; moreover graft versus host (GvH) reactions can impair the efficiencies of SCT or therapy of relapse [3,6]. The reasons for these varying T-cell effects have to be elucidated.

Leukemic blasts are characterized by the expression of myeloid antigens like CD33, CD13, CD117, often together with an aberrant expression of lymphoid antigens (e.g. CD56, CD2) in AML [7]. An insufficient expression of co-stimulatory antigens, MHC molecules and tumor-associated antigens (TAA) on the surface of cancer cells and disturbed mechanisms of apoptosis are the main reasons for an ineffective immune response in oncological diseases [8]. As professional antigen presenting cells (APC), DC specifically stimulate effector T cells, especially tumor-cytotoxic T-cells [9,10]. Therefore, they are regarded as interesting candidates for anti-tumor or

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0008-8749/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2010.06.009

anti-leukemic vaccination strategies [11,12]. In contrast to solid tumors, leukemia-derived DC (DC_{leu}) can be generated without antigen-pulsing by converting leukemic cells from AML-patients *in vitro* directly to leukemia-derived DC co-presenting DC-typical antigens (DCA, e.g. CD40, CD86, CD80, CD1a, CD83), and thereby regaining the stimulatory capacity of mature professional DC [13]. Moreover, we could already show that it is technically possible to generate sufficient DC from any AML-patient under serum-free culture conditions using the best of 3 previously tested DC-generating methods [14–16]. We could show that autologous T-cells, obtained from AML-patients or allogeneic donor-T-cells, can be stimulated by those DC_{leu}, resulting in very efficient cytotoxic effector cells with specific lytic activity (LA) of naïve blasts [15,17]. But in some cases an opposite T cell response pattern was observed; T cells mediating anergy or even supporting blast proliferation *in vitro*. So far, the different T cell response pattern is not predictable. It might be expected, that different precursor-frequencies of leukemia-specific T-cells may result in different subtype-compositions and functional diversities after stimulation with DC_{leu}. In an attempt to elucidate the mechanisms behind, we characterized the compositions of autologous or allogeneic T-cells and their marker-profiles before and after antigenic stimulation via blast-containing MNC ('MNC') or DC_{leu}-containing DC ('DC'). We focused on the role of proliferating-, CD4, CD8, naïve, non-naïve, central-memory-, regulatory, $CCR4^{+}$, $CCR7^{+}$ T-cells in the mediation of anti-leukemia-directed immune-reactions. Moreover, we defined cut-off values for T-cell-subtypes in an attempt to predict the leukaemia lytic efficiency of 'MNC' or 'DC'-stimulated compared to unstimulated T-cells or the clinical course of the patients after immunotherapy (SCT, DLI-therapy). The results of our analysis might contribute not only to the understanding of anti-leukemia-directed immune-reactions in myeloid leukaemia, but also for the prediction of the clinical course of the disease or the selection or development of DC/T-cell based adoptive immunotherapies to treat AML.

2. Materials and methods

2.1. Patients' characteristics, sample collection and diagnostic

Mononuclear cells (MNC) from heparinized blood (PB) or bone marrow (BM) (PB-MNC, BM-MNC) were isolated from the interphase by density-gradient centrifugation (Ficoll-Hypaque, Biochrom), washed and suspended in PBS without Ca^{2+} and Mg^{2+} (Biochrom). Diagnosis of AML- and MDS-cases was based on the basis of French–American–British (FAB) classification [18]. For the generation of DC and the following mixed lymphocyte culture (MLC), samples were collected in active, blast-rich, stages of the disease from 25 AML/MDS patients (8 at first diagnosis, 6 in persisting disease, 1 at relapse, 1 at relapse before stem cell transplantation and 9 at relapse after SCT) after obtaining informed consent. 7 AML patients presented with an undifferentiated leukemia (M0: $n = 3$, M1: $n = 4$), 4 patients with an immature granulocytic leukemia (M2: $n = 4$, M3: $n = 0$), 10 patients with a monocytic leukemia (M4: $n = 4$, M4eo: $n = 2$, M5: $n = 4$) and 1 patient with an erythroid leukemia (M6: $n = 1$). Of the 3 MDS patients, 2 presented with a RAEB and 1 with a CMML. The median age of the patients was 52.2 years (range 12–72 years), the female: male ratio was 1.08. For MLC, 22 AML cases and 3 MDS cases were selected (Table 1). Autologous patients' or allogeneic stem cell donors'-T-cells were separated and prepared for subsequent experiments. In some cases experimental data achieved were correlated with the response to the initiated therapy directly after sample collection without regard to further clinical outcome of the patients.

2.2. DC generation

DC were generated in parallel with three different FCS-free DC-generating methods from blood-samples from 25 AML/MDS-patients in acute stages of the disease as described by us [14,15]

Table 1
Characteristics of patients and samples used for 'MNC'- or 'DC'-stimulation of leukemia specific allogeneic or autologous T-cells in the mixed lymphocyte culture (MLC).

Patient No.	Age at dgn.	Gender	FAB-type	Stage	Blast phenotype [CD]	IC Bla [%]	Source of T-cells
P 362*	53	f	MDS RAEB	Dgn.	13, 33, 34, 117	40	Autologous
P 407	72	m	AML M5	Dgn.	13, 15, 33, 56, 64, 65	58	Autologous
P 419**	68	m	AML M4	Rel. a. SCT	15, 33, 34, 117	94	Allogeneic
P 428	59	f	AML M4	Rel. a. SCT	13, 33,117	47	After SCT
P 436	67	f	AML M4	Dgn.	4, 13, 33, 56, 65, 64	66	Autologous
P 453	40	f	AML M1	Rel. a. SCT	34, 117	20	After SCT
P 454	52	m	MDS CMML	Dgn.	14, 16, 33, 65	37	Autologous
P 460	36	m	AML M0	Rel. a. SCT	7, 15, 13, 33, 56, 65	75	After SCT
P 466*	62	f	AML M6	Rel. a. SCT	13, 33, 34, 65, 117	25	Allogeneic
P 478*	25	f	AML M0	Rel. a. SCT	13, 15, 33, 34, 56, 117	15	After SCT
P 481*	66	f	AML M4/M5	Rel. before SCT	13, 15, 33, 34, 56, 117	93	Autologous
P 481	66	f	AML M4/M5	Rel. a. SCT	13, 15, 33, 34, 56, 117	93	Allogeneic
P 502	41	m	AML M4 eo	Pers. Disease	13, 15, 33, 34, 117	92	Allogeneic
P 502**	41	m	AML M4 eo	Pers. Disease	13, 15, 33, 34, 117	92	Autologous
P 518	55	m	AML M2	Dgn.	13, 33, 117	81	Autologous
P 520*	46	f	AML M1/M2	Dgn.	65, 14, 15, 19, 33, 34	70	Autologous
P 538	12	m	AML M0	Rel. a. SCT	34, 33, 65	93	Allogeneic
P 545**	44	f	AML M1	Pers. Disease	33, 34, 117	83	Autologous
P 546	39	m	AML M2	Dgn.	15, 33, 34, 65, 117	76	Autologous
P 561	43	m	AML M2	Dgn.	13, 15, 33, 65, 117	65	Autologous
P 565	68	f	AML M5	Rel. a. SCT	13, 15, 33, 34, 65	88	After SCT
P 584	52	f	AML M4	Rel.	13, 33, 117	95	Allogeneic
P 618	66	m	MDS RAEBt	Pers. Disease	33, 117	42	Allogeneic
P 652	66	m	AML M1	Pers. Disease	13, 15, 33, 117	68	Autologous
P 655	66	f	AML M2	Pers. Disease	33, 34, 117	95	Allogeneic

m: male; f: female; Rel. a. SCT: Relapse after stem cell transplantation; Dgn.: Diagnosis; Pers. Disease: Persisting Disease; IC BLA [%]: proportions of immunocytologically detected blasts.
* Only MLC-results, no fluorolysis-assay performed.
** No results after MLC available.

Table 2
DC-differentiating methods.

Method/ medium	DC-differentiation stimulating substance	Mode of action	Culture time	References
'MCM-Mimic'	GM-CSF, IL-4, TNFα, FL, IL-1ß, IL6, PGE2	Cytokine based DC-differentiation, PGE2 increases CCR7-expression and improves migration	10–14 d	Kremser (2010), Dreyßig (2010)
'Picibanil'	GM-CSF, IL-4, TNFα, lysat from Streptococcus pyogenes, PGE2	Bacterial lysat and PGE2 stimulate DC differentiation	9–11 d	Kremser (2010), Dreyßig (2010)
' Ca-ionophore'	IL-4, A23187	Bypass of cytokine-driven DC differentiation	3–4 d	Kremser (2010), Dreyßig (2010)

d: days.

(Table 2). Subsequently the method resulting in highest DC-counts was chosen for quantitative generation of DC. All of the substances used for DC-generation are approved for human treatment. Resulting cells were quantified by flow-cytometry using blast-staining (e.g. CD33, CD13, CD65, CD34, CD117) in combination with DC-staining antibodies (e.g. CD1a, CD1b, CD40, CD80, CD83, CD86, CD206) as described [14,15,19]. The generation of DC was defined as successful if >10% DC and >5% DC_{leu} could be generated [15].

2.3. Mixed lymphocyte cultures (MLC)

Positively selected $CD3^+$ T-cells (Milteney Biotech, Bergisch-Gladbach, Germany 1×10^6 cells/well) from MNC from (autologous) patients'-T-cells ($n = 7$), from matched (allogeneic) donor-T-cells ($n = 6$) or from patients at relapse after stem cell transplantation ($n = 4$) were cocultured and stimulated with irradiated (20 Gy) AML-blast-containing MNC (5×10^4 'MNC' (T∗MNC)) and in parallel with irradiated DC_{leu}-containing AML DC (5×10^4 'DC' (T∗DC)) in 1 ml RPMI 1640 medium (Biochrom) containing 15% human serum (PAA) and 50 U/ml IL-2 (Proleukin R5, Chiron). Total DC-counts in the MLC were adjusted to 5×10^4 DC and T-cell counts adjusted to 1×10^6/well. Composition of DC_{leu}-containing DC was evaluated as described above. Cells were harvested after a 10 days-stimulating period of co-cultured T-cells and 2-fold restimulated with 5×10^4 'DC' or 5×10^4 'MNC' and supplementation with IL-2, as described [15,20]. Half medium exchange was carried out every 3–4 days. 6 days after the last restimulation, cells were harvested and the cytotoxicity assay was carried out.

Antigen expressions on (allogeneic or autologous) $CD3^+$ T-cells were evaluated by FACS-analyses comparing the coexpression of CD28, CD154, CD4, CD8, CD45RA, CD45RO, CD62L, CD122, CD25, CD71 before and after blast derived 'MNC'- or 'DC'-contact. This contributed to evaluate proportions of proliferating ($CD71^+$), naïve ($CD45RA^+$), non-naïve ($CD45RO^+$), central-memory ($CD45RO^+CCR7^+$), $CD4^+/CD8^+$ or migratory ($CCR7^+/CCR4^+$) T-cells before or after 'MNC' or 'DC'-co-culture and the functional profile of stimulated/unstimulated T-cells [21–23] were performed for 10 days with 'MNC' or 'DC' followed by (functional) flow-cytometric- and non-radioactive fluorolysis-assays, as described [15,17,21]

2.4. Cytotoxicity fluorolysis assay

The lytic activity of effector T-cells was measured by a fluorolysis-assay by counting viable blast-target cells, labeled with specific fluorochrome antibodies, before and after effector cell (E) contact as described [17]. 'DC'- or 'MNC'-stimulated T-cells from healthy HLA-matched donors (=allogeneic) or from AML and MDS patients (=autologous) as well as unstimulated T cells as a control were co-cultured in 1.5 ml Eppendorf tubes with thawn blasts as target cells (T). The E:T ratio was adjusted to 1:1 and the cells were incubated overnight at 37 °C and 5% CO_2. Before culture, blast-target cells were stained for 15 min with two FITC- and/or PE conjugated 'blast' specific antibodies and co-cultured for 24 h with effector cells (T-cells or DC as target cells were stained with T-/DC-specific antibodies). As a control, target and effector cells were cultured separately and mingled with T-cells shortly before FACS analysis. To evaluate amounts of viable ($7AAD^-$) target cells and to quantify the cell-loss after 24 h of incubation time, cells were harvested, washed in PBS and re-suspended in a FACS flow solution containing 7AAD (BD, Biosciences Pharmingen) and a defined number of Fluorosphere beads (Becton Dickinson, Heidelberg, Germany). Viable cells were gated in a SSC/$7AAD^-$ gate. Afterwards, viable 7AAD negative cells co-expressing a specific blast marker (combinations) were quantified by taking into account defined counts of calibration beads as described. Cells were analyzed in a FACS Calibur Flow-Cytometer using CELL Quest software (Becton Dickinson, Heidelberg, Germany). The percentage of lysis was defined as the difference between proportions of viable blasts before and after the effector cell contact [15,17,23]. As a control in some cases the MHC-indepentent killing of K562 cells by stimulated or unstimulated T-cells was evaluated. In addition in some cases blocking antibodies (anti-HLA-A,B,C and anti-HLA-DR, Acris) were used to block CD8 or CD4 mediated cellular reactions.

2.5. Statistical methods

Mean and standard deviation, median and range and two-tailed *t*-tests were performed with a personal computer using Excel 2000 (Microsoft). Differences were considered as significant, if the *p*-value was <0.05.

3. Results

On average our samples contained 67% myeloid leukemic blasts. With the best of 3 previously tested DC-methods we could successfully generate DC in every given case with on average (ø) 23% (range 10–65%) DC with ø66% (range 16–100%) DC_{leu} and ø43% (range 10–87%) mature DC in the DC-fraction.

We analysed T-cell-characteristics and their predictive effect for the anti-leukemic activity of unstimulated, 'MNC'-or 'DC'-stimulated T-cells. Functional assays showed, that a 'MNC'-stimulation induced anti-leukemic cytotoxicity in 4 of 17 cases, compared to 8 of 17 cases after 'DC'-stimulation. Moreover, 'DC'-stimulated T-cells lysed more blasts compared to 'MNC'-stimulated T-cells. In the remaining cases no blast-lysis or an increase of the leukemic-population was observed.

Eighty to ninety percent viable ($7AAD^-$) T-cells were found after 'MNC' as well as after 'DC'-stimulation (Figs. 1 and 2, right side). Proliferation-rates of T-cells ($CD71^+$ cells) increased significantly from <5% in unstimulated T-cell-fractions to >20% after 'MNC'- or 'DC'-stimulation (range 23–53%, $p < 0.02$) Figs. 1 and 2). Non-naïve ($CD45RO^+$) T-cells increased after 'MNC'- and to a higher extent significantly ($p < 0.01$) after 'DC'-stimulation from 25–40% to 40–60%. With the exception of the allogeneic group, naïve ($CD45RA^+$) T-cells decreased significantly ($p < 0.05$) from about 50% to about 30–40% (Figs. 1 and 2). T-cells co-expressing migration-markers

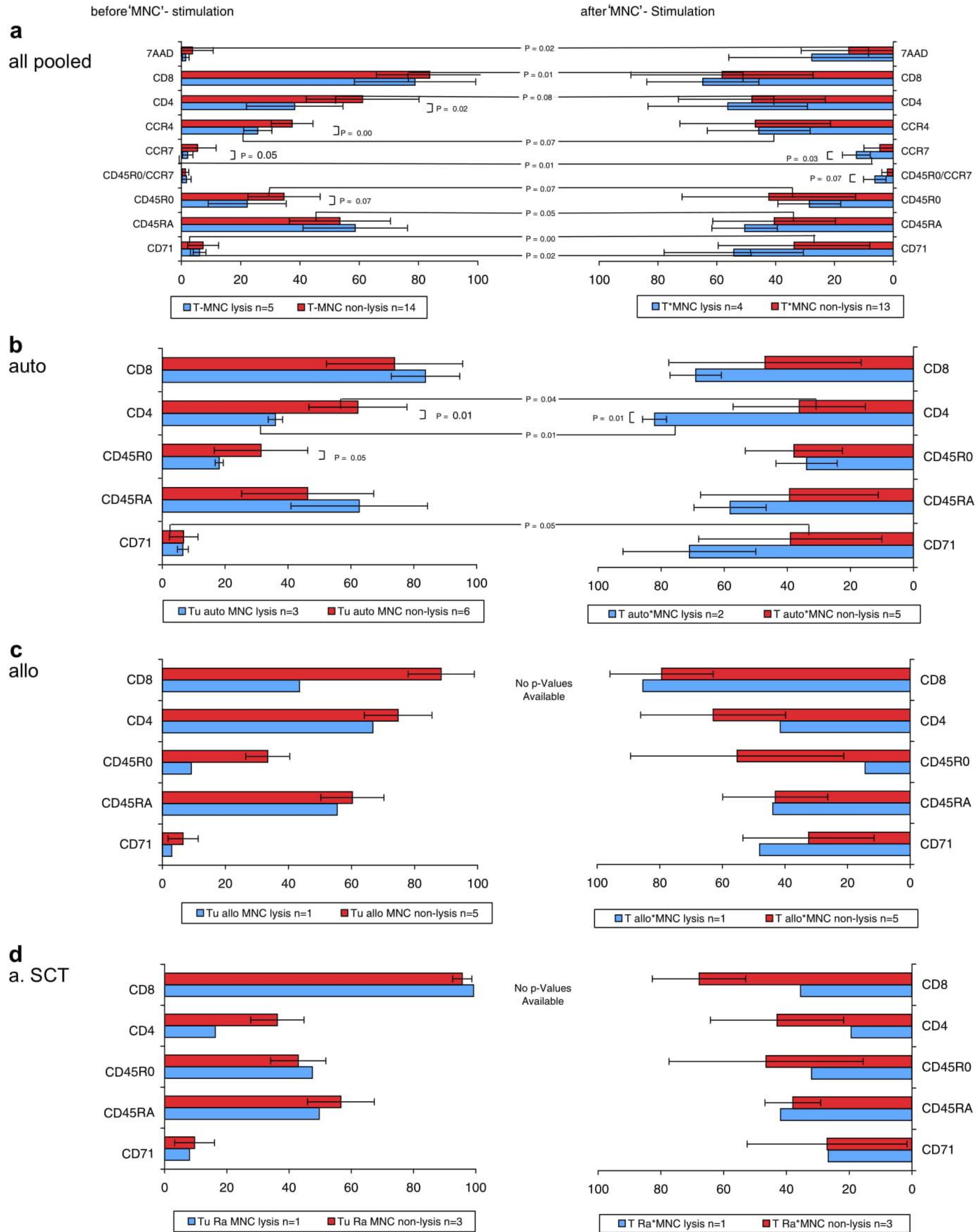

Fig. 1. T-cell compositions, characteristics and correlations with anti-leukemic activity before and after stimulation with 'MNC'. Compositions of *unstimulated* T-cells in cases subdivided in cases before and after 'MNC'-stimulation are given with cases subdivided in cases with or without gain of anti-leukemic lytic activity. Results of different T-cell sources in cases *before* (left side) or *after* 'MNC'-stimulation (right side) are given: (a) T-cell source: autologous, allogeneic T-cells or T-cells at relapse after SCT. (b) T-cell source: autologous T-cells. (c) T-cell source: allogeneic T-cells. (d) T-cell source: T-cells at relapse after SCT.

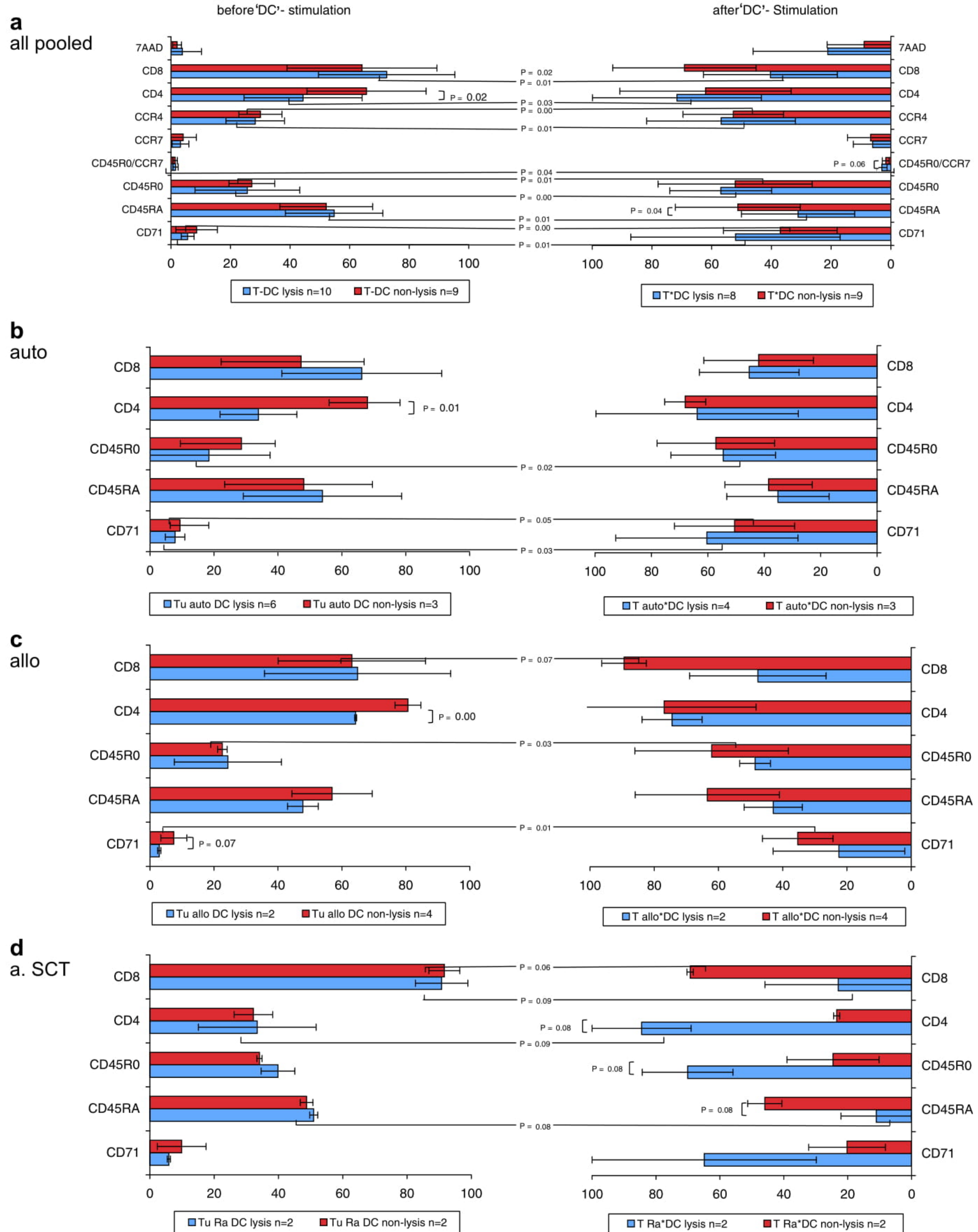

Fig. 2. T-cell compositions, characteristics and correlations with anti-leukemic activity before and after stimulation with 'DC'. Compositions of *unstimulated* T-cells in cases subdivided in cases before and after 'DC'-stimulation are given with cases subdivided in cases with or without gain of anti-leukemic lytic activity. Results of different T-cell sources in cases *before* (left side) or *after* 'DC'-stimulation (right side) are given: (a) T-cell source: autologous, allogeneic T-cells or T-cells at relapse after SCT. (b) T-cell source: autologous T-cells. (c) T-cell source: allogeneic T-cells. (d) T-cell source: T-cells at relapse after SCT.

(CCR7 and especially CCR4) increased after 'MNC'- and 'DC'-stimulation from <5% CCR7$^+$ T-cells to about 10% CCR7$^+$ T-cells and from 30% to 40–50% CCR4$^+$ T-cells with significantly higher values being found in the lytically active groups ($p < 0.07$; Figs. 1 and 2). Low average-proportions of potentially regulatory T-cells (CD62L$^+$/CD122$^+$) were found in *uncultured* CD4$^+$ T-cell-fractions. After 'MNC'- or 'DC'-stimulation, the proportion of potentially regulatory T-cells increased to about 15% in the autologous setting and to about 5% in the allogeneic setting. In the 'relapse-group after SCT' 3% potentially regulatory T-cells were found after 'MNC'-stimulation and 10% after 'DC'-stimulation (data not shown). In uncultured samples proportions of CD4$^+$ T-cells were higher in the groups without lytic activity (Figs. 1 and 2, left side). Minimally higher proportions of T-cells with a regulatory phenotype were found in the non-lytic compared to the lytic group after 'DC'-stimulation (0.5% vs. 0.3% of CD4$^+$ T-cells, data not shown). 'MNC'- or 'DC'-stimulated T-cells (autologous), allogeneic (donor)-T-cells and T-cells at relapse after SCT showed a higher proliferation in the lytic group (55%) compared to 35% in the non-lytic group. The most interesting result was, that cases with anti-leukemic activity after 'DC'-stimulation compared to cases without lytic activity were characterized by CD4:CD8 ratios >1 and CD45RO:CD45RA ratios >1, whereas expressions of migratory markers did not contribute to differentiate cases with from that without lytic activity (Fig. 2, right side).

