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CLINICAL AND PREDICTIVE RELEVANCE OF IN VITRO GENERATION OF DENDRITIC CELLS OF LEUKEMIC ORIGIN IN PATIENTS WITH AML AND MDS

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Inhaltsverzeichnis

Abkürzungsverzeichnis

AML	acute myeloid leukemia
alloSCT	allogeneic stem cell transplantation
APC	antigen-presenting cells
B cells	B lymphocytes
Bla+	leukemic cells expressing blast markers
CCR7	chemokine receptor type 7
CD	cluster of differentiation
CR	complete remission
DC	dendritic cells
DCA	dendritic cell antigen
DC+/MNC	dendritic cells in total mononuclear cell suspension
DCleu	dendritic cells of leukemic origin
DCleu+	cells expressing blast antigens and dendritic cell antigens
DCleu+/MNC	dendritic cells of leukemic origin in total mononuclear cell suspension
DCleu+/DC	dendritic cells of leukemic origin in dendritic cell fraction
CCR7+DC+/DC	mature migratory chemokine receptor type 7 positive dendritic cells in dendritic cell fraction
FACS	fluorescence-activated cell sorting
FLT3	FMS-like tyrosine kinase 3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GvHD	graft versus host disease
GvL	graft versus leukemia effect
IEC	immune effector cells
IL	interleukin
LFS	leukemia-free survival
MAC	myeloablative conditioning
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex

MLC	mixed lymphocyte culture
MNC	mononuclear cells
mo-DC	monocyte-derived dendritic cells
MRD	measurable (“minimal”) residual disease
NMA	non-myeloablative conditioning
OS	overall survival
PGE1, PGE2	prostaglandin E1, prostaglandin E2
RNA	ribonucleic acid
RIC	reduced intensity conditioning
SCT	stem cell transplantation
T cells	T lymphocytes
Th	helper T cells
TGF	transforming growth factor
Tregs	regulatory T cells
TRM	transplant related mortality

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Publication I

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Congress Contributions

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1. Contributions to Original Publications

Contributions to Publication I (as Co-Author)

- Selection of samples in collaboration with main author and co-authors
- Reviewing and evaluation of medical records
- Discussion of results with main author and co-authors
- Support in statistical analyses

Contributions to Publication II (as Co-Author)

- Selection of samples in collaboration with main author and co-authors
- Collecting and compiling clinical findings and reports
- Evaluation of experimental data in a context of clinical data
- Discussion of results with main author and co-authors
- Support in statistical analyses

Contributions to Publication III (as First Author)

- Selection of patients and samples
- Careful selection and compilation of patient data according to defined criteria
- Analysis and interpretation of patients' clinical course of disease
- Complete statistical analysis
- Communication and coordination with co-authors
- Complete preparation of manuscript and finalization of text, figures and references
- Drafting and revision of the manuscript, communication with reviewers and editors

All co-authors permitted the use of the publications listed above for this doctoral thesis by signature.
All co-authors stated that these publications are not part of other doctoral theses.
Co-authors' signed statements were submitted separately along with this thesis.

2. Introduction

My research for this thesis aimed to gain a deeper understanding of the function of (leukemia derived) dendritic cells (DC) in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), particularly their role in therapies based on allogeneic stem-cell transplantation (alloSCT).

2.1. Acute Myeloid Leukemia and Myelodysplastic Syndromes

2.1.1. Overview of AML and MDS

AML and MDS are a heterogeneous group of malignant/premalignant myeloid neoplastic disorders. Gene mutations, changes in gene expression, and chromosomal rearrangements in myeloid precursor cells result in aberrant differentiation and excessive clonal proliferation. Dissemination of leukemic blasts into the bone marrow, blood, and other organs interferes with hematopoiesis, resulting in anemia, neutropenia, and thrombocytopenia, and in cases of AML, if untreated, leads to death within weeks (Dohner, Weisdorf et al. 2015).

With a median age at diagnosis of 69 years (AML) and 76 years (MDS), AML and MDS are diseases mainly affecting the elderly (Goldberg, Chen et al. 2010, Siegel, Giaquinto et al. 2024).
Both affecting the myeloid cell lineage, AML and MDS share significant similarities (e.g., bone marrow involvement, treatment options). Cases of MDS are therefore included in our AML studies.
Whereas only a few decades ago, AML was untreatable, patients now benefit considerably from substantial advancements in diagnostics and therapy (Grimwade and Hills 2009, Dohner, Weisdorf et al. 2015). Treatment outcome, however, is still unsatisfactory.