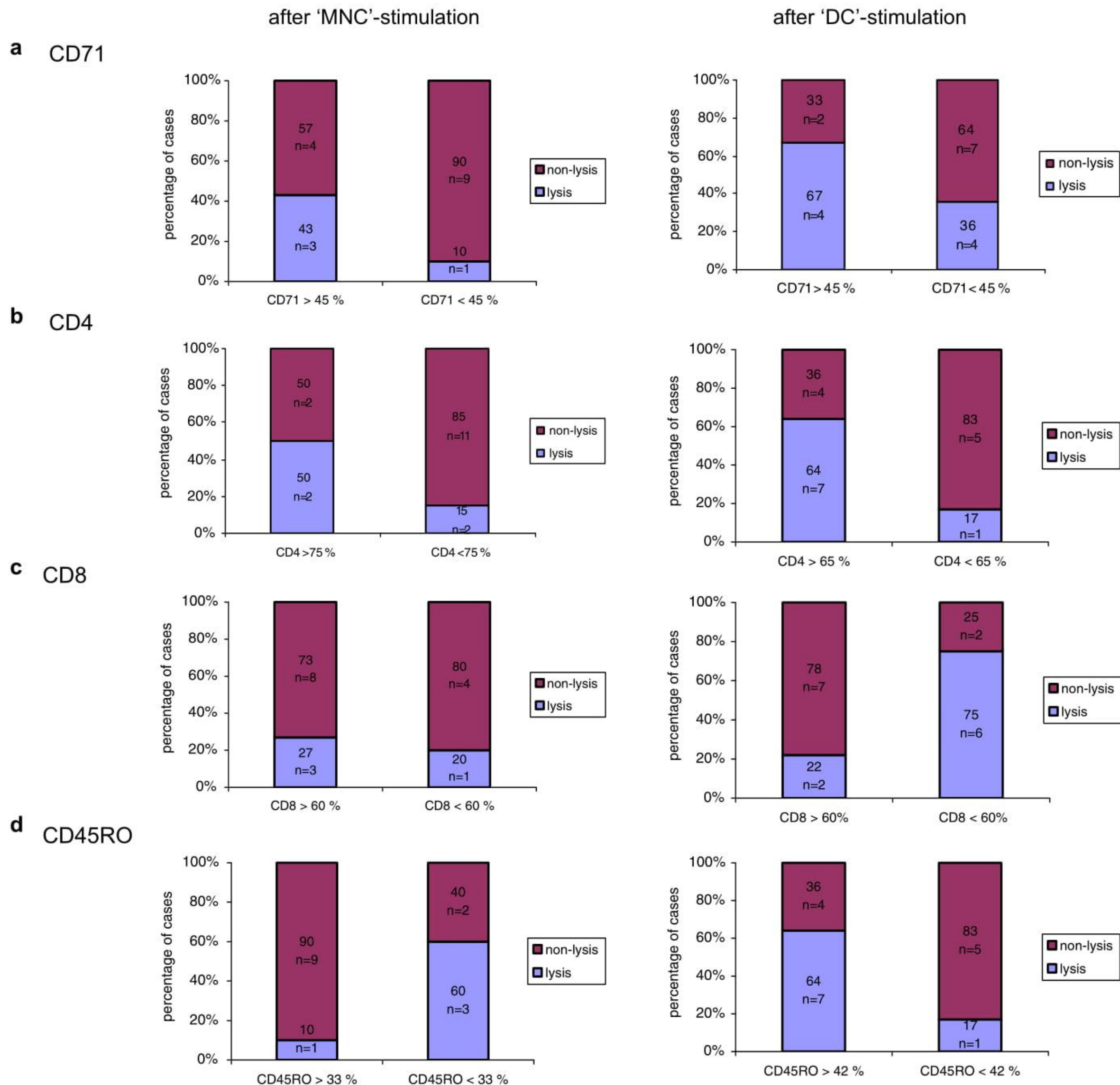

Fig. 3. Predictive values of T-cell surface marker profiles for anti-leukemic lytic capability after stimulation with 'MNC' or 'DC'. Correlations of proportions of T-cell subsets with the anti-leukemic lytic activities are given. T-cells were either stimulated with 'MNC' (left side) or with 'DC' (right side). Cases expressing CD71(a), CD4(b), CD8(c) or CD45RO(d) were separated in two prognostic groups evaluating a cut-off value for the best differentiation of cases into those with or without anti-leukemic activity. Highest probability of lytic activity was shown in cases with >45% proliferating CD3/CD71$^+$ T-cells after 'MNC'- or 'DC'-stimulation (a), in cases with >75% CD3/CD4$^+$ T-cells after 'MNC'- and >65% CD3/CD4$^+$ T-cells after 'DC'-stimulation (b), in cases with >60% CD3/CD8$^+$ T-cells after 'MNC'- and <60% CD3/CD8$^+$ T-cells after 'DC'-stimulation (c) and in cases with <33% non-naive CD3/CD45RO$^+$ T-cells after 'MNC'- and >42% non-naive CD3/CD45RO$^+$ T-cells after 'DC'-stimulation.

Differences were most distinct in cases studied at relapse after SCT. In contrast after 'MNC'-stimulation no correlations of CD4:CD8 ratios or CD45RO:CD45RA ratios were found (Fig. 1, right side). In some cases antibody-mediated blocking experiments were performed: in two cases with DC-stimulated autologous, in one case with DC-stimulated and in one case with 'MNC'-stimulated allogeneic donor-T-cells we could demonstrate, that anti-leukemic-cytotoxic reactions were mediated by the combination of $CD4^+$ and $CD8^+$ T-cells. In another case with unstimulated autologous T-cell a CD8-mediated anti-leukemic T-cell-response could be shown [17].

In an attempt to predict the anti-leukemic behaviour of stimulated T-cells we grouped cases by defining 'cut-off-values', that allowed a most significant separation of lytic and non-lytic groups. We could demonstrate that cases with >45% $CD71^+$ T-cells, >75% $CD4^+$ T-cells, >60% $CD8^+$ T-cells or <33% $CD45RO^+$ T-cells after 'MNC'-stimulation were characterized by a higher probability to belong to the group with lytic activity compared to the remaining cases (Fig. 3, left side). After 'DC'-stimulation cases with >45% $CD71^+$ T-cells, >65% $CD4^+$ T-cells, <60% $CD8^+$ T-cells or >42% $CD45RO^+$ T-cells were characterized by a higher probability to belong to the group with lytic activity compared to the remaining cases (Fig. 3, right side). We could show, that 75% of cases, with >45% $CD71^+$, and in addition >65% $CD4^+$, >42% $CD45RO^+$ and <60% $CD8^+$ cells after 'DC'-stimulation achieved anti-leukemic-lytic activity ($n = 6$) compared to cases with no lysis ($n = 2$). Vice-versa cases with/without lytic activity were characterized by 71/60% DC_{leu}, 52/30% mature DC, whereas DC-counts were not different in the groups compared (22/24%, data not shown). Separating cases according to different T-cell sources (e.g. autologous, allogeneic) comparable results were found as given for the pooled group (data not shown).

Clinical responders to immunotherapy(SCT/DLI-therapy) were characterized by better T-cell-proliferation (50% vs 39%), CD4:CD8 ratios >1 and CD45RO:CD45RA ratios of >1 after 'DC'-stimulation compared to non-responders (data not shown).

In summary that means, that compared to a 'MNC'-stimulation a 'DC'-stimulation regularly, but not in every case, gives rise to leukemia-cytotoxic T-cells with higher lytic activity. T-cell-proliferation-rates, CD4:CD8 ratios and CD45RO:CD45RA ratios after 'DC'-stimulation, but not after 'MNC'-stimulation are not only predictive for the anti-leukemic behaviour of 'DC'-stimulated T-cells *in vitro*, but also for the clinical response to immunotherapy.

4. Discussion

Myeloid leukemic cells can be induced to differentiate into DC_{leu}, that might present the complete leukemic-antigen-repertoire thereby improving (*ex-vivo*-) stimulation of T-cells and *in-vivo*-response to SCT/DLI-therapy [15,17,24]. Requirements to induce an anti-leukemic immune-response *ex-vivo*/*in-vivo* are not well understood [25]. Recently we have demonstrated the central role and superiority of (good-quality) DC for the mediation of leukemia-lytic functions [17]. Anti-leukemic-cytotoxicity was shown in 26% of cases after 'MNC'-stimulation and in 58% after 'DC'-stimulation. Interestingly in 47% of cases *unstimulated* T-cells were able to lyse on average 38% of blasts in the Fluorolysis-assay. This means, that unmanipulated T-cells separated from leukemic samples or from donor cells have different functional properties compared to T-cells stimulated and restimulated with 'MNC' or 'DC' in IL-2 containing media. It might be assumed, that selected T-cells surrounded by blasts during the 10 days' incubation period reflect and simulate the *in-vivo* situation resulting in a production and secretion of soluble response modifiers, cytokines and inhibitory molecules whereas these inhibitory factors are removed by use of unstimulated, pure T-cells. That means, that unstimulated, pure T-cells (without cellular- or micro-environment) must be regarded as highly artificial controls in experimental settings and do not reflect *in-vivo* situations of cells).

Cytotoxic-efficiency was improved, if T-cells were stimulated with 'good-quality-DC' (>45% mature, CD83+ DC or >65% leukemia-derived DC) [17]. Here we characterized the role of unstimulated, 'MNC'- or 'DC'-stimulated T-cells and their quality for the mediation of anti-leukemic reactions. Anti-leukemic 'DC'-(but not 'MNC')-stimulated T-cells were associated with an increase of $CD4^+$ and $CD45RO^+$ non-naïve T-cells and a decrease of $CD8^+$ and $CD45RA^+$ naïve T-cells (ratio of CD45RO:CD45RA and CD4:CD8 T-cells >1) demonstrating, that T-cell-surface-marker-profiles are modulated during stimulation-phases and are associated with different functionalities: 'MNC'-stimulation could impair even the function of healthy-donor-T-cells, e.g. by release of soluble inhibitory factors, expression-patterns of costimulatory-markers or integrin-receptors or activation of regulatory-T-cells [25–27]. Refined analyses showed that 'DC'-stimulated cases with high proportions of proliferating, $CD4^+$, $CD45RO^+$ T-cells and low proportions of $CD8^+$ T-cells had a 75%-chance to gain lytic activity. This could mean, that 'MNC'-stimulation gives rise to cytotoxic-$CD8^+$ T-cells and decrease of effector- or effector-memory-cells and less central-memory-cells, whereas 'DC'-stimulation favors the generation of cytotoxic-$CD4^+$ T-cells with effector- or effector memory-type and probably production of memory-cells, that could be responsible for long-lasting immunity [28]. In some cases $CD4^+$ T-cells with a regulatory-phenotype were detected, although anti-leukemic-stimulated T-cells dominated the immune-response.

Effects of leukemia-cytotoxic $CD4^+$ T-cells have already been shown e.g. $CD8^+$ T-cells can be depleted from the DLI without jeopardizing the GvL effect [29,30]. Both $CD8^+$ T-cells and $CD4^+$ T-cells can suppress ('regulatory-cells') or promote ('cytotoxic-cells') immune-reactions [27,31]. As shown by us $CD4^+$ as well as $CD8^+$ T-cells are involved in the mediation of anti-leukemic functionality [17]. The replicative central-memory-fraction of non-naïve $CD45RO^+$ T-cells (central-memory(T_{CM}) or effector-memory(T_{EM}) or effector-subsets) can mediate protection e.g. after a second challenge with target-cells and are of central significance to prevent patients from relaps [32,33]. We can show, that especially 'DC'-stimulation (and functional-assays) might contribute to prognostic-estimations and to overcome immunological-resistance: (anergic)T-cells at relapse after SCT or even autologous T-cells could be redirected against the leukemic-targets, although not in every case. The analyses of the quantity and quality of soluble and cellular signals or compositions (e.g. T-cell-receptor or cytokine-signals, Tcm, Tem) delivered by the partners involved are in the focus of our ongoing research [34].

Cases with a clinical-response to initiated therapy showed higher proportions of DC_{leu} and higher proportions of $CD4^+$ and $CD45RO^+$ T-cells, as to be measured in CD4:CD8 ratios >1 and in CD45RO:CD45RA ratios >1.

Our findings might contribute to further refine these immunotherapies, especially for patients with relapse after SCT.

In 3 of 9 cases after 'MNC'-stimulation and in 5 of 9 cases after 'DC'-stimulation, a blast lytic activity could be achieved in the clinical responder group; in the clinical non-responders group we observed similar results. This could mean, that the lytic profile of 'DC'- or 'MNC'-stimulated T-cells is *independent* from the clinical response to treatment. However, an improved lytic activity can be achieved after T-cell stimulation with 'DC' irrespective of the state of clinical response to treatment.

The resistance to (immuno)therapy might be overcome by a specific, anti-leukemic 'DC'-stimulation of effector-T-cells—*ex-vivo* or *in-vivo* and/or an isolation of leukemia-reactive T-cells for an adoptive-transfer. However, a careful pretesting of cell compositions and

lytic activities, eventually combined with a selection of CD4+ or CD8+ 'primed' T-cells has to be performed to differentiate functional from dysfunctional T-cells.

Acknowledgments

The authors thank nurses and physicians on the wards for their support and diagnostic laboratories for the contribution of diagnostic reports of the patients. Parts of the results presented in this manuscript were worked out in the course of the doctoral theses of Anja Liepert, Christine Grabrucker, Andreas Kremser, Julia Dreyßig and Markus Freudenreich at the University Hospital Grosshadern of the Ludwig-Maximilian-University Munich. The authors thank Monica Schifferer for support with graphical presentations.

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7 Publication II, Vogt et al. 2014

Profiles of Activation, Differentiation–Markers, or β-Integrins on T Cells Contribute to Predict T Cells' Antileukemic Responses After Stimulation With Leukemia-derived Dendritic Cells

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Summary: Stem cell transplantations and donor lymphocyte infusions are promising immunotherapies to cure acute myeloid leukemia (AML). Leukemia-derived dendritic cells are known to improve antileukemic functionality of T cells. We evaluated the composition and development of distinct T-cell subtypes in AML patients (n = 12) compared with healthy probands (n = 5) before and during stimulation with leukemia-derived dendritic cells–containing DC (DC) or blast-containing mononuclear cells (MNC) in 0–7 days mixed lymphocyte cultures (MLC) by flow cytometry. AML patients' T-cell subgroups were correlated with antileukemic functionality before and after DC/MNC stimulation by functional fluorolysis assays. (1) Unstimulated T cells from AML patients presented with significantly lower proportions of activated, T_{cm}^{+}, CD137^{+}, and β-integrin^{+} T cells, and significantly higher proportions of T_{naive} and T_{eff} compared with healthy probands. (2) After 7 days of DC or MNC stimulation, T-cell profiles were characterized by (significantly) increased proportions of activated T cells with effector function and significantly decreased proportions of β-integrin^{+} T cells. (3) Antileukemic cytotoxicity was achieved in 40% of T cells after MNC stimulation compared with 64% after DC stimulation. Antileukemic activity after DC stimulation but not after MNC stimulation correlated with higher proportions of T_{cm} and T_{naive} before stimulation, as well as with significantly higher proportions of activated and β-integrin^{+} T cells. Furthermore, cutoff values for defined T-cell activation/differentiation markers and β-integrin^{+} T cells could be defined, allowing a prediction of antileukemic reactivity. We could demonstrate the potential of the composition of unstimulated/DC-stimulated T cells for the lysis of AML blasts. Especially, AML patients with high numbers of T_{naive} and T_{cm} could benefit from DC stimulation; proportions of activated and β-integrin^{+} T cells correlated with increased antileukemic functionality and could serve to predict T cells' reactivity during stimulation. Refined analyses in the context of responses to immunotherapies are required.

Key Words: AML, dendritic cells, T-cell differentiation, T-effector cells, T-cell memory, β-integrins

(*J Immunother* 2014;37:331–347)

Received for publication August 19, 2013; accepted March 3, 2014.

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Acute myeloid leukemia (AML) is a neoplastic disorder of the hematopoiesis characterized by clonal proliferation and impaired cell differentiation in the myeloid system. Treatment is usually composed of induction chemotherapy and postremission management, which may contain stem cell transplantation (SCT) followed by transfusion of donor T cells.[1–3] Although 60%–90% of patients achieve a remission, depending on their cytogenetic risk group, the 4-year survival with standard consolidation therapy is only 34%.[4,5] Allogenic SCT is the only curative treatment option in these cases, based on the strong graft-versus-leukemia effect of allogenic lymphocytes.[1,2] However, this treatment is still limited by the occurrence of graft-versus-host disease and thus not successful in every case.[6]

T cells are the main mediators of cellular tumor immunity and are regarded as very promising candidates for immunotherapy.[7] Dendritic cells (DC) are professional mediators of T-cell stimulation by their antigen-presenting capacity. Many different DC-based vaccination strategies to improve the T-cell stimulation have been described.[8] Our group could already show that T cells stimulated by DC generated from AML blasts [leukemia-derived DC (DC_{leu})] are able to overcome their dysfunctional state and regain antileukemic functionality, although not in every case.[9–11]

To explain the different functionality of DC-primed T cells, the composition of T cells, and especially of the T-cell memory in tumor immunology and immunotherapy, has been subject of many studies lately. Memory T cells (T_{mem}) are part of the adaptive immune system and provide long-term immunity. Compared with a naive T-cell (T_{naive})-mediated, the T_{mem}-mediated secondary immune response is faster, stronger, and more effective.[12] There is disagreement concerning the generation of T_{mem} cells. Former investigations proclaimed that a small number of effector T cells (T_{eff}) do not undergo apoptosis after antigen clearance but differentiate into T_{mem} cells, whereas newer models could show a T_{eff}-independent generation of T_{mem}.[13–15] Others proposed that the strength of the stimulus would decide whether a T cell differentiates into a T_{mem} or T_{eff}.[16] In 1999, Sallusto and colleagues divided T_{mem} in 2 subsets. They differentiated CCR7^{+} central memory cells (T_{cm}), which express lymph-node homing receptors, have high proliferation potency, lack effector functions, but respond well to DC stimulation; they can differentiate into T_{eff} and CCR7^{-} effector memory cells (T_{em}), which express homing receptors for the peripheral tissues and display effector function.[17,18] There is evidence that because of their high

proliferation potency, T_{cm} can be regarded as a background pool that supplies lost T_{eff}, whereas T_{em} with their effector functionality are needed for a quick second immune response.[19,20] Moreover, Klebanoff et al[21] could demonstrate the important role of T_{cm} in tumor-antigen vaccination compared with T_{em} and T_{eff}.

In general, these T-cell subsets, as listed previously, can be differentiated by their differential expression of CD45RO and/or CD45RA in combination with CD27, CD28, CD62L, or CCR7.[16,22–24] In addition, Berger et al[25] have provided an alternative strategy to differentiate T_{naive}, T_{cm}, and T_{em} by analyzing their differential expression of CD62L in combination with CD28, FasReceptor, and CCR7.

β-integrins are expressed on all kind of lymphocytes, like T cells, B cells, NK cells, monocytes, macrophages, etc.[26] Adhesion molecule β1-integrin VCAM-1 (CD29) in vessels or β7-integrin MAdCAM-1 in mucosal endothelial cells of the gut[26,27] play an important role in the trafficking of T cells.[28] Moreover, costimulatory signals are transmitted by β1-integrin binding, regulating the proliferation and interleukin 2 (IL-2) production of T cells.[29] β1-integrins are expressed on T_{cm} and act as adhesion molecules providing retention in the bone marrow (BM). After binding to VCAM-1-expressing stroma cells, these cells secrete IL-7 that T_{cm} require for their retention in the BM but not for their long-term survival in general.[30]

The aim of our study was to study the composition of T-cell subtypes in healthy donors or AML patients, with a focus on activated T cells, differently developed T-cell (subtype)-expressing or β-integrin-expressing T cells before/during or after stimulation phases, with leukemic blasts or DC_{leu} in mixed lymphocyte cultures (MLC). We correlated antileukemic functionality of those (stimulated/unstimulated) T cells with T-cell profiles and compositions and deduced predictive values for functionality.

MATERIALS AND METHODS

Patients' Characteristics, Diagnosis, and Sample Collection

After obtaining informed consent, heparinized blood samples were taken from 12 patients with AML in acute stages of the disease (4 at first diagnosis, 1 relapse + 1 in persisting disease before SCT, 4 relapse after SCT, 2 in persisting disease after SCT) and used as sources for the generation of DC. Sources for T cells were before SCT, all of them in active stages of the disease ("autologous," n = 6) or patients after SCT ("after SCT," n = 6), with 4 of them in remission and 2 of them at relapse after SCT (Table 1). Diagnosis of AML and MDS cases was based on the basis of French-American-British classification (Table 1). The median age was 52 years (range, 26–70 y); the male:female ratio was 1:1. Five healthy test persons (fellow students or members of our research group) served as controls. Their median age was 29 years (range, 23–48 y); the male:female ratio was 1:1.5.

Mononuclear cells (MNC) were isolated from whole blood samples by density gradient centrifugation (Ficoll-Hypaque; Biochrom, Berlin, Germany), then washed, and suspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Biochrom). Cell counts were quantified by Neubauer counting chambers. MNCs were frozen with standardized procedures and stored in liquid nitrogen until use.

Flow cytometric analysis of uncultured MNC fractions was performed to quantify the cellular compositions of MNC in 12 AML patients as well as in healthy cell samples. The cellular composition of MNC in AML patients was 3% ± 2% B cells, 9% ± 6% T cells, 2% ± 2% NK cells, 5% ± 6% monocytes, and 56% ± 25% blasts. In cases with (aberrant) expression of $CD19^+$, $CD56^+$, or $CD14^+$ on blasts, B cells, NK cells, or monocyte counts, respectively, were not quantified. The cellular composition of MNC in healthy donors was 9% ± 4% B cells, 42% ± 15% T cells, 13% ± 6% NK cells, and 10% ± 2% monocytes.

DC Generation

Thawed MNCs were pipetted in 12-well tissue culture plates in 1 mL Xvivo 15 (Bio Whittaker Europe, Verviers, Belgium) FCS-free medium. DCs were generated from blood samples according to a refined strategy culturing cells in a minimalized assay with 3 DC-generating methods [MCM-Mimic, Picibanil, Calcium (Ca)-Ionophore, Table 2] in parallel, as described by us.[9,31,32]

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

For analysis of a successful generation of DC, cells were quantified by flow cytometry using patient-specific "blast"-staining antibodies (CD33, CD13, CD15, CD65, CD34, CD117) according to diagnostic reports in combination with DC-staining antibodies (CD1a, CD1b, CD40, CD80, CD83, CD86, CD206) as described.[33] We defined the DC generation as successful if at least > 10% DC and > 5% DC_{leu} could be evaluated in the cell suspension.[9] DC_{leu} were defined by coexpression of blast markers with DC markers. The method resulting in the highest DC and DC_{leu} counts was chosen for quantitative generation of DC.

We generated DC successfully in every given case of our 17 patients' or healthy probands' samples according to procedures already described by members of our group.[9,11] AML samples presented with an average 20% ± 14% DC (range, 10%–51%) and 6% ± 2% DC_{leu} (range, 5%–10%) after cultivation with the best 3 previously tested DC methods in individual patients. We selected 2 times picibanil, 3 times Ca-ionophore, and 7 times MCM-mimic as DC-generating methods (Table 2).

MLC

$CD3^+$ T cells were positively selected (Milteney Biotech, Bergisch-Gladbach, Germany; 1×10^6 cells/well) from MNC from patients (autologous, n = 6) or from patients in remission or at relapse after SCT (n = 6) and cocultured and stimulated with irradiated (20 Gy) AML blast-containing MNC [2.5×10^4 MNC (T'MNC')] and in parallel with irradiated DC_{leu}-containing AML DC [2.5×10^4 DC (T'DC')] in 1 mL RPMI-1640 medium (Biochrom), containing 15% human serum (PAA) and IL-2 50 U/mL (Proleukin R5; Chiron, Munich Germany). Total DC counts in the MLC were adjusted to 2.5×10^4 DC and T-cell counts adjusted to 1×10^6/well, as described.[11,32] After 2-fold restimulation with 2.5×10^4 DC or MNC, supplementation of IL-2 (50 U/mL) and half medium exchange on days 3 and 5, cells were harvested on days 7–8 and the cytotoxicity (fluorolysis) assay was carried out.

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TABLE 1. Patients' and Samples' Characteristics are Given: Age, Sex, FAB Type, and Stages of the Disease of AML Patients and Blast Phenotypes Evaluated by Flow Cytometry, as well as Sources of T Cells Used for Analyses and Their Antileukemic Activity are Given

At Timepoint of DC Generation						At Timepoint of T-cell Preparation			
							Antileukemic Activity (%) of Unstimulated (u), MNC, Stimulated or DC-Stimulated T cells (DC)*		
Patient No.	Age at Dgn (y)/ Sex	FAB Type	Stage	Blast Phenotype (CD)	IC Bla%	Source of T cells and Stage	u	DC	MNC
887	59/F	MDS/ CMML	Pers	33,34,14,64,117	8	Pers, autologous†	−58*	−12	−12
914	66/M	sAML	Rel b.SCT	34,33,15,117	47	Rel b.SCT, autologous	800‡	250	350
824	70/M	M1/M2	Dgn.	7,13,34,117,123	68	Dgn., autologous	ND	−85	100
748	67/M	M2	Dgn.	34,33,117,13,38	50	Dgn., autologous	120	700	800
855	68/M	M4	Dgn.	13,33,34,65,117	37	Dgn., autologous	ND	ND	ND
948	42/F	M5	Dgn.	34,33,117	70	Dgn., autologous	30	−55	20
m569	49/F	M0	pers	15,33,13,34,19	11	CR a. SCT	−60	−76	−63
853	34/M	M0	Rel a. SCT	34,33,117	50	CR a. SCT	ND	−75	ND
m761	38/F	M1	pers	34,117,15,65,2,7	40	CR a. SCT	−30	150	55
880	26/M	M4	Rel a. SCT	33,64,13,15,117	90	Rel a. SCT†	−93	−51	−9
458	47/F	M4	Rel a. SCT	13,33,117	15	CR a. SCT	−40	500	250
938	58/F	M4	Rel a. SCT	34,117,33,4	74	Rel a. SCT†	−60	−77	−80

*Negative values (–) = percentual decrease of blast proportions.
‡Positive values (+) = percentual increase of blast proportions.
†T cells harvested at different time points at relapse (pt 880, 938) or during persisting disease (pt 887) and pooled for MLC.
AML indicates acute myeloid leukemia; DC, dendritic cells; Dgn., first diagnosis; f, female; FAB, French-American-British classification; m, male; MDS, myeloid dysplastic syndrome; MNC, mononuclear cells; pers., persisting disease; pers. rel., persisting relapse; rel, relapse; Rel. a. SCT, relapse after stem cell transplantation.

Cell Characterization by Flow cytometry

Flow cytometric analysis was carried out by using monoclonal mouse anti-human antibodies (moAbs) labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (PC7), or allophycocyanin (APC). Antibodies were provided by Immunotech/Beckman Coulter[a], Becton Dickinson[b], Caltag[c], Serotech[d], and Invitrogen[e]. As FITC-conjugated moAbs we used CD1b[b], CD80[ab], CD83[a], CD86[c], CD3[a], CD27[d], CD154[b], and CD45RO[a]. PE-conjugated moABs used were CD80[a],

TABLE 2. DC-differentiating Methods Selected for Individual AML Patients (After Pretesting)

DC-generating Method/ Medium	DC Differentiation Stimulating Substances	Mode of Action	Culture Time in Days	References	DC Method for Individual Samples
MCM-Mimic	GM-CSF, IL-4, TNFα, IL-1β, IL-6, PGE2	Cytokine-based DC differentiation, PGE2 increases CCR7-expression and improves migration	10–14	Kremser et al[9] Dreyssig et al[31]	887 914 748 M569 853 M761 458
Picibanil	GM-CSF, TNFα, lysate from *Streptococcus pyogenes*, PGE2	Bacterial lysate and PGE2 stimulate DC differentiation	7–8	Kremser et al[9] Dreyssig et al[31]	824 855
Ca-Ionophore	IL-4, A23187	Bypass of cytokine-driven DC differentiation	2–3	Kremser et al[9] Dreyssig et al[31]	948 880 938

AML indicates acute myeloid leukemia; DC, dendritic cells; IL, interleukin; PEG, polyethylene glycol; TNF, tumor necrosis factor.

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TABLE 3. T-cell Characterizations

T-cell Populations	Abbreviation	Surface Marker Profile	Explanatory Note	References
T-cell subsets				
Naive T cells	T_{naive}	CCR7$^+$, CD45RO$^-$, CD4$^+$/ CD8$^+$	Unprimed T cells	Sallusto et al[17] Klebanoff et al[21] Fallen et al[23] Hamann et al[27]
Central memory T cells	T_{cm}	CCR7$^+$, CD45RO$^+$, CD4$^+$/CD8$^+$	Long-term immunity	
Effector memory T cells	T_{em}	CCR7$^-$, CD45RO$^+$, CD27$^+$, CD4$^+$/CD8$^+$		
Effector T cells	T_{eff}	CCR7$^-$, CD45RO$^+$, CD27$^-$, CD4$^+$/CD8$^+$	T cells releasing cytotoxins, perforin, granzymes, granulysin	
Non-naive T cells	$T_{non\text{-}naive}$	CD45RO$^+$, CD4$^+$/CD8$^+$	Memory + effector T cells	
Activation and proliferation				
Transferrin-receptor$^+$ T cells	CD71$^+$ T cells	CD71$^+$, CD3$^+$	Proliferation	Rudd et al[34] Starska et al[35] Myers and Vella[36] Ma and Clark[37]
Type II C-type lectin$^+$ T cells	CD69$^+$ T cells	CD69$^+$, CD3$^+$		
CD80/CD86$^-$R$^+$ T cells	CD28$^+$ T cells	CD28$^+$, CD3$^+$	APC-T-cell interaction	
TNF-receptor superfamily member 9$^+$ T cells	CD137$^+$ T cells	CD137$^+$, CD3$^+$		
CD40 ligand$^+$ T cells	CD154$^+$ T cells	CD154$^+$, CD3$^+$		
β-integrins				
β1-integrin$^+$ naive T cells	CD29$^+$ T_{naive}	CD29$^+$, CD45RO$^-$, CCR7$^+$	Adhesion molecules	Gorfu et al[26] Kamiguchi et al[29] deNucci et al[30]
β1-integrin$^+$ central memory T cells	CD29$^+$ T_{cm}	CD29$^+$, CD45RO$^+$, CCR7$^+$		
β1-integrin$^+$ effector memory or effector T cells	CD29$^+$ T_{em+eff}	CD29$^+$, CD45RO$^+$, CCR7$^-$		
β7-integrin$^+$ naive T cells	β7$^+$ T_{naive}	β7$^+$, CD45RO$^-$, CCR7$^+$		
β7-integrin$^+$ central memory T cells	β7$^+$ T_{cm}	β7$^+$, CD45RO$^+$, CCR7$^+$		
β7-integrin$^+$ effector memory or effector T cells	β7$^+$ T_{em+eff}	β7$^+$, CD45RO$^+$, CCR7$^-$		

Surface marker profiles of T-cell subsets, of T cells expressing activation markers or β-integrins including abbreviations are given.

CD83[a], CD86[bc], CD206[a], CD4[a], CD8[b], CD137[c], and CD29[c]. The PC7-conjugated moABs chosen were the following: CD1a[a], CD40[c], CD3[a], CD4[a], CCR7[b], and 7AAD. CD34[a], CD117[a], CD33[a], CD1a[a], CD40[c], CD206[a] CD3[a], CD8[b], CD28[b], CD45RO[e], CD69[b], CD71[b], and β7-integrin[b] were used as APC-conjugated moAbs (Table 3).

For analysis and quantification monocytes and leukemic blasts, or lymphocytes (including several T-cell subpopulations), before or after culture, we defined a "lympho-gate" surrounding these cells. T-cell subgroups were defined by typical antigen coexpressions (Table 3). In preliminary extensive testing, we compared several T-cell characterization strategies using different panels as provided by Sallusto and colleagues.[16,22,23,25] In general, T-cell subsets can be differentiated by their differential expression of CD45RO and/or CD45RA in combination with CD27, CD28, CD62L, or CCR7. In addition, Berger et al[25] have provided an alternative strategy to differentiate T_{naive}, T_{cm}, and T_{em} by analyzing their differential expression of CD62L in combination with CD28, FasReceptor (CD95), and CCR7. We could demonstrate that strategies, as published by Sallusto and colleagues,[16,17,23–25] (based on CD45RO and/or CD45RA positivity) yielded comparable results to respect to proportions of T-cell subtypes, whereas the Berger strategy (based on CD62L and CD95 positivity) yielded only 10% of positive events compared with results with the strategy as proposed by these 4 groups.[25] Therefore, we decided to select the method as provided by Fallen et al[23] based on expression of CD45RO, CD27, and CCR7, which allows not only a differentiation of $\mathbf{T_{naive}}$, $\mathbf{T_{cm}}$, $\mathbf{T_{em}}$, and $\mathbf{T_{eff}}$ but in addition—by combining another T-cell marker—an attribution to further T-cell subtypes (eg, CD3, CD4, CD8, CD29, β7). T-cell subsets were defined by antigen coexpression as follows: $\mathbf{T_{naive}}$ CCR7$^+$

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$CD45RO^-CD4^+$ or $CD8^+$, **T_{cm}** $CCR7^+CD45RO^+$ $CD4^+$ or $CD8^+$, **T_{em}** $CCR7^-CD45RO^+CD27^+CD4^+$ or $CD8^+$, and **T_{eff}** $CCR7^-CD45RO^+CD27^-CD4^+$, or $CD8^+$; $CD69^+CD3^+$, $CD71^+CD3^+$, $CD28^+CD3^+$, $CD137^+CD3^+$, $CD154^+CD3^+$; **$\beta7^+T_{naive}$** $\beta7^+CD45RO^-CCR7^+$, **$\beta7^+Tcm$** $\beta7^+CD45RO^+CCR7^+$, **$\beta7^+T_{em+eff}$** $\beta7^+CD45RO^+CCR7^-$, **$CD29^+T_{naive}$** $CD29^+CD45RO^-CCR7^+$, **$CD29^+T_{cm}$** $CD29^+CD45RO^+CCR7^+$, **$CD29^+T_{em+eff}$** as $CD29^+CD45RO^+CCR7^-$ (Table 3).

Quantification and characterization of DC and DC_{leu} were carried out by flow cytometry, according to our gating strategy already described by former groups.[11,33]

Proportions of positive events in defined gates compared with the isotype controls were calculated using the Cell Quest software. Flow cytometric analyses were carried out on days 0, 1, 3, 5, and 7 followed by a functional cytotoxic fluorolysis assay on day 7 or 8 as described further.

Cytotoxicity (Fluorolysis) Assay

The lytic activity of (stimulated or unstimulated) effector T cells was measured by a fluorolysis assay by counting viable blast target cells, labeled with specific fluorochrome-labeled antibodies, before and after effector cell contact as described.[11] DC-stimulated or MNC-stimulated T cells from AML patients ("autologous" n = 5) or from AML patients after SCT ("after SCT" n = 6) as well as unstimulated T cells as a control were cocultured in 5 mL FACS tubes with thawed blasts as targets for T cells (T) for 4 or 24 hours. Before culture, blast target cells (T) were stained for 15 minutes with 2 FITC and/or PE-conjugated blast-specific antibodies and subsequently cocultured for 24 hours at 37°C and 5% CO_2 with effector cells (E) in an E:T ratio of 1:1. As a control, target and effector cells were cultured separately and mingled shortly before FACS analysis. To evaluate the amounts of viable ($7AAD^-$) target cells and to quantify the cell loss after 24 hours of incubation time, cells were harvested, washed in phosphate-buffered saline, and resuspended in an FACS flow solution containing 7AAD (BD, Biosciences Pharmingen) and a defined number of Fluorosphere beads (Becton Dickinson, Heidelberg, Germany). Viable cells were gated in a SSC/$7AAD^-$ gate and afterward viable cells coexpressing the patient-specific blast phenotype were quantified by taking into account defined counts of calibration beads as described. Cells were analyzed in an FACS Calibur Flow Cytometer using CELL Quest software (Becton Dickinson). The percentage of lysis was defined as the difference between proportions of viable blasts before and after effector cell contact.

Statistical Methods

Means, SD, and results with 2-tailed t test were evaluated with a personal computer using Excel 2007 (Microsoft). Differences were considered as significant if the P value was <0.05.

RESULTS

Prolog

We compared profiles of autologous T cells from healthy donors or AML patients at first diagnosis/during persisting disease/relapse and allogenic T cells from AML patients harvested after SCT. As T-cell profiles ("autologous" or "allogenic") in AML patients were comparable, we pooled these results for comparison with healthy samples.

Profiles of Unstimulated T Cells From AML Patients Differ From Healthy T Cells

We compared proportions of T-cell subtypes in unstimulated cell suspensions in healthy and AML donors (Fig. 1).

AML patients present with higher proportions of T_{naive} and T_{eff} and lower proportions of T_{cm} compared with healthy donors: Significantly higher proportions $CD8^+T_{eff}$ (20% ± 15% vs. 5% ± 3%, $P = 0.006$) and lower proportions of $CD8^+Tcm$ (22% ± 13% vs. 38% ± 21%, $P = NS$) were found in AML compared with healthy donors; comparable proportions of T_{em} and $T_{non\text{-}naive}$ were found in both groups (Fig. 1A). Similar results could be found in $CD4^+$ T cells. Again, significantly higher values of $CD4^+T_{eff}$ (18% ± 16% vs. 6% ± 3%, $P = 0.02$) and of $CD4^+T_{naive}$ (14% ± 12% vs. 5% ± 5%, $P = 0.04$) and lower values of $CD4^+T_{cm}$ (21% ± 16% vs. 37% ± 15%, $P = 0.08$) were found in AML patients (data not shown).

Unstimulated T cells from AML patients show a lower degree of activation compared with healthy donors: In general, AML patients presented with lower proportions of activated/proliferating T cells: significantly lower proportions of $CD28^+$ (80% ± 13% vs. 96% ± 2%, $P = 0.002$) as well as $CD137^+$ T cells (27% ± 21% vs. 51% ± 26%, $P = 0.01$) were seen, whereas no significant differences were found for $CD69^+$, $CD71^+$, and $CD154^+$ T cells (Fig. 1B).

Unstimulated T cells from AML patients show lower proportions of β-integrin expressions in all T-cell subsets: We found significantly lower proportions of nearly all T-cell subtypes coexpressing β-integrins (β7 or CD29) in AML patients compared with healthy donors before MLC: $\beta7^+T_{naive}$ in T_{naive} (34% ± 21% vs. 77% ± 21%, $P = 0.006$); $\beta7^+T_{cm}$ in T_{cm} (33% ± 15% vs. 53% ± 16%, $P = 0.05$); $\beta7^+T_{em+eff}$ in T_{em+eff} (34% ± 14% vs. 56% ± 17%, $P = 0.04$); $CD29^+T_{naive}$ in T_{naive} (81% ± 14% vs. 98% ± 2%, $P = 0.001$) or $CD29^+T_{cm}$ in T_{cm} (96% ± 2% vs. 100% ± 1%, $P = 0.001$) were seen, whereas no significant differences were found for $CD29^+T_{em+eff}$ in T_{em+eff} (Fig. 1C).

In summary, unstimulated T cells from AML patients were characterized by significantly higher proportions of T_{naive} and T_{eff} and lower proportions of activated/proliferating T cells in $CD4^+$ and $CD8^+T_{cm}$ as well as lower proportions of T-cell subtypes coexpressing β7 and CD29 compared with healthy T cells.

T-cell Profiles in AML Differ From Healthy Samples After Stimulation With MNC or DC in MLC

We compared proportions of T-cell subtypes after 7 days of stimulation with DC or MNC in AML or healthy samples (Fig. 2).

Stimulated T cells from AML patients and healthy are characterized by an effector phenotype: In general, T-cell profiles after both stimulation with DC or MNC were characterized by higher proportions of T cells with effector phenotype. Compared with healthy donors significantly

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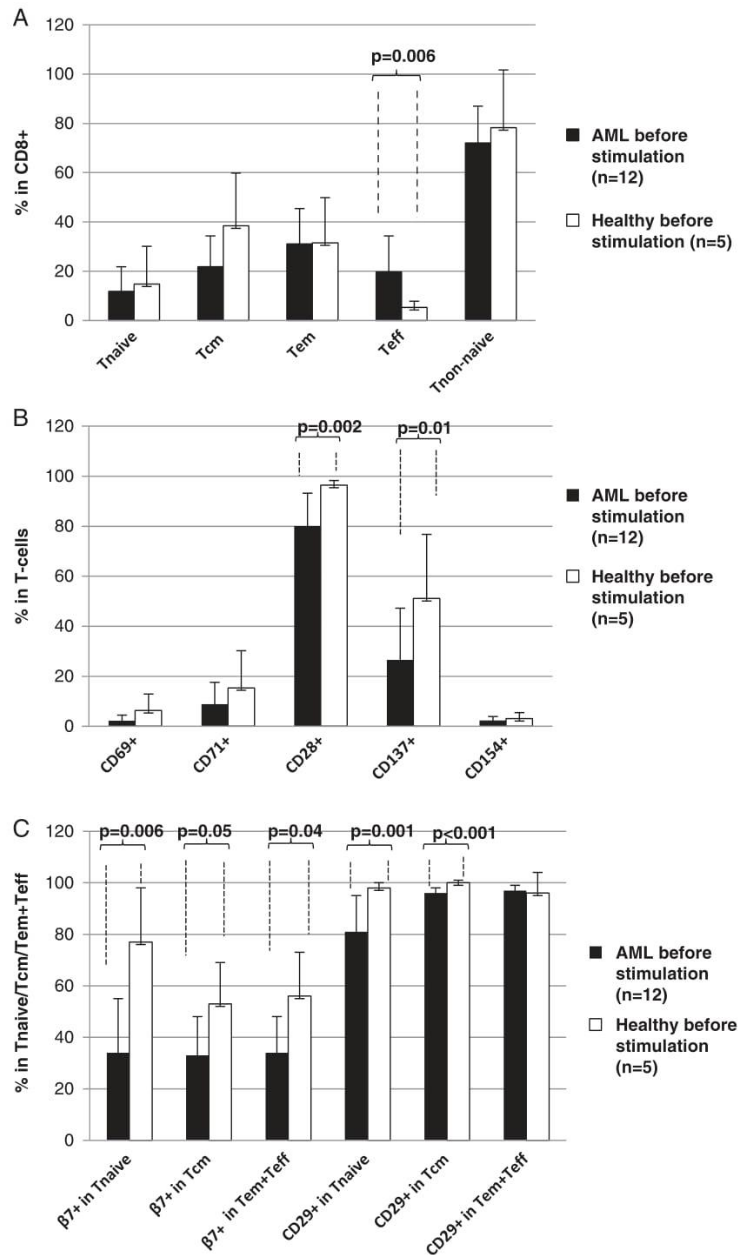

FIGURE 1. T-cell profiles in acute myeloid leukemia (AML) patients and healthy samples **before** dendritic cells (DC) or mononuclear cells (MNC) stimulation. Proportions of (A) T-cell subsets in $CD8^+$T cells, (B) T cells expressing activation markers, or (C) T cell subsets (T_{naive}, T_{cm}, and T_{em+eff}) expressing β-integrins are given.

lower proportions of $CD8^+T_{cm}$ (24% ± 17% vs. 44% ± 13%, $P = 0.03$) and significantly higher proportions of $CD8^+T_{eff}$ (33% ± 21% vs. 16% ± 10%, $P = 0.04$) were found after DC stimulation in AML compared with healthy samples, whereas proportions of $CD8^+T_{naive}$ and $CD8^+T_{non\text{-}naive}$ after DC stimulation did not differ (Fig. 2A). Similar results could be found for $CD4^+$T cells, where AML patients also presented with (not significant) higher proportions of $CD4^+T_{em}$ and T_{eff} and lower proportions of $CD4^+T_{cm}$ (17% ± 15% vs. 39% ± 19%, $P = 0.05$) compared with healthy donors (data not shown). It is interesting to note that we generally could not find any significant differences between DC or MNC stimulation in AML patients. In healthy samples, DC-stimulated T cells showed higher proportions of $CD8^+T_{cm}$ (43% ± 13% vs. 23% ± 8%, $P = 0.02$) and

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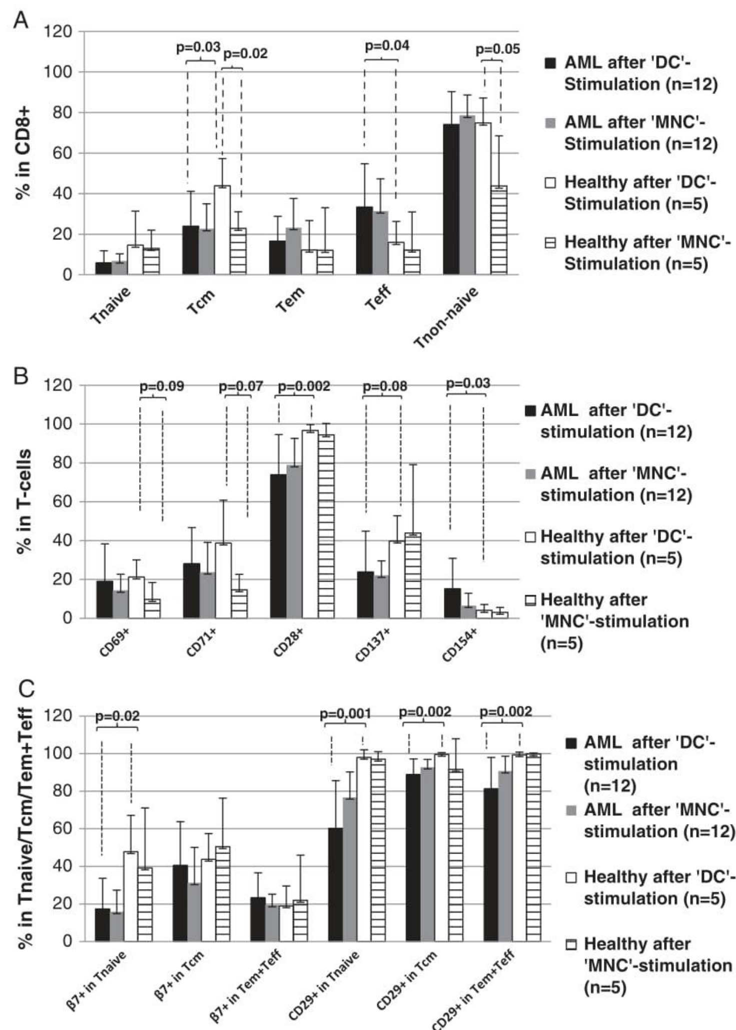

FIGURE 2. T-cell profiles in acute myeloid leukemia (AML) patients and healthy samples **after** dendritic cells (DC) or mononuclear cells (MNC) stimulation. Proportions of (A) T cell subsets in $CD8^+$T cells, (B) T cells expressing activation markers, or (C) T cell subsets (T_{naive}, T_{cm}, and T_{em+eff}) expressing β-integrins are given.

$CD8^+ T_{non-naive}$ (74% ± 12% vs. 44% ± 25%, $P = 0.05$) compared with MNC-stimulated T cells, although differences were not significant (Fig. 2A).

(Significantly) lower proportions of activated/proliferating T cells are found in AML patients compared with healthy donors after stimulation: DC (but not MNC)-stimulated AML T cells showed tendentially significant lower proportions of activated/proliferating ($CD69^+CD3^+$, $CD71^+CD3^{+)}$ T cells compared with healthy donors. Significantly lower proportions of $CD28^+CD3^+$ T cells were found after DC as well as after MNC stimulation in AML compared with healthy samples (DC: 74% ± 21% vs. 97 ± 3%, $P = 0.002$; MNC: 79% ± 14% vs. 95% ± 6%, $P = 0.006$), whereas significantly higher proportions of T cells expressing CD40L were found after DC (but not after MNC) stimulation in AML compared with healthy samples (15% ± 15% vs. 4% ± 3%, $P = 0.03$, Fig. 2B).

Significantly lower proportions of T cells expressing β-integrins are found in AML patients compared with healthy donors after stimulation: After DC (but not after MNC) stimulation, we found significantly lower proportions of nearly all T-cell subtypes coexpressing β-integrins (β7 or CD29) in AML patients compared with healthy donors: $\beta 7^+ T_{naive}$ in T_{naive} (17% ± 16% vs. 48% ± 19%, $P = 0.02$); $CD29^+ T_{naive}$ in T_{naive} (60% ± 23% vs. 98% ± 4%, $P = 0.001$) or $CD29^+ T_{cm}$ in T_{cm} (89% ± 8% vs. 99% ± 1%, $P = 0.002$); and $CD29^+ T_{em+eff}$ in T_{em+eff} (81% ± 16% vs. 99% ± 1%, $P = 0.002$), whereas no significant differences were found for $\beta 7^+ T_{cm}$ in T_{cm} and $\beta 7^+ T_{em+eff}$ in T_{em+eff} in AML compared with healthy samples (Fig. 2C).

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In summary, MNC and, even more, DC-stimulated T cells from AML patients as well as from healthy donors showed increased proportions of T cells with (activated) effector phenotype and decreased proportions of T cells with T_{naive} and/or T_{cm} phenotype. However, significantly lower proportions of T_{cm} were found in AML compared with healthy samples, and especially significantly lower proportions of several β-integrin-expressing T-cell subtypes were found in AML compared with healthy samples.

Kinetic of T-cell Marker Development During DC or MNC Stimulation

We studied proportions of T-cell subtypes (T_{naive}, T_{cm}, T_{em}, T_{eff}, and $T_{non\text{-}naive}$) during the course of MNC and DC stimulation. As AML and healthy samples showed similar kinetics of T-cell subtype proportions during MNC and DC stimulation, we present only data from DC-stimulated T cells in AML samples (Fig. 3).

T_{naive}, T_{cm}, and β7-expressing subtypes decrease and T_{eff} increase during DC stimulation: A decrease of T_{naive} as well as of T_{cm} and an increase of T_{eff} can be seen in CD4$^+$ as well as CD8$^+$ T cells during a 7-day stimulation with DC in AML samples (Fig. 3A). During the 5-day DC stimulation phase, a significant increase of CD69$^+$ T cells between day 0 and day 1 (2% ± 2% vs. 24% ± 14%, $P < 0.001$), of CD154$^+$CD3$^+$T cells (2% ± 2% vs. 13% ± 11%, $P = 0.04$) and of CD71$^+$CD3$^+$T cells between day 0 and day 5 (9% ± 9% vs. 31% ± 13%, $P = 0.01$) was seen (Fig. 3B), whereas proportions of CD28$^+$CD3$^+$ as well as CD137$^+$CD3$^+$T cells stayed stable. All changes in surface marker profiles took place during the first 5 days of stimulation.