2.1.2. AML and MDS Therapy

2.1.2.1. Standard Treatment

Conventional regimens include induction (chemo-)therapy, most commonly with cytarabine and an anthracycline of variable intensity adjusted to patients' risk factors like age or cytogenetics. This is followed by consolidation therapy, usually cytarabine-based chemotherapy, or, in younger patients in a good clinical status, hematopoietic stem cell transplantation (SCT) (Koreth, Schlenk et al. 2009, Dohner, Weisdorf et al. 2015, Dohner, Wei et al. 2022).

Rates of complete remission (CR) reach up to 80% in adults under 60 years of age and up to 60% in patients aged 60 and older. However, two-year survival probabilities are below 50%, and up to 80% of the patients relapse (Dohner, Weisdorf et al. 2015). MDS patients' prognosis is poor as well, with 3-year survival rates below 40% (Ma, Does et al. 2007, Greenberg, Tuechler et al. 2012).

Measurable residual disease (MRD) is widely known as a significant factor contributing to the failure of induction therapy. Eradication or control of MRD remains a crucial factor for long-term remission in AML and MDS (Terwijn, van Putten et al. 2013, Heuser, Freeman et al. 2021).

2.1.2.2. Hematopoietic Stem Cell Transplantation

Allogeneic hematopoietic SCT can successfully eradicate MRD and is therefore regarded as the only potential curative treatment approach. SCT entails replacing the patient's malignant hematopoietic tissue with healthy stem cells from a compatible donor, thereby reconstituting hematopoiesis and eliminating malignant cells through donor immunity (graft-versus-leukemia (GvL) effect) and pre-SCT conditioning therapy (Loke, Malladi et al. 2020).

SCT is linked to high rates of severe side effects and mortality, making this therapy option less appropriate for older patients with comorbidities. It is recommended for patients with high risk of relapse in first CR, with primary refractory disease after the first induction cycle, relapsed disease, or those in a second CR following salvage therapy, particularly in cases with intermediate or high cytogenetic risk (Dohner, Weisdorf et al. 2015, Loke, Vyas et al. 2021, Dohner, Wei et al. 2022).

The antileukemic effect of SCT is promoted by the cytoreductive effect of chemo- and/or radiotherapeutic conditioning prior to SCT and long-term by the GvL effect of transplanted donor-derived immunocompetent cells, primarily mature T lymphocytes (T cells) but also natural killer (NK) cells and B lymphocytes (B cells) (Kolb 2008, Sweeney and Vyas 2019). Donor effector cells, once activated by DC or other antigen-presenting cells (APC), can recognize mismatching antigens (e.g., minor histocompatibility antigens, leukemia associated antigens) expressed via major histocompatibility complex (MHC) on the hosts' residual malignant cells and eliminate them. This mechanism can control MRD and enable stable long-term remissions (Kolb 2008, Kolb, Schmetzer et al. 2014, Sweeney and Vyas 2019, Sterling and Webster 2020).

Pre-SCT conditioning regimens aim to eradicate residual leukemic cells, induce immunosuppression for the prevention of graft rejection and graft-versus-host disease (GvHD) and clearing the hematopoietic stem cell niche in the bone marrow to allow engraftment. Standard myeloablative conditioning (MAC) regimens typically induce permanent bone marrow aplasia without SCT, however, enabling fast engraftment and effective leukemic cytoreduction. The benefits of MAC are primarily evident in individuals aged under 40 years, as elderly patients, especially those with comorbidities have a higher transplant-related mortality (TRM) due to the toxicity of those regimens (Loke, Vyas et al. 2021, Dohner, Wei et al. 2022, Murdock, Ho et al. 2024).

Reduced intensity conditioning (RIC) and non-myeloablative (NMA) regimens utilize lower doses of radio- and chemotherapeutic agents and do not ablate the hematopoietic stem cells completely but still permit engraftment. Residual hematopoiesis and lower toxicity in RIC and NMA have significantly expanded the range of patients eligible for SCT (Estey, de Lima et al. 2007, Kolb and Schmid 2020, Loke, Vyas et al. 2021). As RIC and NMA rely less on the cytotoxicity of high-dose chemotherapy or radiation as in MAC

regimes, the GvL effect emerges as the predominant pathway for eradicating residual leukemia in these approaches. Notably, the GvL effect is evident in all three therapy forms (Sweeney and Vyas 2019, Sterling and Webster 2020).

Despite significant improvements in overall survival (OS) rates and non-relapse mortality over the recent decades, relapse rates remain largely unchanged, with five-year survival ranging from 35–50% and relapse occurring in up to 70% of cases within the first year post-SCT (Canaani, Beohou et al. 2019, Loke, Vyas et al. 2021).

Novel strategies aim on improving efficacy of SCT and overcoming resistance mechanisms utilizing natural killer cell therapy, adoptive T cell transfer, or combination of SCT with targeted immune therapies such as FMS-like tyrosine kinase 3 (FLT3) inhibitors (e.g., Midostaurin) (Kekre and Koreth 2015, Dohner, Wei et al. 2021).

In summary, the long-term effects of stem cell transplantation are mainly mediated by the GvL effect of donor immune cells and are dependent upon effective antigen presentation.

2.1.2.3. New Therapies

Advancements in understanding the disease's molecular and genetic mechanisms allow to target patients' individual abnormalities, resulting in more personalized therapy approaches. New therapies like targeted therapies, epigenetic modifiers, and mitochondrial inhibitors, are the object of ongoing clinical research (Dohner, Wei et al. 2021).

For instance, alterations in the FLT3 gene, which is involved in hematopoietic stem and progenitor cell signaling, are are linked to unfavorable therapy outcomes. By blocking FLT3 receptors' activity, FLT3 inhibitors like Midostaurin restrict the proliferation of leukemic cells and induce apoptosis (Daver, Schlenk et al. 2019, Greiner, Gotz et al. 2022).

Various novel immunotherapies have been developed to induce specific immune responses against tumor cells. Active and passive immunization approaches target leukemia-associated or specific antigens. These include peptide, desoxyribonucleic acid (DNA) or ribonucleic acid (RNA)-based vaccinations and monoclonal antibodies, as well as DC-based vaccinations (see also chapter 2.2.2.) and adoptive T cell transfer (CAR T cell therapy) (Mardiana and Gill 2020, Greiner, Gotz et al. 2022).

It remains challenging to elicit efficient immune responses by vaccination due to difficulties in identifying antigen targets selectively expressed on malignant cells, co-expression of leukemic antigens on healthy progenitor or stem cells and immune evasion mechanisms (Vago and Gojo 2020).

2.1.3. Immune Escape of Leukemic Cells

Various mechanisms can contribute to the leukemic blasts' ability to evade immune surveillance and promote disease progression.

These include the reduced expression or total loss of the MHC complex on blasts, which impairs antigen presentation resulting in ineffective recognition by T cells (Teague and Kline 2013). Malignant cells establish an inhibitory microenvironment, e.g., through adenosine accumulation, which suppresses T and NK cell activity. Additionally, adenosine stimulates regulatory T cells (Tregs) to release immunosuppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β), resulting in impaired DC maturation, downregulation of co-stimulatory molecules, and, in consequence, insufficient T-cell activation (Schmetzer 2012). Moreover, the expression of inhibitory ligands by leukemic cells leads to dysregulation of the T cells' immune checkpoint pathway, resulting in T cell exhaustion or anergy (Vago and Gojo 2020).

Understanding and targeting these processes is of particular relevance for future immunotherapies (Schmetzer 2012).

2.2. Dendritic cells

2.2.1. Dendritic Cells' Biology

DC are a class of professional APC that are crucial in the immune system, especially in anti-tumor immunity. DC are specialized in capturing and processing antigen (e.g., protein-, lipid-, or nucleic acid-fragments) and presenting it on the cell surface to along with costimulatory molecules (e.g., CD86, CD80, CD40). DC can stimulate cells of both the innate and adaptive immunity, thereby inducing and regulating initial and secondary immune reactions (Palucka and Banchereau 1999, Banchereau, Briere et al. 2000, Steinman 2007).