Figure 3C gives proportions of T-cell subtypes coexpressing β-integrins (β7 or CD29) during DC stimulation in AML patients (Fig. 3C, left side) and healthy samples (Fig. 3C, right side). A decrease of β7$^+$$T_{naive}$ in T_{naive} and β7$^+$$T_{em+eff}$ in T_{em+eff} was seen for AML as well as healthy cases, whereas proportions of β7$^+$$T_{cm}$ in T_{cm} stayed rather constant during DC stimulation in AML as well as in healthy cases. Proportions of CD29$^+$$T_{naive}$ in T_{naive}, CD29$^+$$T_{cm}$ in T_{cm}, and CD29$^+$$T_{em+eff}$ in T_{em+eff} decreased in AML patients (Fig. 3C).

In summary, we could show that proportions of activated/proliferating T cells as well as of CD154$^+$T cells and T_{eff} (significantly) increase, whereas proportions of T_{naive}, T_{em} β7$^+$$T_{naive}$ and β7$^+$$T_{em+eff}$ decrease during the first 5 days of DC stimulation in AML cases. These developments could be seen in DC-stimulated and MNC-stimulated T cells from AML samples as well as in DC-stimulated and MNC-stimulated healthy cases.

Correlation of T-cell Profiles of DC, MNC, or Unstimulated T Cells With Their Antileukemic Function

DC stimulation improves antileukemic reactivity: We correlated proportions of T-cell subtypes with their antileukemic functionality after 7 days of DC or MNC stimulation (as demonstrated by a cytotoxicity assay) (Table 1). In 7 of 11 (64%) cases, lytic activity of DC-stimulated T cells was achieved with an average lytic efficiency of 62% ± 25% of blasts (range, between 12% and 85%), compared with 4 of 10 (40%) cases after MNC stimulation with an average lytic efficiency of 40% ± 36% of blasts (range, between 9% and 80%). Of the 9 (56%) cases of unstimulated T cells, 5 showed lytic activity. No differences in antileukemic functionality after stimulation were seen for autologous T cells (T_{auto}, prepared from patients before SCT) compared with T cells prepared from patients after SCT (T_{allo}): 3 of 5 (60%) samples with T_{auto} and 4 of 6 (66%) with T_{allo} gained a leukemia-specific cytotoxicity after DC stimulation compared with 1 of 5 (20%) cases with T_{auto} and 3 of 5 (60%) cases with T_{allo} after MNC stimulation (data not shown).

We could demonstrate that not every DC-stimulated T-cell sample acquired the capability to lyse leukemic cells. We subdivided cases in those with a gain of antileukemic cytotoxic activity (lysis) and those without (no-lysis) and studied the composition of T-cell subsets in the groups compared.

Antileukemic function correlates with high proportions of T_{naive} and T_{cm} in unstimulated cell fractions: T-cell samples with antileukemic reactivities presented with (significantly) higher proportions of CD8$^+$$T_{naive}$ (15% ± 11% vs. 5% ± 5%, $P = 0.06$) as well as CD8$^+$$T_{cm}$ and lower proportions of CD8$^+$$T_{eff}$ and CD8$^+$$T_{non\text{-}naive}$ (68% ± 15% vs. 83% ± 9%, $P = 0.08$) CD8$^+$T cells before stimulation compared with cases without antileukemic activity (Fig. 4A, left side). Similar results could be found for CD4$^+$T cells: after DC stimulation, no differences in proportions of T_{naive}, T_{cm}, or T_{eff} in cases with compared with the group without lysis were found, but higher proportions of T_{em} and $T_{non\text{-}naive}$ in the lytic group, although differences were not significant (Fig. 4A, right side). Compared with DC-stimulated T cells, MNC-stimulated T cells showed a similar composition of T-cell subsets in the lysis and no-lysis group, with the exception that MNC-stimulated cases of the no-lysis group presented with (not significantly) higher proportions of CD8$^+$$T_{em}$ (data not shown). All results were similar in CD4$^+$ and CD8$^+$T-cell subgroups (data not shown).

Antileukemic function correlates with high proportions of activated/proliferating T cells: We compared proportions of activated T cells in unstimulated and stimulated cell suspensions in cases with lysis compared with the group without lysis. Before stimulation, the lytic group showed (not significant) lower proportions of CD71$^+$CD3$^+$ and higher proportions of CD137$^+$CD3$^+$ and CD154$^+$CD3$^+$T cells. Comparable proportions of CD69$^+$CD3$^+$ and CD28$^+$CD3$^+$ were found in the groups compared (Fig. 4B, left side). After DC stimulation, cases with lytic activity showed lower proportions of CD69$^+$CD3$^+$ and CD154$^+$CD3$^+$ and higher proportions of CD137$^+$CD3$^+$ and CD28$^+$CD3$^+$T cells and comparable proportions of CD71$^+$CD3$^+$T cells (Fig. 4B). After MNC stimulation, cases with lysis showed (not significant) higher proportions of CD71$^+$CD3$^+$, CD28$^+$CD3$^+$, and CD154$^+$CD3$^+$T cells and comparable proportions of CD69$^+$CD3$^+$ and CD137$^+$CD3$^+$T cells (data not shown).

As the expression of activation markers on T cells is a dynamic process, we also analyzed the proportions in the lytic compared with the nonlytic group at days 1, 3, and 5 during stimulation. We found that samples with antileukemic functionality at day 7 showed significantly higher proportions of

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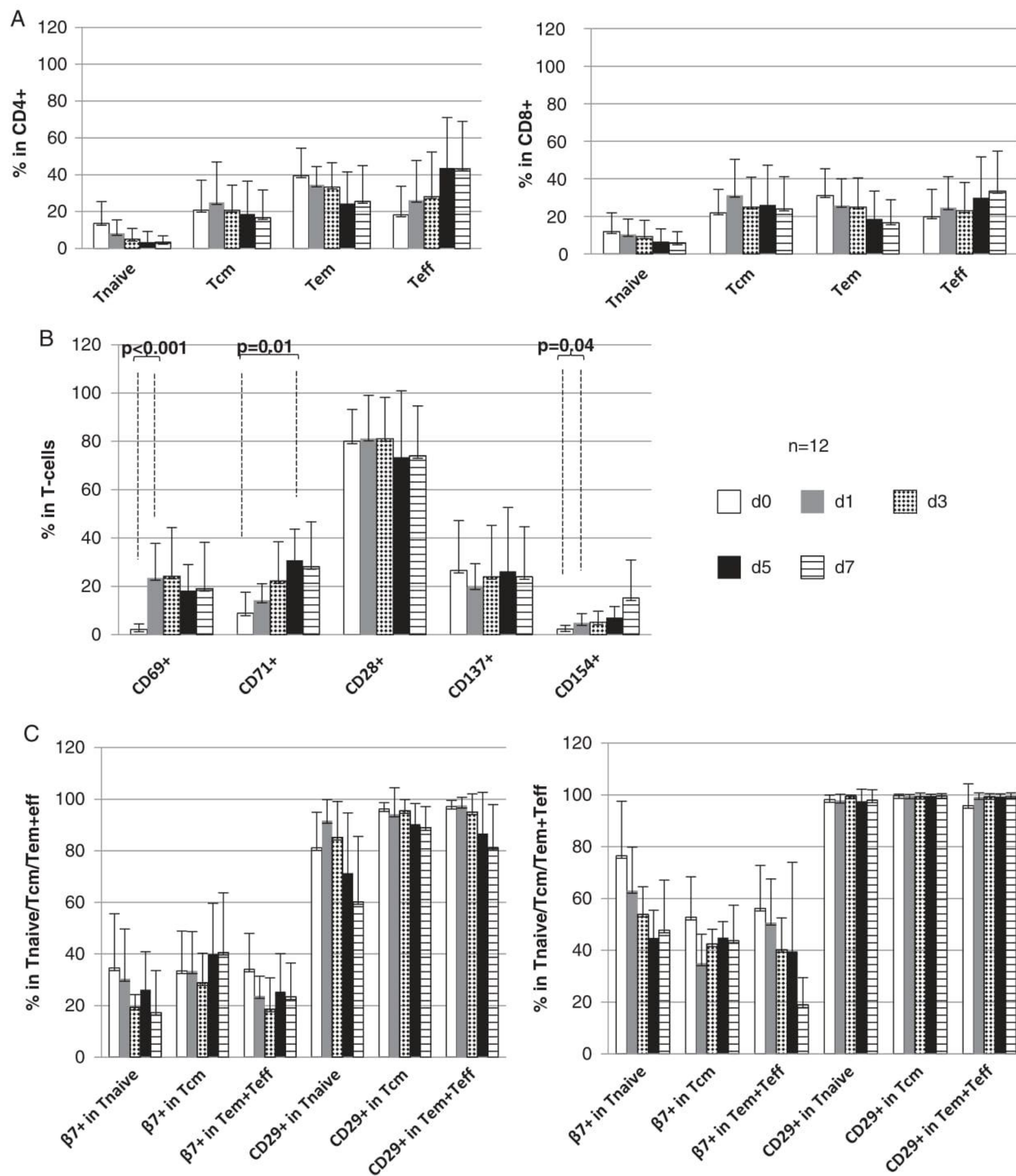

FIGURE 3. Kinetics of T-cell profiles in mixed lymphocyte cultures during stimulation with dendritic cells (DC) from acute myeloid leukemia (AML) patients or healthy donors. Proportions of (A) T-cell subsets (T_{naive}, T_{cm}, T_{em}, and T_{eff}) in CD4$^+$T cells (left side) and CD8$^+$ T cells (right side), (B) T cells expressing activation markers, or (C) T-cell subsets (T_{naive}, T_{cm}, and T_{em+eff}) expressing β-integrins in AML patients (left side) and in healthy samples (right side) are given.

CD69$^+$ and CD137$^+$T cells during DC stimulation compared with T cells without lytic function. Lytically active T cells showed higher proportions of CD69$^+$CD3$^+$T cells at day 5 (26% ± 7% vs. 10% ± 6%, P = 0.006) and of CD137$^+$CD3$^+$T cells at days 1 (27% ± 6% vs. 11% ± 6%, P = 0.006), 3 (32% ± 25% vs. 10% ± 6%, P = 0.08), and 5 (35% ± 30% vs. 7% ± 3%, P = 0.05). Moreover, lytically active T cells showed higher proportions of CD71$^+$CD3$^+$T cells at day 5 and CD28$^+$CD3$^+$T cells at days 1, 3, and 5, although differences were not significant (data not shown). It is interesting to note that there were no significant differences between lytic and nonlytic groups during MNC stimulation.

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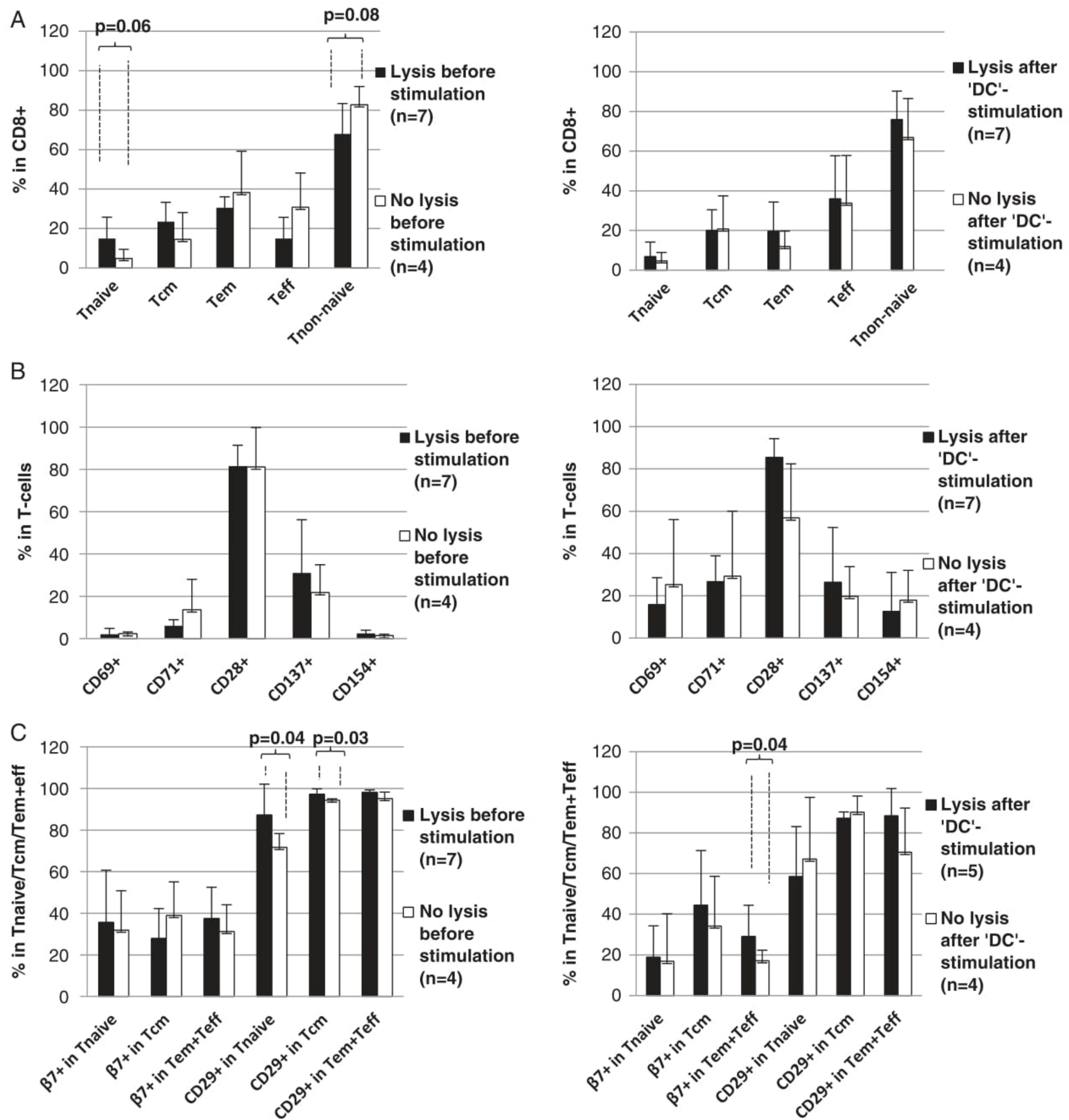

FIGURE 4. T-cell profiles and antileukemic functionality of T cells [prepared from acute myeloid leukemia (AML) patients before or after stem cell transplantation (SCT)] before or after stimulation with dendritic cells (DC). T-cell profiles of AML samples with lytic activity (lysis) compared with AML samples without lytic activity (nonlysis) before (left) or after (right) DC stimulation are given: (A) T-cell subsets in CD8$^+$T cells, (B) T cells expressing activation markers or (C) T-cell subsets (T_{naive}, T_{cm}, and T_{em+eff}) expressing β-integrins.

Antileukemic function correlates with high proportions of $CD29^+T_{naive}$, $CD29^+T_{cm}$, and $β7^+T_{em+eff}$ T cells before stimulation as well as $β7^+T_{em+eff}$ and $CD29^+T_{em+eff}$ after DC and MNC stimulation: We compared proportions of β-integrin expressions on T-cell subtypes in unstimulated and stimulated cell suspensions in the lytic and nonlytic group. Before stimulation, lytic samples showed significantly higher proportions of $CD29^+T_{naive}$ in T_{naive} (87% ± 14% vs. 72% ± 7%, $P = 0.05$) and $CD29^+T_{cm}$ in T_{cm} (97% ± 3% vs. 94% ± 1%, $P = 0.03$) as well as higher proportions of $CD29^+T_{em+eff}$ in T_{em+eff} and $β7^+T_{naive}$/$β7^+T_{em+eff}$ in T_{naive} and T_{em+eff}, and lower proportions of $β7^+T_{cm}$ in T_{cm}, although differences were not significant. After DC stimulation, lytic samples showed higher proportions of $β7^+T_{naive}$ in T_{naive}, $β7^+T_{cm}$ in T_{cm}, and $β7^+T_{em+eff}$ in T_{em+eff} as well as $CD29^+T_{em+eff}$ in T_{em+eff}, although differences were not significant and comparable proportions of $CD29^+T_{cm}$ in T_{cm} (Fig. 4C). In general, MNC-stimulated samples showed similar results (data not shown). In contrast, lytically active samples showed lower proportions of $CD29^+T_{naive}$ in T_{naive} after DC stimulation, whereas higher proportions of $CD29^+T_{naive}$ in T_{naive} after MNC stimulation could be found.

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In summary, cases with antileukemic cytotoxicity presented with higher proportions of activation marker positive T cells (expressing, eg, CD69, CD28, CD137) during DC stimulation. Moreover, cases with antileukemic activity showed higher proportions of $CD29^+$ and $\beta7^+$ non-naive T cells before and after both DC and MNC stimulation, as well as higher proportions of T_{naive} and T_{cm} before stimulation.

Proportions of T-cell Subsets Predict Their Antileukemic Activity as well as the Clinical Course of the Patients

Proportions of unstimulated and even more DC-stimulated T_{naive}, T_{cm}, nonproliferating, $\beta7^+$, or $CD29^+$ T-cell subsets contribute to predict the T cells' antileukemic reactivity: To predict the lytic activity, we created cutoff values that allow a separation of cases in 2 groups with higher or lower proportions of certain T-cell subpopulations. It is interesting to note that cutoff values could be evaluated for unstimulated and DC-stimulated, but not for MNC-stimulated T cells. We found that cases with higher proportions than >7.5% of $CD8^+T_{naive}$ and >12% $CD8^+T_{cm}$ and lower proportions than <24% $CD8^+T_{eff}$ before stimulation showed in 75% to 86% of cases antileukemic T-cell activity (Fig. 5A, left side). Vice versa, only 40% and 25% of cases with proportions of $CD8^+T_{naive}$ and $CD8^+T_{cm}$ below these thresholds gained the capability to lyse blasts. The same cutoff values could be evaluated for DC-stimulated T cells. However, after DC stimulation, 71% of cases with <7.5% $CD8^+T_{naive}$ showed lytic activity in ex vivo settings, whereas 71% of cases with proportions >12% $CD8^+T_{cm}$ and 71% of cases with proportions >24% $CD8^+T_{eff}$ went along with antileukemic T-cell reactivity, as demonstrated in the cytotoxicity assay (Fig. 5A, right side). Similar results could be found for $CD4^+T_{naive}$ and $CD4^+T_{cm}$ (data not shown).

Moreover, we could demonstrate that low counts of activated T cells before stimulation correlated with higher rates of cytotoxicity: 100% of cases with <1.5% $CD69^+CD3^+$ T cells, 83% of cases with <6% $CD71^+CD3^+$ T cells, and 83% of cases with >1.8% $CD154^+CD3^+$ T cells before DC stimulation were able to lyse blasts (Fig. 5B, left side). In general, cases with higher proportions of activated T cells after the DC stimulation showed higher rates of lytic functionality, although cutoff values could not be defined for all activation markers: 80% to 100% of cases gained blast lytic functionality, when samples contained >19% $CD69^+CD3^+$, >18% $CD71^+CD3^+$, >85% $CD28^+CD3^+$, and >11% $CD137^+CD3^+$ T cells after 5 days of DC stimulation (Fig. 5B, right side). Similar cutoff values could be found on day 1 (CD69, CD71, and CD137), day 3 (CD137), and day 7 (CD69, data not shown).

Furthermore, we observed that higher proportions of T cells expressing β-integrins before DC stimulation were predictive for antileukemic functionality: 71% to 100% of cases with >26% $\beta7^+T_{em+eff}$, >79% $CD29^+T_{naive}$, >95% $CD29^+T_{cm}$, or >97% $CD29^+T_{em+eff}$ before DC stimulation were able to lyse blasts (Fig. 5C, left side). Similar results could be found for T_{em+eff} after stimulation: All cases with >26% $\beta7^+T_{em+eff}$ or >90% $CD29^+T_{em+eff}$ showed lytic activity. It is interesting to note that we found inverse results for T_{naive} and T_{cm} after DC stimulation: none of the cases with >79% $CD29^+T_{naive}$ or >95% $CD29^+T_{cm}$ was able to lyse blasts, whereas lower proportions of $CD29^+T_{naive}$ and T_{cm} went along with a high probability of antileukemic functionality (Fig. 5C, right side).

In summary, cases with higher proportions of $CD8^+T_{naive}$, $CD8^+T_{cm}$, $CD3^+CD154^+$, and β-integrin$^+$ T-cell subsets as well as cases with lower proportions of $CD8^+T_{eff}$ or activated T cells before DC stimulation went along with high probabilities of antileukemic functionality. After DC stimulation, higher proportions of $CD8^+T_{cm}$, $CD8^+T_{eff}$, higher proportions of T cells expressing activation markers and β-integrins on T_{em+eff} or lower proportions of $CD8^+T_{naive}$, $CD29^+T_{naive}$, and $CD29^+T_{cm}$ were predictive for antileukemic activity.

High proportions of T_{cm} before and T_{eff} after DC stimulation correlate with the clinical response to immunotherapy: We correlated our findings with the clinical course of the patients: 9 of our 12 patients (75%) received immunotherapy, of which 2 patients (22%) did not respond, 6 patients (67%) responded, and 1 patient (11%) died of SCT-related complications. The 3 remaining patients did not achieve a remission by chemotherapy. In 4 of 6 responders to immunotherapy (67%) and 2 of 3 nonresponders (67%) a blast lytic activity in the cytotoxicity test after DC stimulation could be demonstrated.

In general, patients responding to immunotherapy presented with (significantly) higher proportions of $CD8^+T_{cm}$ before (20% ± 12% vs. 11% ± 1%, $P=0.13$) and $CD8^+T_{eff}$ after DC stimulation (46% ± 21% vs. 20% ± 6%, $P=0.03$) compared with nonresponders. Concerning other T-cell subgroups, especially activated or β-integrin$^+$ T cells, no significant differences in response rates to immunotherapy were found (data not shown).

DISCUSSION

Role of T Cells in Tumor Immunity

Allogeneic SCT is the only curative treatment option for patients, with AML and donor T cells mediating a strong antileukemic effect, although relapses occur.[38–40] T cells are main mediators of anti-tumor reactivity. In healthy persons, they are mobilized by DC, presenting phagocytized and processed tumor antigens—resulting in antitumor reactive effector T cells. Moreover, T-memory cells allow an immediate response upon repeated challenge with known antigens.[12] A retreatment of AML patients who underwent donor lymphocyte infusion is a transfusion of donor T cells that results in complete remission in a great proportion of patients.[9,10,39–41]

Few data are available about the development and activation of T cell subsets under different stimulation conditions and their correlations with antileukemic functionalities. Additional knowledge in this area could contribute to prepare or manipulate defined T-cell subsets and improve immunotherapy of AML.

The Best Strategy for T-cell Characterization

In preliminary experiments, we have evaluated and compared different strategies to characterize T cells and their subtypes, as described in the literature.[16,42,43] The aim was to establish a reliable T-cell panel, simple in application for qualitative and quantitative T-cell characterization under different stimulatory conditions. As mentioned in the Materials and methods section CD45RO, CD27, and CCR7 proved to be ideal markers for characterization of

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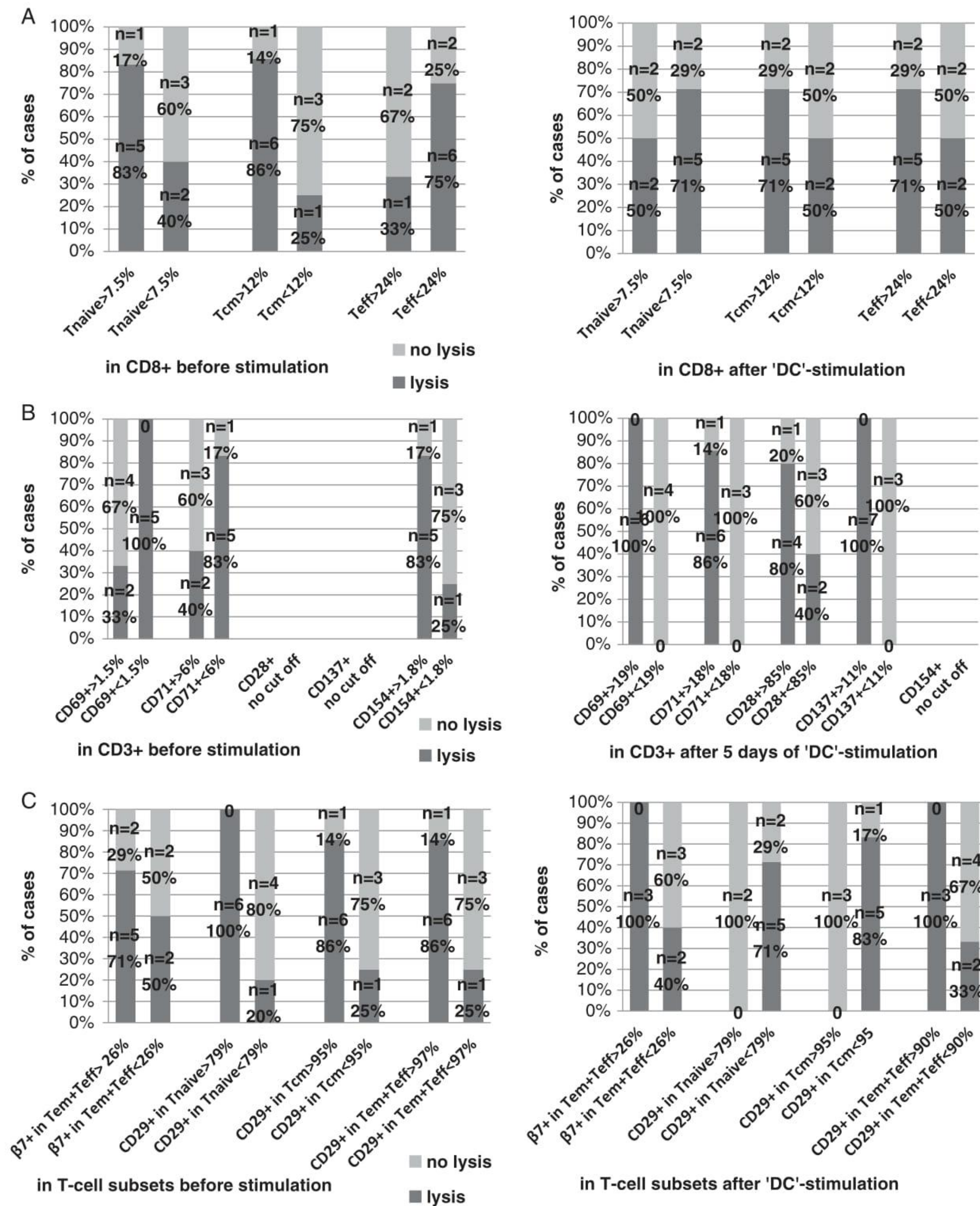

FIGURE 5. Predictive "cutoff" values of T-cell subset proportions separating samples' cohorts in cases with/without antileukemic functionality before stimulation (left) or after (right) dendritic cells (DC) stimulation. Proportions of (A) T-cell subsets in CD8$^+$T cells, (B) T cells expressing activation markers or, (C) T-cell subsets (T_{naive}, T_{cm}, and T_{em+eff}) expressing β-integrins are given.

T-cell subsets T_{naive}, T_{cm}, T_{em} T_{eff}, and $T_{non-naive}$. Results of preliminary experiments showed comparable proportions of T-cell subtypes using panels as recommended by Sallusto and colleagues.[16,23,42] The "Fallen" strategy offers the possibility to combine a fourth surface marker (eg, $CD4^+$,$CD8^+$,$CD29^+$, etc.)—for example, in a 4-Channel Flow cytometer that enables further subtyping of T-cell subsets. Some groups prefer a CD45RA-based strategy, addressing the protein tyrosine phosphatase CD45RA instead of CD45RO to discriminate between $CD45RA^+$

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CD45RO$^-$T$_{naive}$ and CD45RO$^+$CD45RA$^-$T$_{non\text{-}naive}$.[43] We decided to use CD45RO to distinguish T$_{naive}$ and T$_{non\text{-}naive}$ as the presence of CD45RA$^+$ T$_{em}$ (T$_{emRA}$), as described in the literature, could interfere with our results.[44]

Unfortunately, we could not confirm the methodological strategy (in preliminary experiments), as recommended by Berger et al[25]: in our hands only 10% of several T-cell subsets were found with the "Berger" protocol compared with the 3 different strategies as described by Fallen, Sallusto, or Klebanoff, as previously shown. This could be because of a highly specialized gating/quantification strategy of T-cell subsets in the "Berger" protocol, which we could not reproduce. Alternatively, different T-cell subtypes could be discussed that might be detected by the "Berger" protocol but not by protocols of the remaining groups.

In consequence, we decided to use a modified T-cell characterization strategy as provided by the "Fallen"-"Sallusto"-"Klebanoff" protocols.

T-cell Subtypes in AML Compared With Healthy Samples

Compositions of unstimulated T cells in AML patients differ from healthy T cells: Our data show that uncultured T cells, either AML patients' T-cells before (autologous) or after SCT, were characterized by (significantly) higher proportions of T$_{naive}$ and T$_{eff}$ and lower proportions of CD4$^+$ and CD8$^+$T$_{cm}$, activated/proliferating as well as β-integrin/CD29-expressing T cells compared with healthy donors. Inappropriate stimulation, but also different immune escape mechanisms, induced by leukemic cells, can lead to an insufficient T-cell response and anergic T cells poorly responding to antigenic stimulation and activation.[8,45] Our results confirm these former findings and could be interpreted in the biological context of AML, characterized by permanent stimulation of T cells by residual leukemic cells, resulting in increased (probably ineffective) effector cell proportions. Moreover, increased proportions of T$_{naive}$ and reduced proportions of activated T cells could point to a T-cell anergy or insufficient specific stimulation in the context of antileukemic immunity.

Stimulation of T cells from AML patients with MNC and DC increases proportions of activated T cells with effector function: We and others could already show that the leukemia-induced T-cell anergy could be overcome, especially by DC stimulation.[7–9,11,45] Our data show that T-cell profiles from AML and healthy donors showed a change toward a profile with higher proportions of T cells with effector phenotype after DC stimulation as well as MNC stimulation. As before stimulation, T cells from AML patients showed lower proportions of T$_{cm}$-expressing, activation marker-expressing, and β-integrin-expressing T cells but higher amounts of T$_{eff}$.

This could mean that, compared with healthy probands, AML patients present with higher proportions of (ineffective?) T$_{naive}$ and T$_{eff}$ and in consequence lower proportions of T$_{cm}$. This could be interpreted as a reactive overproduction of T$_{eff}$ possibly because of permanent confrontation with leukemic antigens compared with healthy donors.

In contrast, the reduced number of T$_{cm}$ points to an impaired generation of T$_{cm}$ or T$_{memory}$ in general. The importance of T$_{memory}$ for the immune response is undisputed.[12] Spranger et al[46] could demonstrate that CD8$^+$ T$_{em}$ are characterized by increased cytotoxin expression and contribute to an improved killing of tumor cells, and Klebanoff et al[21] could show that T$_{cm}$ are even more important than T$_{em}$ for adoptive immunotherapies. Possibly an impaired T$_{cm}$ generation in AML patients could contribute to tumor escape, outbreak of the disease, or its relapse.

The T cells' grade of activation can be measured by several activation markers. CD69 and CD71 are described as suitable proliferation markers, as they are not expressed on unstimulated T cells and show a strong upregulation after stimulation.[35] CD28, CD137, and CD154 are expressed on T cells during activation and transmit costimulatory signals.[34,36,37] Some groups use CD28 to characterize T$_{eff}$ as CD28$^-$CD8$^+$CD57$^+$ because it gets lost after stimulation.[47] In general, AML patients presented with lower proportions of activated cells during stimulation with DC or MNC. That could possibly indicate a T-cell anergy or diminished specific antileukemic T-cell activation in AML samples. Interestingly, high proportions of activated T cells were found after mixed lymphocyte culture of T cells with DC or MNC as well as after stimulation of healthy T cells with healthy DC. The upregulation of activated T cells in healthy samples might be explained by antigenic stimuli caused by infectious antigens or inflammatory stimuli. Alternatively, the T-cell stimulating cytokine IL-2 might induce an upregulation of CD69 and CD71. The only activation marker that increased exclusively in AML samples was CD154, the receptor for CD40. Houtenbos et al[48] have already shown that DC$_{leu}$ express CD154L (= CD40), and that T-cell proliferation and priming efficacy of tumor-specific cytotoxic T cells can be improved by αCD40/αCD28 diabodies. Vice versa, Lee et al[49] could show that additional CD40L improves DC generation. We suggest that T-cell stimulation by DC is not dependent on CD154 but can be enhanced by mutual CD154-CD40 DC-T-cell interactions. CD28 and CD137 did not seem to be useful activation markers in our setting—at least not in the time range used—as they were highly expressed before stimulation and remained at the same level in healthy samples or were even slightly downregulated in AML. Taken together CD69 and CD71 qualify as general proliferation markers for healthy as well as AML samples (as already shown before)[9,11,32] and CD154 might be a promising marker to characterize specific activation marker after DC stimulation in AML.

Decreased proportions of β-integrin-expressing T cells after stimulation as a specific sign of antileukemic T-cell activation?: We found significantly lower proportions of CD29$^+$T cells in AML patients after stimulation compared with healthy donors. CD29 is involved in the migration of leukocytes to BM or inflamed tissues, β7-integrin in the migration to gut-associated lymphoid tissues.[50] Vermeulen et al[51] could confirm the hypothesis that CD29 also plays a role in the stem cell homing to the BM. DeNucci et al[30] found that T$_{cm}$ lacking CD29 lose their adhesion to stroma cells in the BM and emigrate from BM. As in general, β-integrins can be regarded as adhesion molecules and play a role in T-cell trafficking and stimulation,[26–29,52] our results could mean that T cells in AML patients not only have a reduced recruitment of T-cell precursors (T$_{em+eff}$) expressing β-integrins but also in general lower proportions of β-integrin$^+$T cells. This could go along with a modified trafficking and stimulation of these T cells in AML patients: possibly β-integrin-expressing T cells are found in

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comparable proportions as T cells expressing costimulatory surface molecules (eg, CD28), with lower proportions found in AML patients compared with healthy donors. Alternatively, T cells expressing β-integrins could resemble healthy T cells presenting "anchors" that are found in high proportions and necessary for every kind of antigen contact, but are reduced in AML patients.

Development of T-cell subtypes over time: Concerning T-cell developments under stimulation, we confirm former data of the literature: MNC-stimulated and even more DC-stimulated T cells from AML patients as well as from healthy donors showed increased proportions of T cells with effector and/or T_{cm} phenotype and decreased proportions of T cells with T_{naive} phenotype. Sallusto and colleagues described the alteration of the T-cell profile during activation toward a T-cell profile with higher proportions of T cells with effector function.[16,21,25]

According to the literature, immune responses are divided into 4 phases: an "innate," "contraction," "expansion," and "memory phase." In virus-specific immune responses, the maximum level of proliferation and differentiation is achieved 8 days after infection, at the end of the "expansion phase."[53,54] In contrast, we could show that a stable ratio with maximum proportions of T_{eff} is achieved after 5 days of stimulation. We suggest that the target-orientated activation of T cells through DC, but also MNC in our approach speeds up the T-cell proliferation and activation. Alternatively, our findings could be explained by the occurrence of T_{cm} in the cell suspensions, which might differentiate faster and stronger into T_{eff} than T_{naive}.[12]

The generation of T-cell memory over time is still not completely understood. It could be shown that after stimulation, T cells differentiate from T_{naive} to T_{cm}, and from T_{cm} to T_{em} and T_{eff} depending on strength and duration of the stimulus.[42] These T-cell subtypes can be measured/quantified by their subtype-typical expression of surface molecules, telomere length, and secretion of cytokines and perforines.[16,17,42,55] Others suggest that a small proportion of T_{eff} does not undergo apoptosis but differentiate into T_{cm} after antigen clearance and remains as a memory cell population.[13,14] As we could detect a little increase of proportions of T_{cm} at day 1, we rather suggest that T_{naive} differentiate directly into T_{cm} and have not passed through the effector stage, even if our setting is not sufficient to prove that.

Antileukemic Functionality Correlates With T-cell Subtypes

Our results confirm former findings of our group that ex vivo stimulated T cells can gain antileukemic functionality and that DC-stimulated T cells are superior to MNC or unstimulated T cells, with respect to their antileukemic activity[9,11]: 64% of cases after DC stimulation gained antileukemic functionality compared with 40% after MNC stimulation. We suggest that this difference can be explained by the capability of DC_{leu} to present the whole leukemic antigen repertoire and to interact with T cells,[8] although at a first glance no significant differences in T-cell proportions after DC or MNC stimulation were found.

DC stimulation improves antileukemic functionality of T cells and goes along with different T-cell profiles in cases with compared with cases without antileukemic functionality: We could identify and correlate patterns of T-cell subset compositions with their antileukemic activity. Cases with antileukemic cytotoxicity presented with (significantly) higher proportions of $CD4^+/CD8^+$ T_{naive}, $CD4^+/CD8^+$ T_{cm} and lower proportions of $CD4^+/CD8^+$ T_{em} and $CD4^+/CD8^+$ T_{eff}. That means that cases with higher proportions of T cells with a lower grade of differentiation before stimulation develop better immunoreactions: T_{naive} and T_{cm} are cells with a high potential to undergo a stimulation, proliferation, and differentiation into T cells with effector function. In contrast, cases with high proportions of T_{eff} before stimulation have a higher chance to fail in the lysis test. However, not only the proportion of T_{cm} *before* DC stimulation, but also after stimulation seems to be important for the efficacy of the immune response. "Cutoff" analyses showed that higher proportions of T_{cm} after stimulation correlated with good antileukemic functionality. As T_{cm} are defined by the lack of or reduced effector functions,[16,17,56] it is not clear how T_{cm} influence immune reactions after stimulation. It is possible that the increased occurrence of T_{cm} in the lysis group is a sign of successful T_{cm} generation, which correlates with a successful antigen clearance. Vice versa patients without sufficient immune response are unable to produce T_{cm}. According to that, T_{cm} could be used as a predictive marker to monitor antileukemic activity in a course of immunotherapy such as allogenic SCT.

Besides, T_{cm} T_{eff} are the main mediators of cellular immune response: high proportions of T_{eff} after stimulation correlated with good antileukemic functionality. However, not only the quantity of T_{eff} is important but also their quality. We found that AML patients present with higher proportions of (probably anergic) T_{eff} before stimulation compared with the healthy control group, what might be because of AML patients', but not healthy donors', permanent contact with leukemic cells in vivo. This proves that the quantity of T_{eff} alone does not provide a successful antigen clearance. In contrast with our findings, after stimulation patients with lower proportions of T_{eff} before stimulation had a higher chance to develop an antileukemic response after DC stimulation, what might be explained by the patients' as low proportions of T_{cm}/T_{naive} before stimulation, that could be primed for antileukemic response. In addition, patients with high proportions of T_{naive} and T_{cm} showed highest lytic activity after DC stimulation, pointing to the central role of a pool of unprimed or memory cells for antileukemic reactivity.

High proportions of β-integrins correlate with antileukemic functionality: We found higher proportions of $CD29^+/β7$-integrin$^+$ T_{cm} and T_{naive} before stimulation to be associated with good antileukemic activity. As β-integrins, especially CD29, transmit costimulatory signals and play a role in the proliferation and IL-2 production of T cells,[29] a lack of β-integrins might correlate with a lower capability to stimulate T cells. Kim et al[57] showed that costimulatory signaling through CD29 augments T-cell stimulation, and Kuklin et al[58] suggested that α4β7 facilitates immune response in the gut. That could explain why lytically active T cells (that require IL-2 for optimal proliferation) present with higher proportions of $CD29^+$ T_{naive} before stimulation.

Moreover, our cutoff analyses showed a correlation of high proportions of $CD29^+/β7$-integrin$^+$ T_{em+eff} after DC stimulation with antileukemic functionality. We conclude that $CD29^+/β7$-integrin$^+$ T_{naive}/T_{cm} cells, which differentiate into $CD29^+/β7$-integrin$^+$ T_{eff}, are the T_{eff} subpopulation with the best antileukemic functionality. According to that, CD29 and β7-integrin could serve as a

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marker for newly generated T_{eff} with antileukemic functionality.

Proportions of activated T cells during DC stimulation correlate with antileukemic functionality: Antileukemic activity correlated clearly with proportions of activated T cells. However, this correlation could not be found at day 7. That can be explained by the fact that activation markers are often only temporarily expressed and some of them were already downregulated after 7 days of DC stimulation. In contrast, during the stimulation process, patients with antileukemic activity (lysis group) after 7 days showed higher proportions of T cells expressing activation markers at days 1, 3, and 5 compared with those without lytic activity (nonlysis group). We conclude that patients with higher proliferation capabilities (in between 5 d of stimulation) are superior to develop antileukemic functionality. These results indicate the predictivity of an expression of activation markers for the antileukemic functionality of T cells.

Clinical Relevance of DC Subtypes for Immunotherapy of AML

We could show that antileukemic functionality can be predicted by the T-cell profile of AML patients before and after DC stimulation. More detailed cutoff analyses revealed that the composition of T-cell subsets and activation markers are useful to predict antileukemic activity and could therefore contribute to predict a response to immunotherapy.

In the previous chapter, we could show that patients with higher proportions of T cells with high proliferation potency such as T_{cm} and T_{naive} benefit more from DC stimulation than patients with high proportions of (probably dysfunctional) T_{eff}. That would mean that the immune system of patients who suffer from a relapse after SCT, but present with an adequate high number of T_{cm} could possibly be reactivated through a specific stimulation, for example, through DC. In addition, we could show that higher proportions of T_{cm} and T_{eff} after stimulation correlate with good antileukemic functionality and could therefore be used as predictive markers in immunotherapy. Similarly, CD69, CD71, CD137, and β-integrin could be used in analogy as predictive markers: our data suggest that $CD69^+$ and $CD71^+$ T cells, as well as $CD28^+$ and $CD137^+$ or $CD29^+$/β-integrin$^+$ T cells are responsible and are required for antileukemic responses. In consequence, strategies for adoptive immunotherapy could be deduced from our data: an adoptive transfer of T_{cm} or T_{eff} as discussed by Klebanoff et al[21,25] or Berger et al[25] could provide a pool of cells with high proliferative potency and high resistance to apoptosis, that are—in contrast to T_{em}—able to mediate a longtime memory against leukemic cells without inducing GvHD reactions.[16,17,59] Moreover, our data again point to the promising role of DC stimulation and/or the role of an in vivo-induced DC-mediated immune stimulation (without requiring additional costimulatory signals), which may result in specific (re)activation of antileukemic T cells from T_{naive} or T_{cm} pools and could improve T-cell-based immunotherapies such as SCT or donor lymphocyte infusion.[12,14]

CONCLUSIONS

In conclusion, we could show that T-cell profiles from AML patients differ from T-cell profiles from healthy samples. We suggest that these differences are due to a permanent stimulation of T cells by residual leukemic cells and reflect the inappropriate immune response to AML. In addition, our data show that this state can be overcome by DC-based T-cell stimulation giving rise to functional T cells: in samples presenting with high proportions of T cells with high proliferation potency such as T_{cm} and T_{naive} as well as of β-integrin$^+$ T cells' antileukemic reactions could be more likely be induced.

This could mean that the immune system of patients who suffer from a relapse after SCT, but present with a high number of T_{cm}, could possibly be reactivated through stimulation with leukemia-derived DC in vivo or in vitro DC stimulation and, in addition, those markers as as listed previously can be used as predictive markers to monitor the immune reactive potential of T cells and their subsets in the course of the disease.

ACKNOWLEDGMENTS

The authors thank the nurses and physicians on the wards for their support. They also thank the employees of the diagnostic laboratories for the patients' diagnostic reports. The results presented in this manuscript were worked out in the doctoral theses of J.S. and V.V. at the University Hospital Großhadern of the Ludwig-Maximilian-University, Munich.

CONFLICT OF INTEREST/ FINANCIAL DISCLOSURE

All authors have declared that there are no financial conflicts of interest with regard to this work.

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8 Publication III, Ansprenger et al. 2018

Cellular Immunology 328 (2018) 33–48

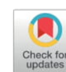
Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Paramunity-inducing Factors (PINDs) in dendritic cell (DC) cultures lead to impaired antileukemic functionality of DC-stimulated T-cells



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ARTICLE INFO

Keywords:
AML
Paramunity
Dendritic cells
T-cells
Immunotherapy
PIND
Zylexis

ABSTRACT

Introduction: Paramunity-inducing-Factors (PINDs) consist of attenuated/inactivated viruses of various pox-virus-genera, used in veterinary medicine as non-antigen-specific, non-immunising stimulators of the innate immune system against infectious and malignant diseases. Their danger-signaling-interactions were tested for their capacity to improve leukemic antigen-presentation on DC generated from AML-patients' blasts ('DC_{leu}') and DC-stimulation/activation of antileukemic T-cells.
Methods: We analyzed, whether the addition of PINDs during DC cultures (15 healthy, 22 leukemic donors) and mixed lymphocyte culture (MLC, n = 15) with autologous (n = 6), allogeneic (n = 2) or T-cells after stem cell transplantation (SCT; n = 7) would alter the quality and quantity of DC, the composition of T-cell-subsets, and/or their antileukemic functionality (AF) as studied by FACS and functional Fluorolysis-cytotoxicity-assays.
Results: **Effects on 1. DC-cultures:** PINDs in DC-cultures lead to increased proportions of mature DC and DC_{leu}, but reduced proportions of viable and overall, as well as TLR4- and TLR9-expressing DC. **2. MLC:** PINDs increased early (CD8+) T-cell activation (CD69+), but reduced proportions of effector-T-cells after MLC **3. AF:** Presence of PINDs in DC- and MLC-cultures reduced T-cells' as well as innate cells' antileukemic functionality. **4. Cytokine-release profile:** Supernatants from PIND-treated DC- and MLC-cultures resembled an inhibitory microenvironment, correlating with impaired blast lysis.
Conclusions: Our data shows that addition of PINDs to DC-cultures and MLC result in a "blast-protective-capacity" leading to impaired AF, likely due to changes in the composition of T-/innate effector cells and the induction of an inhibitory microenvironment.

PINDs might be promising in treating infectious diseases, but cannot be recommended for the treatment of AML-patients due to their inhibitory influence on antileukemic functionality.

1. Introduction

Allogeneic hematopoietic cell transplantation (allo-SCT) represents the only potentially curative therapeutic strategy in acute myeloid leukemia (AML) [1], with donor T cells being the most important mediators of antileukemic reactions [2,3], though relapses after SCT occur. Restoring remission in relapsed patients after allogeneic SCT can be augmented by a transfusion of donor T cells (DLI), thereby emphasizing the central role of T-cells. However, not all relapsed patients respond to a DLI-based therapy, moreover, graft versus host reactions can impair the effectiveness of SCT and immunotherapy of relapses [4]. Reasons for varying T-cell effects and methods to restore or improve the anti-leukemic capacity of effector-cells, such as T cells engineered to express chimeric antigen receptors (CARs) or engineered T cell receptors (TCRs) have provided promising results and are the subject of ongoing research [5].

DCs, amongst other tasks, act as key controllers of antigen-specific effector T cell responses, stimulating tumor cytotoxic T cells in particular. They thereby serve as an essential link between the innate and the adaptive immune systems. This makes DC-based tumor

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https://doi.org/10.1016/j.cellimm.2018.03.005
Received 30 July 2017; Received in revised form 14 January 2018; Accepted 13 March 2018
Available online 16 March 2018
0008-8749/ © 2018 Published by Elsevier Inc.

immunotherapies a highly interesting target for anti-tumor or anti-leukemic vaccination strategies, summarized under the term "DC vaccines". Molecular identification of tumor-specific antigens recognized by T lymphocytes has led to different strategies of their presentation through DC, e.g. antigen-pulsing [1,6,7]. A different approach, which is pursued by our group, employs DC of leukemic origin (DC_{leu}), which can be generated by directly converting leukemic cells from AML patients in vitro (as described below), thereby co-presenting DC-typical antigens (e.g. CD40, CD86, CD80, CD1a, CD83), and thus regaining the stimulatory capacity of mature professional DC [6,8,9].

Our group has been able to further elicit DC_{leu}-containing-DC induced T cell response patterns after mixed lymphocyte culture (MLC) of DC with T cells: T-cells, obtained from AML-patients (autologous) or donors (allogeneic), can be stimulated by those DC_{leu}, resulting in very efficient cytotoxic effector cells with specific lytic activity against naïve blasts, although not in every case [10,11], which prompted us to search for means to further improve the method.

It has been shown, that in addition to disease-specific effects, vaccines against infectious diseases can have nonspecific effects on the ability of the immune system to react to other targets [12]. The so called "Paramunity inducers" (PINDs) utilize this as their functional principal.

PINDs consist of highly attenuated (by 0.05% β-propiolactone) and inactivated virus strains of various poxvirus-genera with closely linked protein complexes in the envelopes of the virus particles being responsible for their efficacy. Their development was based on observations of positive side-effects of smallpox vaccinations repeatedly described from the late 18th century onwards: healing of chronic skin rashes and reduced susceptibility to various infectious diseases, e.g. measles, scarlet fever and whooping cough. Even the prophylactic use of the vaccination, e.g. against syphilis, is described [13–15]. An early case report on long-term remission of chronic lymphatic leukaemia after smallpox revaccination dates back to the late 1970s [16].

The first *experimental* proof of non-specific effects from a specific trigger came from the observation of the so called "ring-zone-phenomenon". This was based on the production of soluble antiviral substances in infected chicken embryos and cell cultures after being exposed to avi-pox-viruses, thereby protecting neighbouring cells from infections [17]. Based on these experiments Mayr et al. began to develop PINDs as non-immunising vaccines, with the capability of generating endogenic protective, non-antigen specific ("paramunising") mechanisms. PINDs lead to an activation and regulation of the paraspecific, i.e. innate and unspecific immune system against noxious substances from the outside (e.g. bacteria) or the inside (tumours cells) [14,15]. Ahne et al. [18] showed, that the PIND "CONPIND" initiated the production of major inflammatory mediators, most notably TNF-α, in whole blood and in human mononuclear cell cultures. The most notable evidence of their potential efficacy was shown with cats positive to feline leukaemia virus (FeLV), which recovered within two weeks after PIND-treatment [19]. Regardless of the promising results, there is a scarcity of non-veterinarian studies on PINDS and there are none in a malignant setting.

Interactions between pathogen-associated molecular patterns (PAMPs) on the surface of pathogens, and innate immune pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), control for both, innate and adaptive immunity, thereby offering a possible mechanism of action of PINDs. Cancer immunotherapy may utilize immune responses against PAMPs. For example TLRs are known to be able to activate a complex signaling cascade, leading to regulation of DC-activity, including phagocytosis, chemokine receptor expression, migration from peripheral tissue to draining lymph nodes, enhanced antigen presentation by antigen presenting cells (APCs), eventually resulting in increased production of cytokines, chemokines, adhesion molecules and antimicrobial peptides [20–24]. TLR4 and TLR9 (which we analyzed in this study) are receptors involved in immune reactions against bacterial and/or viral 'danger signals' [25,26].