Being part of the innate immunity itself, DC can activate other components of innate immunity, such as NK cells, to bridge intervals until the antigen-specific adaptive immune response is initiated. Additionally, activated NK cells can stimulate the maturation of immature DC (Reschner, Hubert et al. 2008).

After capture of antigens, immature DC undergo maturation, enhancing their ability to activate T cells. While migrating towards afferent lymphoid organs, alterations in their surface markers (e.g., costimulatory molecules, cytokine receptors) and cytokine production (e.g., IL-12) can be seen (Palucka and Banchereau 1999). When an antigen specific T cell encounters a DC presenting matching antigen, the formation of immunological synapses between both is initiated by the interaction between the T cell receptor and MHC-peptide complex. Additional mandatory interactions involving costimulatory molecules (e.g., CD86 on DC), induce the upregulation, clonal proliferation, and differentiation of naïve T cells, specific for the presented antigen, into non-naïve effector and memory T cells (Grakoui, Bromley et al. 1999, Schmetzer 2012). Antigen-specific effector T cells, stimulated by DC, are key mediators of anti-tumor immunity by recognizing MHC I-antigen complexes on tumor cells and can directly eliminate them or induce apoptosis (Banchereau, Briere et al. 2000).

DC induce naïve CD4+ T cells to differentiate towards various subtypes of Th cells or Tregs via MHC II-antigen receptor complex and cytokine signaling. Th cells activate cytotoxic T cells through cross-priming, mediated by interactions with DC, and promote recruiting and activation of macrophages, eosinophils and B cells, or in the case of Tregs, prevent excessive immune reactions or even suppress immune reactions (Banchereau, Briere et al. 2000, Berard and Tough 2002).

In humoral immunity, activated DC significantly influence in the process of B cell differentiation towards antibody-secreting plasma cells by production of cytokines like B cell-activating factor and IL-12 (Banchereau and Steinman 1998, Palucka and Banchereau 1999, Ueno, Schmitt et al. 2010).

DC stimulate and regulate both innate and adaptive immunity by reciprocal activation, rendering them essential in the crosstalk and interface of both systems (Banchereau, Briere et al. 2000, Steinman 2003, Wan and Dupasquier 2005).

2.2.2. Dendritic Cell-Based Therapy

2.2.2.1. Vaccination with Monocyte Derived Dendritic Cells

DC-based immunotherapies aim at initiating strong targeted immune responses by enhancing or reestablishing impaired APC- and effector cell functionality (Claxton and Choudhury 2001). One approach entails the in vitro cultivation of DC from patients' autologous monocytes (mo-DC), followed by pulsing with tumor antigens (Yu, Sun et al. 2022).

DC can be cultured from isolated monocytes using cytokines (e.g., Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)), followed by activation with proinflammatory mediators, resulting in mature dendritic cells (Dauer, Obermaier et al. 2003).

Pulsing with antigens can be achieved through several methods like exposure to tumor lysates, transfection with antigen-encoding messenger RNA or loading of peptides corresponding to specific tumor antigens. Those DC can be administered as a vaccine to the patient. This cumbersome process is sensitive and requires a high level of expertise, and outcomes vary widely among patients (Van Tendeloo, Van de Velde et al. 2010, Anguille, Van de Velde et al. 2017, Fritah, Rovelli et al. 2022). Limitations might be due to the limited migratory capacities of mo-DC and risk of immune escape in single antigen approaches (Lee, Yam et al. 2023).

2.2.2.2. Therapies Based on Dendritic Cells Generated from Leukemic Blasts

A different strategy aims to directly induce differentiation of leukemic blasts into leukemia derived DC (DCleu) ex vivo. Those DCleu express patient-specific blast antigens (e.g., CD34, CD117, CD65 or lineage discordant markers like CD56, CD7), as well as DC antigens (DCA) (e.g., CD80, CD86, CD40, CD1a, CD206, CD137L, CD209) (Kremser, Dreyssig et al. 2010, Van Acker, Versteven et al. 2019, Ansprenger, Amberger et al. 2020).

DCleu have the unique ability to display the whole range of known and unknown patient-specific leukemic antigens. As a result, DCleu do not require pulsing with tumor antigens to stimulate immune responses. A larger variety of presented antigen targets is expected to make DC-based therapy approaches less sensitive to potential antigen loss or antigen drift (Van Acker, Versteven et al. 2019, Amberger and Schmetzer 2020).