The aim of our study was to determine, whether the addition of PINDs to DC/DC_{leu} cultures could 1) optimize their antigen-presenting potential, 2) improve the composition and function of DC-stimulated T-cells in MLC (in the presence of additionally added PINDs) and 3) improve the function of cells of the innate immune system.

Therefore we added PINDs to DC-cultures of AML blasts to allow PIND-phagocytosis/processing in maturating DC differentiating to 'leukemia-derived DC', as well as to MLC-cultures to additionally stimulate T-cells. Our hypothesis was that immunogenicity, enhanced this way, would lead to an improved activation and subsequently superior antileukemic functionality of T-cells. Therefore, we compared the composition, quantity and quality of defined DC- and T-cell-subtypes before and after respective cultures, and correlated the data with the antileukemic functionality of stimulated T-cells in the presence and absence of PINDs. In addition we performed some experiments using mononuclear cells, containing T- as well as NK cells, as effector cells.

According to our hypothesis based on physiological functions of DC, PINDs administered to AML patients could be phagocytized by patients' spontaneously arising DC/leukemia-derived DC in vivo, thereby leading to 'improved leukemic antigen presentation' and induction of antileukemic T-cell-immunity in vivo. Moreover PINDs – based on observations of Horber and Mayr [14,19] – could directly induce cells of the innate immune system. These mechanisms could contribute to stabilization of disease or remissions in AML patients.

2. Materials and methods

2.1. Patients

Heparinized blood samples were taken by aspiration of peripheral blood (PB) or bone marrow (BM), after obtaining informed consent. Samples were collected from healthy test persons and from AML-patients. Clinical characteristics of samples which were used in cytotoxicity fluorolysis assays are given in Table 1.

2.2. Diagnostics

Diagnostic reports were provided by accredited laboratories of the patients' treating institution. Diagnosis of AML was based on the French-American-British (FAB) classification [27] and flowcytometry to define a blast phenotype [28,29].

2.3. Sample collection

Mononuclear cells (MNCs) were separated from whole blood samples by density gradient centrifugation (Ficoll-Hypaque, Biochrom, Berlin, Germany), then washed and suspended in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany). After quantification MNC were frozen with standardized procedures and stored in liquid nitrogen until use.

T-cells were obtained through positive selection by $CD3^+$ antibodies (Milteney Biotech, Bergisch-Gladbach, Germany 1×10^6 cells/well) from MNC from AML patients or from healthy stem cell donors, as described [10,11].

2.4. DC generation

MNCs were pipetted in 12-well tissue culture plates in 1 ml Xvivo (Bio Whittaker Europe, Verviers, Belgium) FCS-free medium: Dendritic cells (DCs) were generated (in a preliminary experiment) in parallel with 4 different DC generating methods: MCM-Mimic ('MCM') [30], Ca-Ionophor ('Ca') [31], and Picibanil ('Pici') [32], Intron ('Int') [33]. Subsequently, the method resulting in highest DC counts was chosen for quantitative generation of DC as described by us [6,8,9] in the main experiments. These were performed in parallel settings with or without added PINDs, as given in the experimental workflow (Fig. 1).

Table 1
Characteristics of patients and samples used for PIND experiments and functional assays.

Patient-No.	FAB-Type	Stage	Source of T-Cells	DC-Generating-Method *	Selected Blast Markers [CD]	IC-Blast in %	Lysis/Blast-Proliferation (24 h)**		
							Tu	T (DC)	T (DCz + Z)
504M	MDS RAEB2	pers.	T after SCT in CR	Ca	117	32	+30	+120	+70
P980	sAML M0	rel. after SCT	T after SCT at rel.	Pici	34	38	+35	+40	+90
P898	AML M1	Dgn	T auto at Dgn	MCM	117	87	n.d.	+40	+80
P1011	AML M1	rel.	T auto in CR	Ca	117,34,65	86	n.d.	+100	+80
P1231	AML M1	rel.	T auto in CR	MCM	117	48	n.d.	−53	+10
P837	AML M2	Dgn	T auto at Dgn	Pici	13, 34, 117, 15	78	+400	+500	+600
P1144	AML M2	Dgn	T after SCT in CR	MCM	34,117,56	65	n.d.	+880	+980
774M	AML M2	rel.	T after SCT in CR	MCM	15,65,117	92	−65	+250	+200
P984	sAML M2	rel. pers.	T auto at rel. pers.	Int	34, 117	57	+100	−30	+80
P1252	AML M2	rel. after SCT	T after SCT at rel.	MCM	34	31	n.d.	−70	+80
724M	AML M4eo	rel.	T donor	MCM	34,117	12	+60	−30	+40
P757	sAML M4	Dgn	T auto at Dgn	MCM	34,117	45	n.d.	+45	+390
P1001	AML M4	rel. after SCT	T after SCT at rel.	Pici	34, 117	75	−75	−3	+20
P1001	AML M4	rel. after SCT	T donor	Pici	34, 117	75	+60	−30	+40
P998	AML M5	rel.	T after SCT at rel.	Ca	64,117,34	95	n.d.	−30	+430
P935	sAML	Dgn	n.d.	Ca	34,117,65,15	40	n.d.	n.d.	n.d.
P948	sAML	rel.	n.d.	Ca	34,117	36	n.d.	n.d.	n.d.
P534	AML M1	Dgn	n.d.	Int	117,34	40	n.d.	n.d.	n.d.
P890	AML M2	rel.	n.d.	Pici	34,117	28	n.d.	n.d.	n.d.
P819	AML M2	rel. pers.	n.d.	MCM	65,34,117,7,15	15	n.d.	n.d.	n.d.
P650	AML M5	rel.	n.d.	MCM	19, 15	74	n.d.	n.d.	n.d.
P721	sAML M5	rel.	n.d.	Ca	65,15,56	80	n.d.	n.d.	n.d.

Dgn first diagnosis, rel. relapse, rel. pers. persisting relapse, CR complete remission, IC-Blast immunocytologically detected blasts, Tu uncultured T cells, T(DC) T cells stimulated with DC, T(DCz + Z) T cells stimulated with DC in the presence of Zylexis, n.d. not done; * 'best' of four DC-generating-methods, selected for DC generation (Ca Ca-Ionophore, Int Intron, MCM MCM-Mimic, Pici Picibanil). **results of functional fluorolysis are given in percental proportions of lysed blasts (−X%) or blast proliferation (+X%) after incubation with different T effector cells for 24 h.

"MCM": DC were generated from 2 to 2.5 × 10^6 MNC/ml in 'MCM-Mimic' medium containing 800 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF; Sandoz, Holzkirchen, Germany), 500 U/ml Interleukin 4 (IL-4; Cell Concepts, Umkirch, Germany) and 40 ng/ml FLT3-Ligand (FLT; PromoCell, Heidelberg, Germany) for 10–14 days, adding the same cytokines after 4–5 days again. On day 7–8 half medium exchange was performed, and 150 ng/ml IL-6 (Cell Concepts, Umkirch, Germany), 5 ng/ml IL-1β (Cell Concepts, Umkirch, Germany), 1 µg/ml Prostaglandin E2 (PGE_2, Pfizer, Vienna, Austria) and 200U/ml Tumor necrosis factor (TNF) α (Cell Concepts, Umkirch, Germany) were added. After 10–14 days, cells were harvested for subsequent experiments [6,8,30].

"Ca": DC were generated from 7 × 10^5 MNC/ml in 'Ca-Ionophore' medium adding 375 ng/ml A23187 (Sigma-Aldrich, Thum, Germany) and 250 U/ml IL-4. After 3–4 days, cells were harvested for subsequent experiments [6,31].

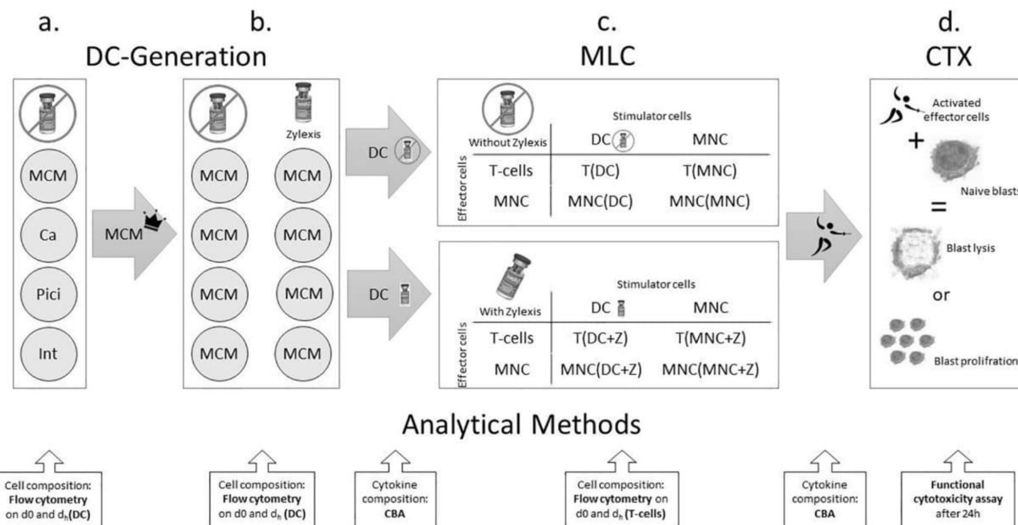


Fig. 1. Experimental workflow to study the influence of Zylexis on DC-generation as well as cells in MLC and cytotoxicity: a. Pretesting with few cells and identification of the 'best' () DC-generating method for individual samples (without PINDs). Here MCM was chosen (exemplarily) as 'best method' b. Quantitative DC-generation with the previously chosen 'best DC-generating method' with and without the addition of Zylexis in parallel(evaluated by flowcytometry). Asservation of culture-supernatans for CBA. c. MLC of effector cells (T-cells or MNC) with stimulator cells (DC or MNC), with and without the (repeated) addition of Zylexis (resulting in the effector cells as detailed in Table 3b, evaluated by flowcytometry). Asservation of culture-supernatans for CBA. d. Functional cytotoxicity assay of various MLC-cell compositions to detect antileukemic capacity. The Various analytical methods and their timing are given below.

Table 2
Parapox-Virus Stem(s) in different PINDS used for experiments.

PIND	Parapox Virus Stem(s)	Abbrev. DC*	Availability
none (w/o)	none	$DC_{w/o}$	
Zylexis	ovis	DC_Z	Commercially available (Pfizer AH, Inc.)
3P	ovis	DC_{3P}	Prepared and provided by I. Harawacz
HP	avi	DC_{HP}	Prepared and provided by I. Harawacz
HP-3P	ovis, avi	$DC_{HP\text{-}3P}$	1:1 combination of HP and 3P
Conpind**	ovis, avi	DC_C	Prepared and provided by B. and A. Mayr

* Abbreviation for DC after culture with different PINDs, ** listed as Patents No DE3504940 A1 and US 6,162,600 A of Anton Mayr.

"Pici": DCs were generated with 'Picibanil', a lysis product of *Streptococcus pyogenes* which has nonspecific immunomodulatory effects, from 2 to 2.5 × 10^6 MNC/ml in the presence of 500 U/ml GM-CSF and 250 U/ml IL-4. After 7–8 days in culture, 10 µl/ml OK-432 (Chugai Pharmaceuticals, Kamakura City, Japan) and 1 µg/ml PGE_2 were added. Cells were harvested after 9–11 days in culture [8,32].

"Int": DC were generated from 2 to 2.5 × 10^6 MNC/ml in 'Intron'-medium containing 800 U/ml GM-CSF, and 5 µl/ml Interferon alpha (Int; PromoKine, Heidelberg, Germany) for 10–14 days, adding the same cytokines after 4–5 days for a second time. On day 7–8 half medium exchange was performed, and 800 U/ml GM-CSF, 5 µl/ml Int and 200 U/ml TNFα were added. After 10–14 days, cells were harvested for subsequent experiments [33,34].

"PINDs": In order to potentially further improve their T-cell stimulating capacity, we cultured DC using the methods described above, in parallel adding PINDS consisting of Parapox-ovis, Parapox-avis or a combination of both virus stems (see Table 2), to the respective medium. Zylexis is commercially available from Pfizer AH, Inc. (formerly Baypamun from Bayer, Inc.). HP and 3P were prepared and provided by I. Harabacz, HP-3P is their 1:1 combination. Conpind is also a combination of both virus stems, prepared and provided by B. and A. Mayr.

Lyophilized products were resolved in aqua ad injectionem as recommended, resulting in PIND-concentrations of 25 mg/ml (46 IFN-Units). Dilutions of 1:5, 1:10 and 1:20 were used, resulting in end-concentrations of 9.2, 4.6 or 2.3 IFN-Units in cultures. Dilutions of 1:5 and 1:10 of our settings were chosen according to personal recommendation of Prof. A. Mayr and used for previous experiments. Finally we used the 1:20 dilution because of an increased accumulation of dead cells in the 1:5 and (to a lesser extent) in the 1:10 concentrations (see "Results – 1.2 Usage of PINDs in a concentration of 1:20" for further information).

All experiments were performed according to the strategy given in chapter 11 (experimental setup).

2.5. Flow cytometry

Flow cytometric analyses with a panel of mouse monoclonal antibodies (moABs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem CY7-PE-conjugation (PC7), or allophycocyanin (APC) was performed to evaluate and quantify amounts and phenotypes of leukemic cells, DCs, B-cells, T-cells and NK-cells in the PB/BM samples as described [10]. Antibodies were purchased from Immunotech/Beckman Coulter ([a]), Becton Dickinson ([b]), Caltag ([c]), Serotech ([d]) and Invitrogen ([e]). The following conjugated moABs were used: **FITC:** CD40[c], CD80[ab], CD83[a], CD86[c], CD3[a], CD25[a], CD39[d], CD45RO[a] and CD122[d]; **PE:** TLR9[a], CD4[a], CD8[b], CD56[a],CD73[b], CD80[a], CD83[a], CD86[bc], CD127[a], CD152[a] and CD206[a]; **PC7**: CD1a[a], CD3[a], CD4[a], CD25[a], CD40[c], CD56[a], CD83[bc], CD86[c], CCR7[b], TLR4[a]and 7AAD[a]; **APC:** CD1a[a], CD3[a], CD8[b], CD25[e], CD33[a], CD34[a c], CD40[c], CD45RO[e], CD69[b], CD71[b], CD117[a] and CD206[a].

Cells were suspended in PBS with 20% FCS (Biochrome) and incubated with the moABs according to manufacturer's instructions. For intracellular staining, we used FIX & PERM reagents (CALTAG Laboratories, manufactured by An Der Grub Bio Research GmbH, Austria) according to manufacturer's instructions for fixation and permeabilization, in order to facilitate antibody access to intracellular structures, leaving the morphological scatter characteristics of the cells intact. Proportions of positive events in the defined gate compared with the isotype controls were calculated using CellQuest Software (BD), as described [6,8,10].

2.6. Quantification and characterization of DC by flow cytometry

DCs were generated, harvested, counted and quantified by flow cytometry as described above. Before culture mononuclear cell samples were characterized to identify the blast marker with the highest expression, as well as DC-markers with low/no expression in MNC. After culture these blast and DC markers were combined to identify the DC-method which yielded the highest DC-count (characterized by expression of DC-markers, that were not expressed on uncultured cells) and DC_{leu} (characterized by co-expression of DC- and blast markers). DCs were characterized by the proportionally highest expressed DC-marker (DC_{opt}). For further analysis and quantification of DCs, and especially leukemia-derived DC, a refined gating strategy was applied [9]. This strategy takes into consideration different scatter profiles of blasts and DC and enables a sensitive detection and quantification of blasts, which were not converted to DC_{leu}, of DC without proof of leukemic derivation and of DC with leukemic derivation (DC_{leu}). In addition viable (DC_{opt} not co-expressing 7AAD; DC_{via}) or mature (DC_{opt} co-expressing CD83; DC_{mat}) DC in DC-fractions were identified (Table 3a).

2.7. Mixed lymphocyte culture (MLC)

Previously selected $CD3^+$ ***T-cells*** from MNC from AML patients or from healthy stem cell donors were co-cultured and stimulated with irradiated (20 Gy) AML-blast containing MNC in the presence/absence of Zylexis (5 × 10^4 'MNC'; T(MNC), T(MNC + Z)) and in parallel with irradiated DC_{leu}-containing DC (5 × 10^4 'DC'; T(DC)), irradiated DC_{leu}-containing DC_z and additional Zylexis (5 × 10^4 'DC'; T(DC_z +Z)) in 1 ml MLC-Medium (RPMI 1640 medium (Biochrom) containing 15% human serum (PAA) and 50U/ml IL-2 (Proleukin R5, Chiron)). In these comparative settings the influences of Zylexis should be optimized: Zylexis was not only added to DC cultures (giving DC the chance to phagocyte, process and present Zylexis-antigens), but also to MLC in order to benefit from DC-mediated as well as direct influences of Zylexis on T-cells. Various experimental settings are given in Table 3b. Total DC-counts in the MLC were adjusted to 5 × 10^4 DC per 1 × 10^6 T-cells. Cells were restimulated with 5 × 10^4 'DC' or 5 × 10^4 'MNC' and supplementation with IL-2, as described [8] with a half-medium-exchange on day 4. On day 7 cells were harvested and the cytotoxicity assay was carried out. Some experiments were performed using ***MNC*** fractions (containing T- as well as NK or other cells of the cellular immune system) as effector cells. For better comparability with results obtained with T-cells as effector cells cell counts in the MLC using MNC as effector cells were adjusted to T-cell counts in the effector cell suspension using only T-cells; the remaining experimental settings were performed in analogy to MLC using T-cells with/without addition of Zylexis and stimulation with DC_{leu} containing DC (MNC(DC), MNC(DC_z + Z) or blast-containing MNC (MNC(MNC), MNC(MNC + Z); Table 3b).

Antigen expressions on (allogeneic or autologous) $CD3^+$ T-cells were evaluated by FACS-analyses comparing the co-expression of CD28, CD154, CD4, CD8, CD45RA, CD45RO, CD25, CD71 before and after blast derived 'MNC'- or 'DC'-contact. This let us evaluate proportions of proliferating, costimulatory, naïve, memory, central memory or $CD4^+$/

Table 3
Abbreviations of a. DC-subtypes, b. different MLC-settings by type of effector cells c. T-cell-subtypes as evaluated by flow cytometry with the respective surface-marker profiles after staining with fluorochrome-labelled antibodies.

DC-Type	Surface Marker	Abbreviation
a. DC-subtypes		
Proportionally highest expressed DC-marker in the MNC-fraction	DC^+	DC_{opt}
Viable DC (DC_{opt} not dyed by 7AAD)	DC^+, $7AAD^-$	DC_{via}
Mature DC (DC_{opt} co-expressing CD83)	DC^+, $CD83^+$	DC_{mat}
DC of leukemic derivation (DC co-expressing blast markers)	DC^+, Bla^+	DC_{leu}
DC_{leu}-proportions in the blast cell-fraction	DC^+, Bla^+	DC_{leu}/Bla
DC_{leu}-proportions in the DC-fraction	DC^+, Bla^+	DC_{leu}/DC
DC_{leu}-proportions in the MNC-fraction	DC^+, Bla^+	DC_{leu}/MNC
TLR-expressing DC in the MNC-fraction	DC^+, TLR^+	TLR^+ DC

Effector cells by preceding mixed lymphocyte culture (MLC)	Abbreviation
b. Mixed lymphocyte culture settings by type of effector cells	
Unstimulated T-cells (no MLC)	T_u
T-cells cultured/stimulated with AML-blast containing MNC	T(MNC)
T-cells cultured/stimulated with AML-blast containing MNC in the presence of Zylexis	T(MNC + Z)
T-cells cultured/stimulated with DC (containing DC_{leu})	T(DC)
T-cells cultured/stimulated with DC_z (containing DC_{leu}) in the presence of Zylexis	T(DC_z + Z)
MNC cultured/stimulated with DC (containing DC_{leu})	MNC(DC)
MNC cultured/stimulated with DC_z (containing DC_{leu}) in the presence of Zylexis	MNC(DC_z + Z)
MNC stimulated with MNC	MNC(MNC)
MNC stimulated with MNC in the presence of Zylexis	MNC(MNC + Z)

Names of T-cell populations	Abbreviation	Surface-Marker-Profile	Explanatory Note	References
c. Subtypes of T-cells as evaluated by flow cytometry				
CD3+ T-cells	CD3+ T-cells	CD3+	entire T-cell population	[69], Lanzavecchia et al. (2004)
CD4+ expressing T-cells	CD4+ T-cells	CD4+, CD3+	CD4 positive subpopulation	
CD8+ expressing T-cells	CD8+ T-cells	CD8+, CD3+	CD8 positive subpopulation	
T-cell subsets				
Naive T-cells (co-expressing CCR7 = CD197)	T naive	CD197+, CD45RO−	T-cells before (naive) and after (non-n.) encountering its cognate antigen	[67,68,69,36]
Non-naive T-cells (not expressing CCR7)	T non naive	CD197−, CD45RO+		
Effector T-cells	T eff	CD197−, CD27−, CD45RO+/−	T-cells mediating the immune response, "reactive effector cells"	
Ro+ Effector T-cells	T eff Ro+	CD197−, CD27−, CD45RO+		
Ro-Effector T-cells	T eff Ro−	CD197−, CD27−, CD45RO−		
Effector memory T-cells	Tem	CD197−, CD27+, CD45RO+	fast reactivation through antigen contact, "reactive memory"	
Central memory T-cells	Tcm	CD197+, CD45RO+	"slow" reactivation in the lymphatic system, "protective memory"	
Regulatory T-cells	T reg			
IL-2-R + IL-7-Rlow expressing CD4+ T-cells	CD4+ Treg	CD4+, CD25++, CD127low	Important for maintaining immunological balance, "regulatory effectors"	Vignali et al. (2008), Schick et al. (2012)
IL-2-R + IL-7-Rlow expressing CD8+ T-cells	CD8+ Treg	CD8+, CD25++, CD127low		
Activation & Proliferation				
Transferrin-Receptor+ T-cells	CD71+ T-cells	CD71+, CD3+	proliferating T-cells	Ned et al. (2003)
Type II C-type lectin+ T-cells	CD69+ T-cells	CD69+, CD3+	early proliferating T-cells	Sancho et al. (2005)
IL-2-Receptor+ T-cells	CD25 + CD4+ T-cells CD25 + CD8+ T-cells	CD25+, CD4+ CD25+, CD8+	activated T-cells	Letourneau et al. (2009)
CD80/86-R+ T-cells	CD28+ T-cells	CD28+, CD3+	APC-T-cell-interaction	Rudd et al. (2009)
TNF-Receptor superfamily member 9+ T-cells	CD137+ T-cells	CD137+, CD3+		Myers et al. (2005),
CD40Ligand+ T-cells	CD154+ T-cells	CD154+, CD3+		Mackey et al. (1998)

DC^+ Cells stained with a DC-marker (in this setting: the highest expressed), **Bla^+** Cells stained by blast markers, **$7AAD^-$** Cells not reactive for 7AAD, **$CD83^+$** cells stained with CD83, **TLR +** cells stained with a conjugated antibody against a TLR (TLR4 or TLR9).

$CD8^+$ or migratory T-cells before and after 'MNC' or 'DC' co-culture [10,35,36]. Subtypes of T cells are given in Table 3c.

2.8. Cytotoxicity Fluorolysis assay

The lytic activity of effector T-cells or MNC (containing T- and NK-cells) was measured by a Fluorolysis-Assay by counting viable blast target cells, labeled with specific fluorochrome antibodies, before and after effector cell contact as described [10].

Stimulated effector-cells (T-cells or MNC) from healthy HLA-matched donors (= allogeneic) or from AML and MDS patients (= autologous) as well as non-stimulated T-cells (= uncultured, T_u) as a control (see Table 3c) were co-cultured with thawn blasts as target cells, which were stained for 15 min with two FITC- and/or PE conjugated 'blast' specific antibodies before culture. The effector- to target-cell ratio was adjusted to 1:1 and the cells were co-cultured for 24 h. To evaluate amounts of viable ($7AAD^-$) target cells and to quantify the cell-loss after 24 h of incubation time, cells were harvested, washed in PBS and re-suspended in a FACS flow solution containing 7AAD (BD, Biosciences Pharmingen) and a defined number of fluorosphere calibration-beads (Becton Dickinson, Heidelberg, Germany). Viable cells were gated in a SSC/7AAD-gate. Afterwards, viable, 7AAD-negative cells co-expressing a specific blast marker (combinations) were quantified by taking into account defined counts of calibration beads as described. Cells were analyzed in a FACS Calibur Flow Cytometer using CELL Quest software (Becton Dickinson, Heidelberg, Germany). The percentage of lysis was defined as the difference between proportions of viable blasts before and after the effector cell contact [8].

2.9. Cytometric bead assay (CBA) for cytokine and chemokine analysis

During DC-generation (n = 11) and MLC (n = 5) 500 µl-supernatants of each sample were taken and stored at −80 °C until analysis. The TH1/TH2-CBA-kit II with antibodies for IL-4, IL-6, IL-10, TNF-α and IFN-γ was used for analysis. As a control cytokine levels in cell culture media (RPMI + 20% human serum, Biochrom, Berlin, Germany) without cells added were measured. The standard serial dilutions of positive and negative controls of the respective cytokines were included. 50 µl mixed human TH1/TH2 Cytokine capture beads and 50 µl PE Detection Reagent were incubated with 50 µl of each test sample in assay tubes (12 × 75mm, BD Falcon) for 3 h at room temperature. After a washing step with 1 ml Wash Buffer the assay tubes were centrifuged at 200*g* for five min, the bead pellets resuspended in 300 µl Wash Buffer and analyzed by flow cytometry. Each sample was tested once, as recommended. Cytokines or chemokines measured in the samples were quantified using the standard curve [37].

2.10. Statistical methods

Mean and standard deviation, median and range, two-tailed *t*-tests and analyses of variance (ANOVA) were performed with a personal computer using Excel 2010 (Microsoft) and Graph Pad PRISM (GraphPad Software, Inc.). In detail we used the ANOVA-test when comparing parameters from *more than two* sets of data, e. g. values of a DC-parameter obtained in a given experiment without PINDs (w/o) and with several different PINDs (e.g. Zylexis, Conpind, etc.). The best result among any of the four DC-generating-methods was individually chosen for w/o and each PIND and selected for comparison.

We used the *t*-test when comparing parameters from *two* sets of data, e.g. w/o and a single PIND (Zylexis) or differently pooled averages of DC-parameters from different PINDs (Ø). In both cases we either used the value obtained with the best DC-generating-method in the presence of the PIND, or selected the value in analogy to the best method obtained w/o.

Differences were considered as significant, if the p-value was < 0.05.

2.11. Experimental setup

Influences of various PINDs on peripheral blood-cells (from healthy or AML-donors) was to be studied in various experiments: 1) DC were generated in the presence versus absence of PINDs. 2) Compositions of DC- (or MNC) stimulated T-cells after MLC (again in the presence vs absence of PINDs) were evaluated. 3. T-cells' antileukemic effects after MLC (cytotoxicity assay).

Preliminary experiments were performed to compare 1) efficiency of freshly prepared ('new') vs up to ten day old PIND-solutions ('old'), as well as 2) the efficiency and toxicity of different PIND-dilutions (1:5, 1:10, 1:20). Additionally we performed 3) multiple comparative analyses of different combinations of PINDs and DC-generating methods.

Experimental workflow (Fig. 1): Since cell quantity for all experiments was limited, we had to determine the 'best method' for every given sample. 1) In a first step a small number of DC were generated in parallel with 4 different DC generating methods, but without the addition of Zylexis. 2) Subsequently, the method resulting in highest DC counts was chosen for quantitative generation of DC (in the presence vs absence of Zylexis, analysis of cell composition by flow cytometry and cytokine profiles by CBA). 3) Subsequently a MLC was performed by coculture of effector cells (T-cells or MNC) with stimulatory cells (DC and MNC), with addition of Zylexis to MLCs with stimulatory cells previously exposed to Zylexis (resulting in various effector cell settings as detailed in Table 3b). Cellular compositions (in comparison to unstimulated Tcells) as well as cytokine profiles were evaluated by Flowcytometry and CBA-assays. 6) Antileukemic functionality of various settings was assessed through Cytotoxicity Fluorolysis Assay.

3. Results

3.1. Preliminary testing

3.1.1. Dissolved ten-day-old Zylexis shows similar activity compared to Zylexis prepared directly before use

In a clinical setting, PINDs, such as Zylexis, are used immediately after preparation from lyophilisates ("new"). In an experimental setting only small quantities are used. Therefore we compared results from PINDs being prepared ten days in advance and stored at 8 °C until use ("old") to its corresponding "new" preparation.

In this trial we used Zylexis as a representative of all PINDs, and analyzed quantity (DC_{opt}), maturation (DC_{mat}), and viability (DC_{via}) of DC generated from healthy donors through the Ca-, MCM-, Pici-, and Int-methods with "old" or "new" Zylexis in concentrations of 1:10 and 1:20. Statistical analysis showed no significant differences between the use of "old" or "new" Zylexis in 1:10 and 1:20 settings in either DC-generating-method (data not shown).

We decided to use Zylexis, and the other PINDs, up to ten days after preparation in our future experiments.

3.1.2. Usage of PINDs in a concentration of 1:20

Treatment of animals with PINDs is performed by injecting one vial (containing 25 mg/ml) into one animal, independent of weight, species or body mass index. Following personal communication with Mayr et al. (no publications on this topic are available) we diluted the out of the box Zylexis-preparation (intended for injection in animals) of 25 mg/ml (46 IFN-Units) 1:5, 1:10 and 1:20. This resulted in end-concentrations of 9.2, 4.6 or 2.3 IFN-Units in cultures. We observed numerous dead cells in light microscopy during our pre-preliminary testing when using 1:5 and to a lesser extent 1:10 dilutions. Due to the extraordinary accumulation of dead cells in the 1:5 dilutions, we refrained from further testing of 1:5 dilutions and further compared 1:10 and 1:20 concentrations. Analysis of proportions of DC subtypes generated from 9 healthy donors under the influence of PINDs (Zylexis, Conpind, HP, 3P, HP-3P) in the aforementioned concentrations showed less dead cells and in addition (non-significantly) higher levels of DC_{opt},

DC_{mat} and DC_{via} in the 1:20 dilution (data not shown). Therefore we conducted our further experiments using 1:20 dilutions.

3.1.3. None of the four methods is favored or disadvantaged by the addition of PINDs to DC-generation

We determined if certain combinations of PINDs and DC-generating methods, would generally result in superior/inferior DC-counts.

We compared DC- subtypes (as listed in Table 3a) from 14 healthy and 9 leukemic donors after culture with MCM, Ca, Pici and Int. Each method was employed five times with the individual addition of either Zylexis, Conpind, HP, 3P or HP-3P. No significant differences in proportions of DC-subtypes could be found for any specific combination (ANOVA, data not shown).

Therefore we continued to use all four DC-generating methods in combination with all PINDs in our further experiments.

3.2. Comparison of quality and quantity of DC cultured with and without PINDs

3.2.1. The presence of PINDs in DC-cultures with healthy or leukemic MNC decreases DC's viability and increases their maturation

Our first step was to determine the effects of PINDs on the proportions of DC-subtypes (by flowcytometry) obtained from the 4 standard DC-generating-methods in general. Therefore we compared results obtained with the different DC-methods w/o, with Zylexis or with a pooled average from all PINDs (øPINDs). For the latter each sample's best results from any of the four methods were used.

In healthy donors ($n = 15$) a decrease in DC_{opt} and significantly in DC_{via} ($p = 0.02$) could be observed in the presence vs. absence of PINDs in DC-cultures. Proportions of DC_{mat} increased on a non-significant scale (Fig. 2a).

The addition of PINDs to DC generation from leukemic donors ($n = 12$) resulted in decreased DC_{opt}, DC_{via} and DC_{leu}/bla, while DC_{leu}/DC or DC_{leu}/MNC remained similar. Again DC_{mat} increased significantly ($p = 0.01$, *t*-test; see Fig. 2b).

Additional refined analyses comparing DC-parameters through different modes of selection underlined the overall observation of decreased viability and increased maturation in DC generated in the presence of PINDs (data not shown).

In conclusion the presence of any PIND decreased viability and increased maturation of DC from healthy and leukemic samples compared to DC generating methods w/o the addition of PINDS.

3.2.2. Zylexis benefits maturation of DC

Due to limited cells available we focused further testing on Zylexis, as the only currently commercially available PIND.

We analyzed the influence of Zylexis on DC-cultures from healthy ($n = 15$) and leukemic samples ($n = 12$) in analogy to Section 2.1. In both cases we observed a (significant) decrease of DC_{via} (healthy samples: $p = 0.004$, *t*-test) and DC_{opt} as well as a significant increase in DC_{mat} (healthy samples: $p = 0.04$, leukemic samples: $p = 0.02$, *t*-test) in the presence of Zylexis (Fig. 2a and b). Comparisons between Zylexis and the other PINDs did not show significantly different influences on DC-proportions (ANOVA, data not shown).

3.2.3. The presence of Zylexis in DC cultures decreases TLR4- and TLR9-expression on DC/DC_{leu}

TLR4 and TLR9 are known to facilitate "danger signaling" in DC-mediated T-cell activation. Therefore we studied their expression under the influence of PINDs.

DC were generated in parallel with all four DC-generation methods. Similar average proportions (no statistical differences, ANOVA) of $TLR9^+$ and $TLR4^+$ DC were found in all settings in the *absence* of Zylexis, (data not shown).

In parallel experiments the *presence* of Zylexis in all DC-culture-media (significantly) decreased expression levels of TLR9 and TLR 4 on blasts and DC_{leu}, while their expression on DC was not affected (Fig. 3 a and b; *t*-test).

3.3. Effects of Zylexis on compositions and functions of T-cells in mixed lymphocyte cultures (MLC)

We compared different surface marker profiles on T-cells (autologous and HLA-matched T-cells after SCT from AML-patients, $n = 8$) cultured with DC generated in the absence (T(DC)) or in the presence of Zylexis, which was additionally added to the culture-medium of the latter (T(DC_z + Z)), as described in the materials and methods section, chapter 7.

3.3.1. Zylexis induces early T-cell Activation, but impairs their differentiation to effector T-cells

We analyzed influences of Zylexis on T-cell activation and proliferation in MLR. No significant differences could be found in these T-cell-subtype proportions (*t*-test), although $CD69^+$ T-cells as well as $CD25^+CD8^+$ T-cells increased under the influence of DC_z + Z (arrows, Fig. 4a).

We analyzed the influence of Zylexis on proportions of the following T-cell-subtypes: naïve-, central-memory-, effector-memory-, effector-T-cells. We observed a lower decrease of T_{naive} as well as a lower increase of T_{eff} under the influence of Zylexis in MLC (Fig. 4b), whereas proportions of T_{cm}, T_{em} and T_{reg} were similar in both groups (data not shown).

In conclusion this means that the presence of Zylexis in MLC increases early CD8+ T-cell proliferation, but impairs differentiation to effector T-cells.

3.4. Effects of Zylexis on T-cells' antileukemic functionality

Prior results from our group have shown, that DC/DC_{leu} stimulated T-cells (T(DC)) are the most efficient antileukemic effector cells (although not effective in every given case). We studied, whether the presence of Zylexis in MLC would increase antileukemic activity compared to cultures w/o Zylexis or untreated T-cells (T_u).

3.4.1. T-cells stimulated with DC_z or MNC in the presence of Zylexis lose their antileukemic functionality (Fig. 4)

We performed parallel investigations of **DC/DC_{leu}** stimulated T-cell-cultures *without* [MLC (T(DC)] or *with* Zylexis added to DC cultures and [MLC (T(DC_z + Z)] as well as T-cells stimulated with blast-containing MNC *without* [MLC T(MNC)] and *with* Zylexis added to MLC [MLC (T(MNC + Z)], and evaluated T-cells' antileukemic activity after 24 h of incubation with blast target cells.

Our results showed, that the presence of Zylexis in the culture decreased the antileukemic reactivity of T-cells (Fig. 5a, arrows): In the Zylexis-groups only 1 of 15 T(DC_z + Z)-samples (6.7%) showed lytic activity, whereas there was none in the T(MNC + Z)-group. At the same time lysis was highest with T(DC) (53.3%, 8 of 15 cases), while unstimulated T-cells (T_u) or blast-stimulated T-cells T(MNC) displayed blast-lysis in 2 of 8 (25.0%) and 2 of 14 (14.3%) cases after 24 h-contact with blast-target-cells ($p = 0.01$; ANOVA). Especially direct comparison between matching effector cells – T(DC) and T(DC_z + Z), as well as T(MNC) and T(MNC + Z) – statistically pronounced the decreased antileukemic functionality, especially under the influence of DC ($p = 0.004$; $p = 0.165$; *t*-test). In cases in which lysis occurred, similar decreases of blast-proportions were observed. T(DC) showed an average of −41% lysed blast-cells and the one case in which Lysis occurred in T(DC_z + Z) showed −44% lysed blasts. In cases without Lysis, the amount of blast proliferation did not show significant differences among T_u, T(DC), T(DC_z + Z), T(MNC) and T(MNC + Z) after 24 h (ANOVA).

To obtain more detailed information on T-cells' compared to MNCs' antileukemic effector cell reactivity and efficacy, we used T-cells or

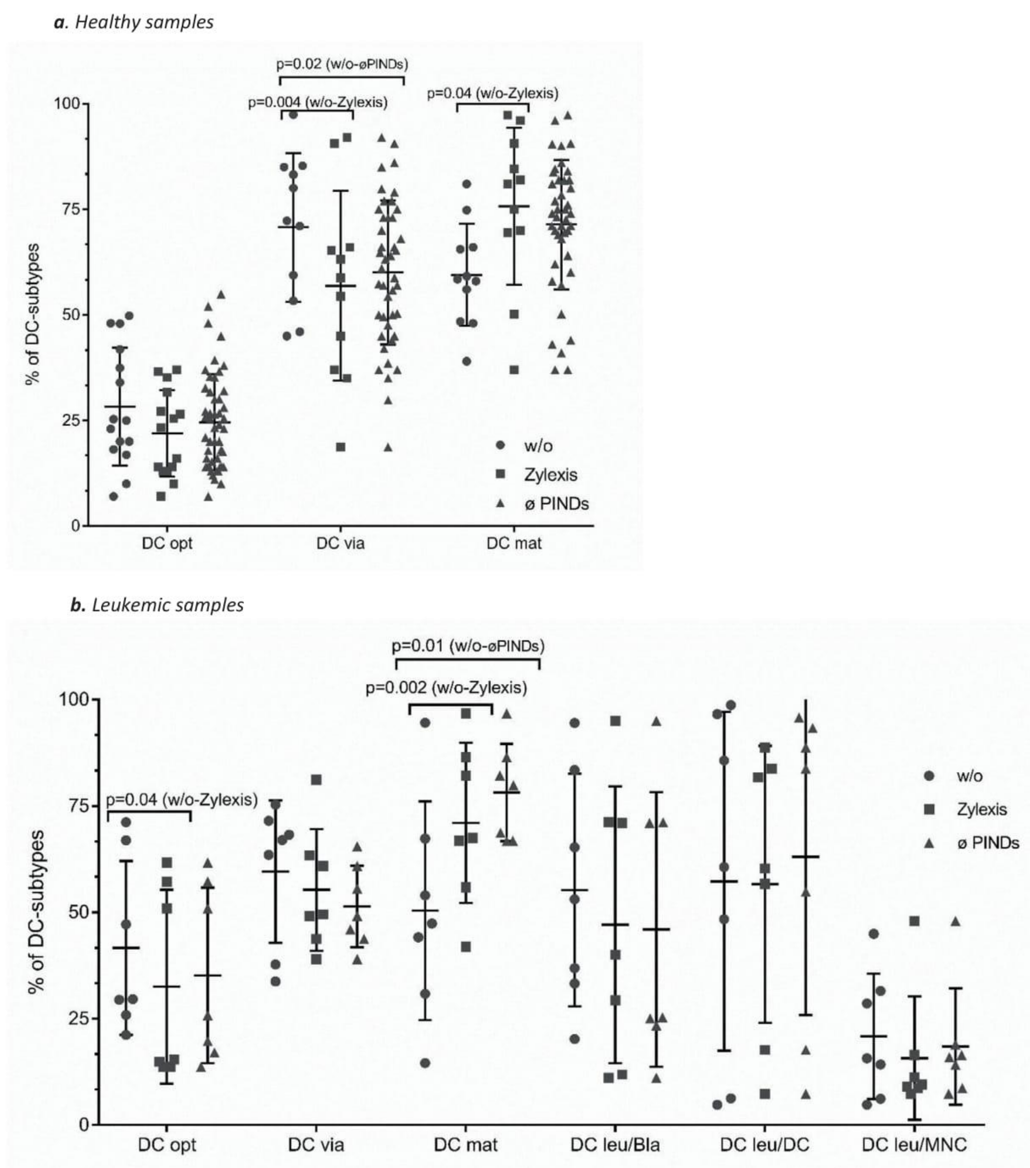


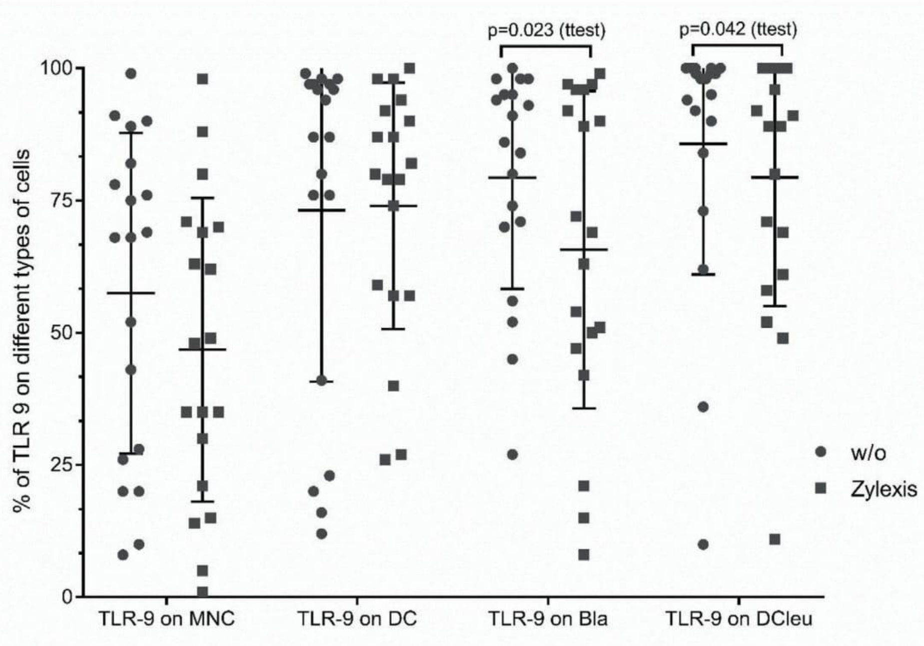
Fig. 2. Comparison of proportions of DC-subtypes (in % of cells) obtained with DC-methods w/o, with Zylexis or with average of pooled results from all PINDs (øPINDs;): Each sample's best results from any of the four methods in (a.) healthy (n = 15) and (b.) leukemic samples (n = 12) were used. Statistically significant values from *t*-tests between w/o and Zylexis as well as w/o and øPINDs are given (*t*-test). No significant differences between DC-values under the influence of Zylexis and øPINDs could be found.

MNC (containing T-cells as well as cells of the innate immune system; six cases) as *effectors* (stimulation with MNC and DC, in the presence/absence of Zylexis): T-cells led to lysis in 4 of 24 cases, while antileukemic activity could only be observed in 1 of 24 cases using MNC as effector cells. Use of the latter also resulted in a significantly higher rate of proliferating blasts in cases without lysis (p = 0.01, *t*-test, data not shown). Proportions of cases with antileukemic activity from differently stimulated effector cells (MNC or T-cells) are given in Fig. 5b. No antileukemic reactivity in the presence of Zylexis could be demonstrated, regardless of the effector cells (T-cells and MNC). A significant increase of lysis from DC-stimulated T-cells compared to all other effector/stimulator cell combinations regardless of the presence of Zylexis could be shown (p = 0.04, ANOVA). This increase was also significant when directly comparing DC-stimulated T-cells' antileukemic activity in the absence compared to the presence of Zylexis (p = 0.05, *t*-test). Again, in cases without lysis, the amount of blast proliferation (if lysis did not occur) did not show significant differences in the groups compared (data not shown).

So although comparable effector-cell counts were added to MLC cultures in both settings, antileukemic reactivity of those using MNC, which additionally contained other immune reactive cells, showed decreased antileukemic function compared to "pure" T-cells. Addition of Zylexis to MNC-settings did not result in lysis in any case.

We conclude that the presence of Zylexis lead to a significant loss or inhibition of antileukemic T- as well as antileukemic innate- (MNC)cell functionality, potentially benefitting blast-proliferation. No special benefit on the "innate immunity" could be observed under the influence of Zylexis.

a. TLR-9 expression in the presence or absence of Zylexis



b. TLR-4 expression in the presence or absence of Zylexis

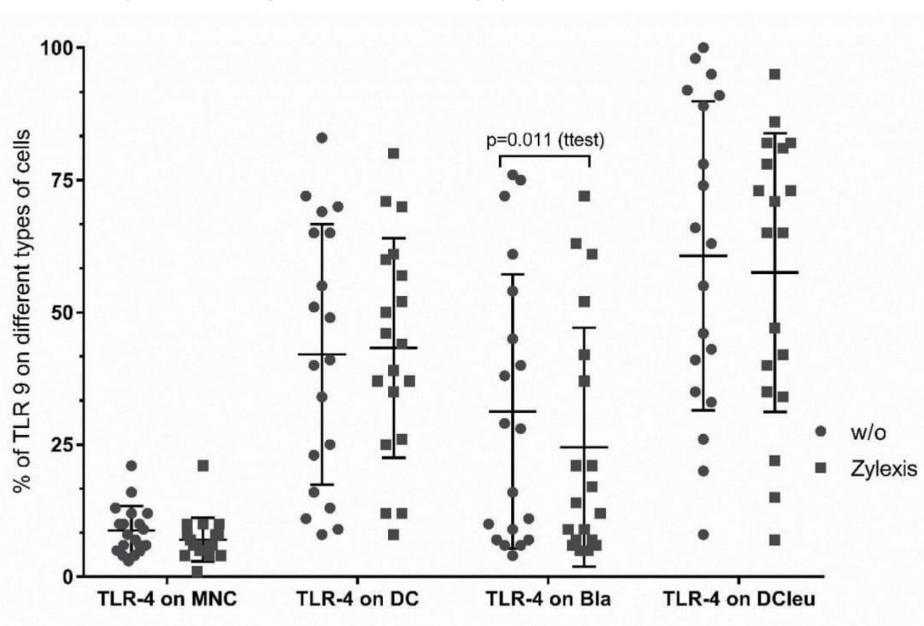
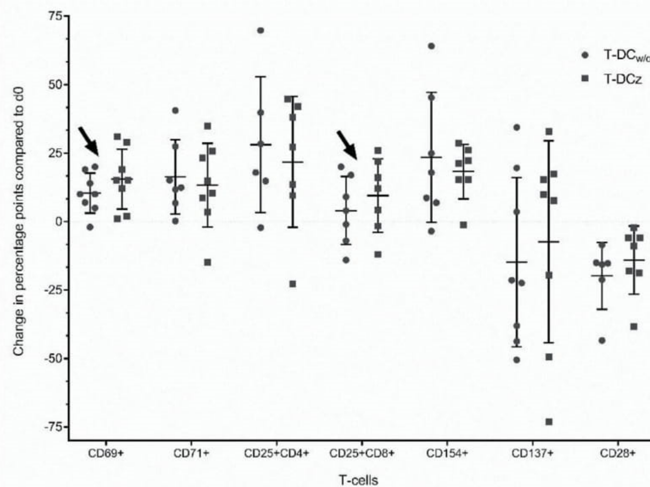


Fig. 3. Expression of TLR on cells of leukemic origin (blasts and DC_{leu}) (significantly) decreased after culture in different DC-generating media in the presence of Zylexis (*t*-test). Proportions of all cells ("TLR-9/4+"), DC, blasts and DCleu expressing TLR9 (a.) and TLR4 (b.) in cultures w/o or with Zylexis are given. Results obtained after culture with 4 different DC-generating methods showed similar average proportions of TLR-expression and are pooled.

3.4.2. Cases without lysis presented with higher T_{reg} and T_{eff} counts in MLC-samples of T(DC) and T(DC_Z + Z) on the day of harvest (n = 8)

We correlated T-cells' composition with their antileukemic functionality in the presence or absence of Zylexis. We observed similarly increased proportions of CD4+ and CD8+ T_{reg} in cases *without* lysis after stimulation with DC in the presence or absence of Zylexis (Fig. 6a and b). However, in cases *with* antileukemic activity, DC-stimulated T-cells were able to mediate antileukemic activity *despite* upregulated CD8+ T_{reg} counts, but not in the presence of Zylexis (Fig. 6a). Cases *with* antileukemic activity also showed upregulated T_{eff} in the presence or absence of Zylexis (Fig. 6c).

***a.** Upregulated proportions of early proliferating CD3⁺ and activated CD8⁺ T-cells found in the presence of Zylexis after MLC*



***b.** Downregulated production of Teff (and lower decrease in Tnaive) in the presence of Zylexis*

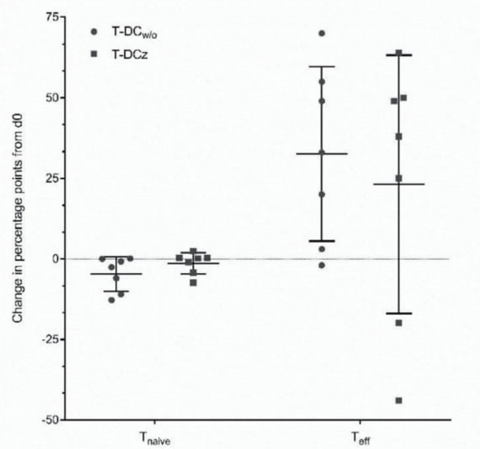


Fig. 4. Changes of different T-cell subtypes after MLC with DC in the presence or absence of Zylexis, compared to day 0 (n = 8) in percentage points.

This data points to a different influence on the T-cell composition through Zylexis in DC-cultures, than DC alone.

3.4.3. Effects of Zylexis on Cytokine-Profiles in DC- or MLC-culture supernatants

In previous studies we have shown, that high cytokine-concentrations (especially IL-6, IFN-γ) correlated with antileukemic T-cell-activity [37]. We analyzed whether the addition of Zylexis would alter cytokine release profiles in **DC**-culture supernatants. We measured the levels of IL-2, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ and MCP1 in *supernatants after DC-generation* from different DC-generating methods (Ca; MCM; Pici; Int), with and without Zylexis (n = 11). Pooling the results of the DC-generating methods we were able to show (significantly) lower levels of IFN-γ, TNF-α and IL-2 in supernatants containing Zylexis (Fig. 7a). Levels of the inhibitory IL-10 were (non–significantly) higher under the influence of Zylexis (3.0 ± 7.9 vs. 3.6 ± 14.2, p = 0.83).

In analogy we analyzed cytokine-release-profiles (IL-2;IL-4; IL-6; IL-8; IL-10; IL-12p70; IL-17A; TNF-α; IFN-γ; MCP1) in supernatants taken from **MLC**-media containing different effector cells (n = 5). The presence of DC vs. blast containing MNC increased the release of IL-4 after MLC, especially under the influence of Zylexis (p = 0.001; ANOVA; Fig. 7b). Levels of the remaining cytokines did not differ in the groups compared (data not shown).

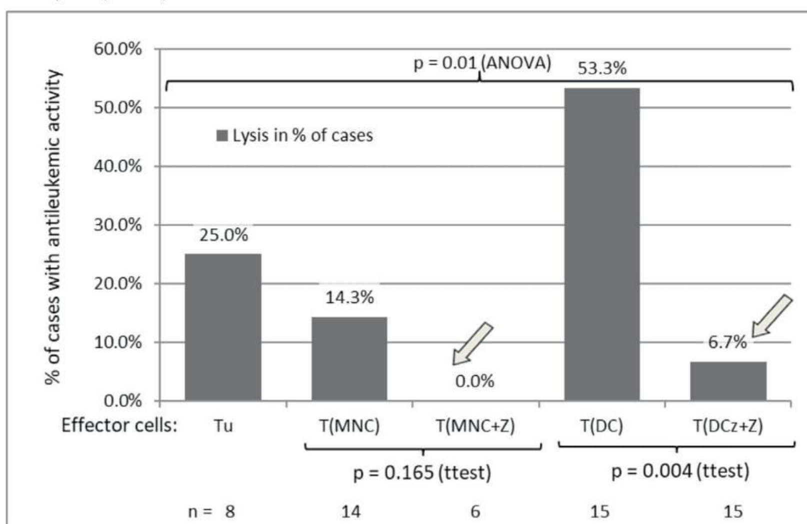
We conclude, that the presence of Zylexis influences the cytokine-release-profiles of cells in DC- as well as DC-stimulated MLC-cultures by favoring a more inhibitory cytokine-environment.

4. Discussion

4.1. How to improve antileukemic T-cell-activity – With a special focus on DC

A better prognosis has been reported for combinations of chemotherapy and immunotherapy than for chemotherapy alone to treat leukemia [38,39]. However, allo-HCT remains the only curative treatment option for many haematological malignancies, although long-term follow-up data beyond 5 years remains scarce. Its curative potential is mainly attributed to the graft-versus-leukemia effect, which in turn is primarily driven by donor derived immune-effector-cells, mainly donor-T-cells primed against leukemic targets. Their concerted infusion after allo-SCT in order to treat relapsed leukemia has become known as

a. Proportions of cases with antileukemic activity from untreated T-cells (T_u), and T-cells stimulated with MNC or DC - without or with Zylexis (arrows).



b. Proportions of cases with antileukemic activity from 6 parallelly tested cases with T-cells or MNC as effector cells, either stimulated with blast-containing MNC or DC_{leu} containing DC and in the absence or presence of Zylexis.

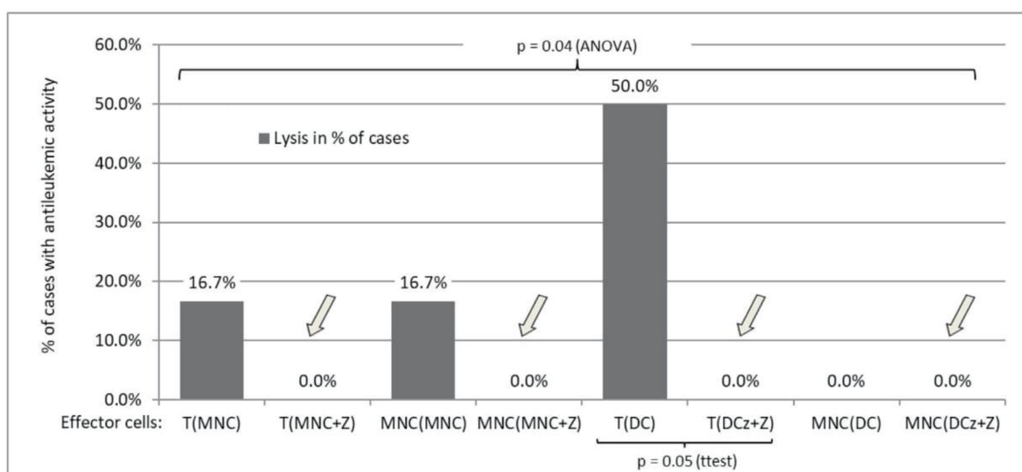


Fig. 5. The presence of Zylexis significantly decreased the effector cells' antileukemic activity **DC** dendritic cells, MNC Mononuclear cells; Tu untreated T-cells; T (MNC) blast-stimulated T-cells; T (MNC + Z) blast-stimulated T-cells with Zylexis; T(DC) DC stimulated T-cells; T (DCz + Z) DC stimulated T-cells with Zylexis; MNC(DC) MNC cultured/stimulated with DC (containing DCleu); MNC(DCz + Z) MNC cultured/stimulated with DCz (containing DCleu) in the presence of Zylexis; MNC(MNC) MNC stimulated with MNC; MNC(MNC + Z) MNC stimulated with MNC in the presence of Zylexis.

DLI [4,40,41]. Dendritic Cells (DC) are known to be the main T-cell stimulators due to their professional antigen presenting functionality [42]. DC-based immunotherapy involves their activation and subsequent presentation of tumor-antigens to effector-lymphocytes, initiating an immune response against cells expressing the equivalent antigens [7].

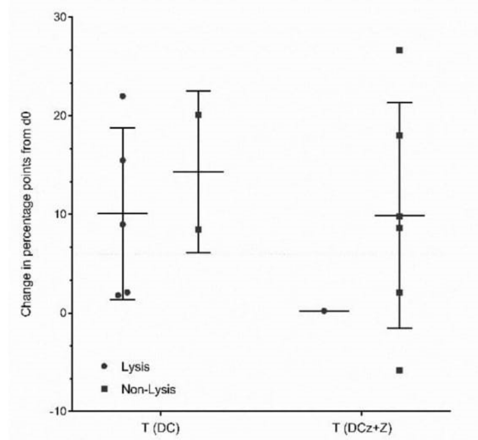
Due to DC's unique antigen presenting capacity immunosuppressive features of the leukemic blasts can be circumvented [43].

Different methods of loading antigens in or onto DC have been used in order to optimize antitumor responses. One strategy involves pulsing DC with leukemic cells or fusion of blasts with DC. Those DC are then able to present their tumor antigens to (donor) lymphocytes and thereby convert them into cytotoxic T cells [44]. Although several concepts exist, the identification and low immunogenicity of tumor-specific peptides remain a problem [7].

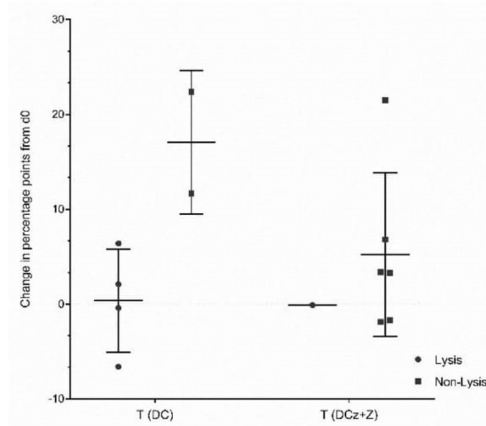
A strategy reserved to myeloid malignancies is to convert leukemic blasts directly to leukemia-derived dendritic cells (DC_{leu}), thereby creating APCs presenting the whole leukemic antigen repertoire and possessing the stimulatory capacity of mature professional DC – thereby bypassing the highly complex procedures mentioned above. We could show that T-cells, obtained from AML-patients (autologous) or donors (allogeneic), can be stimulated by these DC_{leu}, resulting in very efficient cytotoxic effector cells with specific lytic activity against naïve blasts, although not in every case [10,11].

Observations of tumor remissions occurring in a context of infectious diseases have been known for centuries. Many physicians followed the credo of the ancient Greek philosopher Parmenides (ca. 500BCE): "Give me the power to induce fever and I will cure all diseases" [45]. According to a medieval legend, a priest called Peregrine Laziosi was immediately cured of his cancerous leg ulcer after a serious

a. CD8+ Treg



b. CD4+ Treg



c. Teff

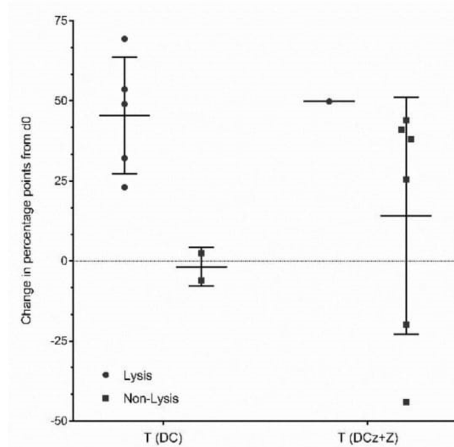


Fig. 6. Gain or loss in percentage points of selected T-cell proportions in MLC-samples (n = 8) of T(DC) and T(DCz + Z) on day of harvest (compared to d0) in correlation with achieved Lysis/non-Lysis. DC-stimulated T-cells are able to mediate antileukemic activity and compensate function of upregulated CD8+ Tregs (a.), but not in the presence of Zylexis, whereas CD4+ Tregs (b.) and Teff (c.) are not affected.

local infection occurred; because of this "miracle healing" he became the patron saint of all cancer victims [46]. Several surgeons of the 18th and early 19th century induced "targeted" infections by cutting wounds directly at the sites of tumors, especially in breast cancer. Jean Cruveilhier, the first professor of pathological anatomy in Paris, recommended that inflammation may be induced "by incisions or irritating applications" [47]. Around 1900, the New York surgeon William Coley refined this empirical approach by systematically using a bacterial vaccine ("Coley's vaccine") which is said to have induced regression of metastatic cancer in a considerable number of his patients [48]. With the emergence of aseptic surgery, antibiotics, antipyretics and modern therapeutic options for cancer, Coley's vaccine fell into oblivion.

In recent years research on therapeutic strategies involving the nonspecific, innate immune activation has become more important. For example clinical data shows a correlation between improved outcome and tumor-infiltration of both, innate natural-killer- and adaptive T-cells. Recently a role of iNKT (invariant natural killer cells), CIK (cytokine induced killer cells) or NK (natural killer)-cells in the mediation of DC-stimulated antileukemic responses was described [49].

One of the few cancer immunotherapies in widespread clinical use, the intravesical administration of Bacillus Calmette–Guerin for superficial bladder cancer, is innate and nonspecific in its action, utilizing antimicrobial immunity for antitumor effects [50,51]. Efforts to turn on the immune system against cancers with inactivated tumor vaccines or intra-tumor injections of bacterial products to induce local inflammation and recruit an antitumor immune response have led to anecdotal successes [52].

A biological employing a nonspecific activation of the innate immune system as its mode of action is Ipilimumab. It is an antibody, which blocks CTLA-4, a receptor on T-cells transmitting inhibitory signals during antigen-presentation through DC, and thereby leads to activation of the immune system [53]. Another example of an immune response modifying drug is Imiquimod, a synthetic imidazoquinoline that activates TLR7, currently used for the treatment of actinic keratosis, superficial basal cell carcinoma and condylomata acuminate [54]. The drug does not exhibit direct antiviral or antiproliferative activity when tested in a number of cell culture systems. Cells activated by Imiquimod secrete cytokines (primarily INF-α, IL-6, and TNF-α), thereby stimulating several other aspects of the innate immune response, as well as cellular immunity (e.g. Langerhans-cells, natural killer cells or macrophages) [55].

4.2. Pinds have a negative impact on antileukemic T-cell activity

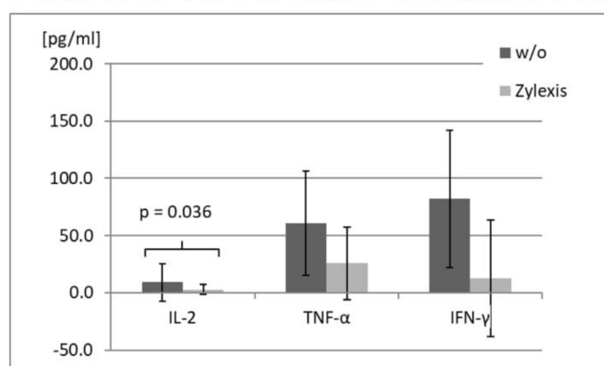
PINDs are known as (pox-derived) substances "optimizing" immune responses not only in infectious, but also in malignant diseases of animals due to stimulating/co-stimulating capacities [12,15]. Similarly, PINDs are known to act as non-immunising vaccines, with the capability to activate and regulate the paraspecific, i.e. innate, immune system, as shown in veterinarian fields. Their mechanism of action has not been analyzed in depth [15].

The aim of this study was to determine, whether the addition of PINDs to DC-generating media or to MLC-cultures (going along with PIND-phagocytosis/processing during DC- differentiation) would lead to an increased antileukemic stimulatory capacity of DC/DC_{leu}, in MLC with T-cells.

In the past our group was able to demonstrate DC_{leu} containing DC could be generated ex vivo in every given case with at least one of the DC-generating methods presented above, and that the composition and quality of DC is predictive for a successful ex vivo antileukemic response, especially with respect to proportions of mature and leukemia-derived DC [10].

An astonishing finding was, that the presence of PINDs in DC cultures benefitted maturation of DC, but decreased DCs' viability as well as TLR4- and 9-expression on blasts *and* DC DC_{leu} during culture. This

***a.** Cytokine levels (IL-2, TNF-α and IFN-γ) in DC-culture supernatans in the presence or absence of Zylexis (n=11)*



***b.** IL-4 levels in MLC-culture supernatans after T-cells' stimulation with blast-containing MNC or DCleu-containing DC in presence or absence of Zylexis (n=5)*

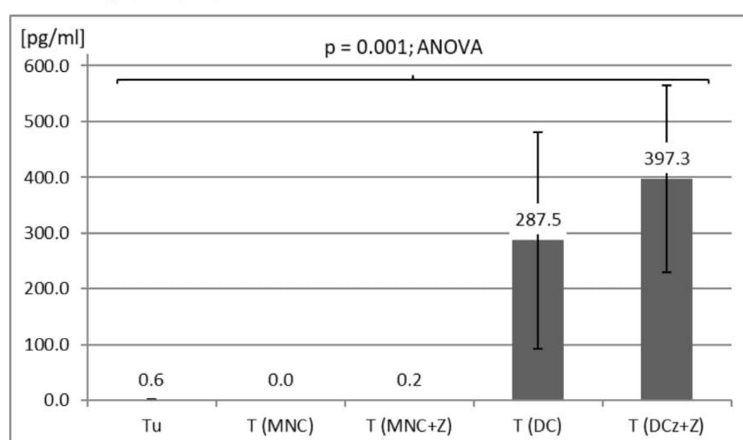


Fig. 7. The presence of Zylexis decreased release of IL-2, TNF-α and IFN-γ in DC-culture supernatans (a.) and increased release of IL-4 in MLC-supernatans after MLC of Tcells with DC-stimulated T-cells in the presence of Zylexis (b.)

was true for all kinds of PINDs tested, whether they were derived from "ovis" or "avi" virus strains, or the combination of both. TLRs have been linked to virus detection and the induction of immune responses. As PINDs consist of highly attenuated and inactivated virus strains, we expected an increase in TLR-expression, although it is known, that inactivated Parapoxvirus ovis mediates its immunostimulatory properties by TLR-dependent as well as TLR-independent pathways [56]. We conclude that PINDs either act through different TLRs/PRRs than tested, or exclusively on TLR-independent pathways [57].

We found, that the presence of Zylexis induced early (CD8+) T-cell activation, while differentiation to effector-T-cells was impaired. Moreover, no special activation of cells of the innate immune system (using the total MNC-fraction as effector cells) under the influence of Zylexis could be observed.

Applying Zylexis to the DC- and MLC-culture-medium resulted in a loss of antileukemic functionality, almost producing a "blasts protective capacity". This finding was further supported by the detection of an "inhibitory soluble microenvironment" (low Il-2, TNF-α, IFN-γ, high IL-4) in DC- or MLC-culture supernatants in the presence of Zylexis. Such an inhibitory microenvironment through soluble factors was described before by Vignali et al. [58]. Although alternatively an induction of a (antileukemic) B-cell-/antibody- response can be discussed [59] in the presence of PINDs in DC/MLC-settings, we suggest a minor role of IL-4 (in the presence of low IL-2, TNF-α, IFN-γ and higher IL-10 and T_{reg}-subtypes). An interesting finding was, that DC stimulated T-cells were able to lyse AML-blasts despite high CD8+ T_{reg}, however not in the presence of Zylexis – pointing to an influence of Zylexis on the composition of (regulatory) T-cells. Further analyses are necessary to characterize these (regulatory) T-cells in more detail: $CD39^+$, $CD73^+$ or other T_{reg}-subtypes might mediate the inhibitory effects described.

MUTZ-3 is a myeloid leukemia cell line, that can acquire a DC-like phenotype and function as potent antigen presenting cells (MUTZ-3-derived DC = MuDC). Kim et al. [60] were able to demonstrate, that MuDC did not show an enhanced level of CCR7 or cytokine production after lipopolysaccharid (LPS) stimulation, and LPS-stimulated MuDC ultimately failed to stimulate primary NK cells. On the other hand our group could show recently, that cells of the innate immune system (NK-, CIK- and iNKT-cells) are characterized by different cellular profiles in patients with AML, ALL or CLL, and play a role in the mediation of antileukemic reactions after DC-stimulation [49]. This suggests, that in analogy the leukemia derived DC used in our experiments might as well show an impaired reaction from stimulation through other PAMPs (e.g. PINDs). The underlying mechanism to this remains unknown.

In the past we were able to show that an inhibitory microenvironment as established by blasts can impair an antileukemic T-cell response, but can be overcome by the conversion of leukemic blasts to DC_{leu} [61]. Recent findings of our group support these cellular effects on a soluble level: we showed that 'DC'-/'MNC'-stimulation of T-cells

resulted in increased cytokine-levels in culture-medium compared to serum. High cytokine-concentrations (especially IL-6, IFN-γ) in MLC –supernatants after T-cells' 'DC'-/'MNC'-stimulation correlated with lytic T-cell-activity [37]. In the context of this study we could (at least in part) confirm the following observations: in the presence of Zylexis lower concentrations of IL-2, TNF-α and IFN-γ were found in MLC supernatants. This might add to the blast-protective effect described above, as these cytokines have been described as important mediators of the immune response [62]. This is in contrast to the findings of Ahne et al., who described an increase in inflammatory mediators, most notably TNF-α, when studying effects of the PIND Conpind [18]. These cytokines decreased in our experiments, which might be due to other factors influencing PIND-dependent immunomodulation.

'Viral therapies' using smallpox-, herpes zoster-, hepatitis-, influenca-, varicella-, measles-, rabies- or other viruses used in the treatment of cancer were shown to induce (transient) tumor regressions [63]. Moreover, our group could show, that a 'danger signaling' via PGE-2, Picibanil or PGE-1 gives rise to improved DC-generation, T-cell composition and soluble factor release after MLC, as well as an improvement of the antileukemic reactivity of DC-stimulated T-cells in MNC or even whole blood culture settings, containing the total cellular and soluble microenvironment of the individual leukemic patient [64].

Various escape mechanisms (inhibitory cytokines, regulatory cells), or unknown 'blast protective mechanisms' can be overcome by carefully selected immunomodulatory strategies: oncolytic viruses were shown to limit the efficacy of an virus based immunotherapeutic approach [65]. Combinations of viral-based therapeutic strategies as listed above with low-dose chemotherapies or IL-2, improved strategies concerning application (timing, route of administration and dosage) in combination with thorough quality controls in manufacture of uniform virus products and monitoring of unexpected or adverse events (e.g. elevated antibodies against the viral products, inhibitory cytokine profiles in serum, clinical events) could contribute to improve the viral products and their clinical use in a context of antitumor- or immune-therapy [62,63].

Our findings support the central role of T-cells as the most important mediators of antileukemic reactions by showing their superior antileukemic functionality as effector cells before and/or after SCT. However, these T-cell effects can be variable due to several 'influences' in the microenvironment [2,66]. Additional assays to quantify leukemia-specific cells (e.g. by Tetramer-, CSA-, ELISPOT, intracellular cytokine or degranulation assays) could further contribute to understand the whole functional repertoire of PINDs.

5. Conclusions

In summary our data points to Zylexis' induction of early T-cell activation and reduced effector cell function – resulting in a down-regulated antileukemic T-cell functionality in general, that might be caused by altered DC-characteristics (e.g. decreased TLR-expression in combination with increased maturation, decreased viability) and/or changed microenvironment – protecting blasts from attacks by immune-reactive cells. This means that although the use of PINDs might be promising in curing patients with infectious diseases, in patients with myeloid leukemias the "blast protective capacity" induced by PINDs might lead to unspecific and/or inhibitory influences in the cellular/innate immunity. The need for a thorough analysis on PINDs' whole range of effects in different settings (e.g: in/ex vivo environment, infectious/malignant) arises.

Therefore PIND-supported antileukemic trials cannot be recommended for the treatment of AML-patients.

6. Funding and declaration of interests

This project was supported by RIEMSER pharmaceuticals.

Parts of the results presented in this manuscript were worked out in the course of the MD thesis of Christian Ansprenger, Dr Julia Schick, Dr Valentin Vogt, Annika Hirn-Lopez and Yvonne Vokac at the University of Munich and the Helmholtz- Centre Munich.

The project-idea is summarized and listed as intentional report of Prof. Hans-Joachim Kolb and Prof. Helga Schmetzer at the University Hospital of Munich; CONPIND is listed as Patents No DE3504940 A1 and US 6,162,600 A of Anton Mayr.

Acknowledgements

The authors thank nurses and physicians on the wards for their support and diagnostic laboratories for the contribution of diagnostic reports of the patients. Moreover, we thank all of our colleagues for helpful discussion. We would like to thank Prof Barbara and Prof Anton Mayr as well as Dr Ihor Harabacz for providing us with Conpind, HP and 3P as well as guidance and discussion regarding PINDs and their employment.

7. Obituary

On the death of Prof. Dr. Barbara Mayr (died 2015) and Prof. Prof Anton Mayr (died 2014).

Prof Mayr was appointed to the Chair of Microbiology and Animal Diseases at the LMU in Munich in 1963. His later wife Prof Barbara Mayr became a professor at the same institute in 1972. In scientific terms, the Mayr couple worked mainly in the field of immunization in infectious diseases, especially of viral diseases in animals and the development of live vaccines. The MVA virus (Modified Vaccinia Ankara Virus) developed by Prof Mayr finally created the basis for the development of safe pox vaccines.

Prof B. and A. Mayr provided all their knowledge about 'paramunity inducers' (PINDs) made from highly attenuated poxvirus products, as well as the substances themselves for the project described here, as well as discussed the first results together with us. Unfortunately, the two could not experience the completion of our project. We, the authors of the manuscript thank them for all the enthusiasm, all the advice and accompaniments in the project. The expertise of the two we have to owe the execution of this very special project.

My special thanks go to Prof B. Mayr for many encouraging and personal words throughout the project phase.

Helga Schmetzer (in the name of all authors).

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10 Danksagung

Mein größter Dank gilt Frau Prof. Dr. Helga Schmetzer, die mir das Thema überlassen, mich hervorragend betreut und mir darüber hinaus unendliche Geduld geschenkt hat. Vielen, vielen Dank liebe Helga, Du bist die Beste!

Herzlicher Dank gebührt auch den inzwischen verstorbenen Prof. Barbara Mayr und Prof. Anton Mayr sowie Dr. Ihor Harabacz für die zur Verfügung gestellten Chargen Conpind, HP and 3P und noch viel mehr für die Diskussionen und Anregungen zum Thema und zu PINDs im allgemeinen.

Darüber hinaus möchte ich dem lieben Team/Mitdoktoranden in „unserem" Labor (vor allem Tanja Kröll, Dr. Valentin Vogt, Dr. Julia Schick) ganz herzlich für die gute Zusammenarbeit und die gegenseitige Unterstützung, aber auch die vielen gemeinsamen Stunden danken.

Gewürdigt werden sollen auch die vielen Patientinnen und Patienten sowie die gesunden Spender, die durch ihre Bereitschaft uns Probenmaterial zu überlassen die vorliegenden Untersuchungen überhaupt erst ermöglicht haben.

Auf privater Seite gilt mein Dank meiner Familie und Freunden, die über die Jahre oft auf mich verzichten mussten, weil noch „was" gemacht werden musste. Insbesondere meiner Frau Florina und meinen Eltern Christine und Alfred Ansprenger haben mir die Freiheiten ermöglicht, so dass ich ungestört arbeiten konnte.

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