DCleu can be generated ex vivo from peripheral blood mononuclear cells (MNC) as well as whole blood, utilizing a variety of strategies ex vivo, including various cytokines (e.g., GM-CSF), prostaglandins (e.g., prostaglandin E1 (PGE1), bacterial lysates (e.g., Picibanil), danger signals, and others to modulate differentiation, maturation, and function (Choudhury, Liang et al. 1999, Woiciechowsky, Regn et al. 2001, Lee, Truong et al. 2002, Houtenbos, Westers et al. 2003, Sato, Takayama et al. 2003, Westers, Stam et al. 2003, Rouas, Lewalle et al. 2004, Kremser, Dreyssig et al. 2010, Hirn Lopez, Deen et al. 2019).

As shown by us and others, successful generation of substantial amounts of DCleu in serum-free culture is feasible any AML and MDS patient (Lee, Truong et al. 2002, Houtenbos, Westers et al. 2003, Sato, Takayama et al. 2003, Kremser, Dreyssig et al. 2010, Dreyssig, Kremser et al. 2011). These DC express costimulatory molecules and can elicit effective immune responses against leukemic cells ex vivo (Grabrucker, Liepert et al. 2010, Liepert, Grabrucker et al. 2010, Dreyssig, Kremser et al. 2011).

DC, converted from leukemic blasts, can be administerd to patients as a vaccine and have shown various immunologic effects in clinical trials, such as an increase of antileukemic cytotoxic T cells or central memory T cells (Li, Giannopoulos et al. 2006, Dong, Liang et al. 2012, Van Acker, Versteven et al. 2019).

2.2.2.3. Therapy Based on In Vivo Generation of DC

It is long known that myeloid leukemia cells can differentiate towards DC in vivo (Mittermuller, Kolb et al. 1986). A direct differentiation of leukemic blasts towards DCleu in vivo could leverage their capacity to effectively present antigen, consequently inducing specific antileukemic immune responses without the cumbersome process of adoptive cell transfer (Kolb, Rank et al. 2004).

In order to generate DC under physiological conditions that can induce antileukemic T cell reactions, our workgroup developed an ex vivo culture system that utilizes heparinized whole blood samples. Whole blood most closely mimics in vivo conditions, as it contains a variety of inhibitory or activating cytokines and molecules (Amberger and Schmetzer 2020, Kugler 2020, Schwepcke, Klauer et al. 2022).
The system is based on three kits containing two clinically approved response modifiers or cytokines: GM-CSF for differentiation and PGE1, PGE2, or Picibanil for maturation (European patent EP3217975, US-Patent US10912820, Modiblast GmbH, inventor Prof. H. Schmetzer).

It could be seen that these kits effectively provided adequate amounts of DCleu and mature DC ex vivo without causing blast expansion and, after mixed lymphocyte culture (MLC), enhance antileukemic immune effector cell (IEC) reactions and reduce frequencies of immune suppressive Tregs (Amberger, Doraneh-Gard et al. 2019, Hirn Lopez, Deen et al. 2019, Amberger and Schmetzer 2020, Pepeldjiyska, Li et

al. 2022). First animal trials and single individual clinical trials showed promising results in terms of specific immune responses, notably increased rates of DCleu, mature DC, potentially AML-specific NK- and T cells, and a decrease in Tregs without blast expansion (Atzler, Rank et al. 2019, Plett, Klauer et al. 2022, Anand, Filippini Velazquez et al. 2024).

2.3. Outline of This Thesis

We hypothesize that induced or spontaneous in vivo conversion of blasts to mature DC, originating from leukemic cells, can initiate potent immune responses against leukemia (Schmetzer 2012). In consequence, the response to alloSCT could be explained by improved DC-mediated IEC activation (Kolb, Schattenberg et al. 1995, Schmid, Labopin et al. 2007).

In return, patients without response to such immunotherapies might have impaired APC or effector cell functionality, resulting in insufficient AML-specific antigen presentation and subsequently impaired IEC activation and co-stimulation. This could imply that therapeutic administration of substances that enhance DC-differentiation and maturation can enable and facilitate the in vivo conversion of leukemic blasts towards DCleu (Sachs 1978, Choudhury, Liang et al. 1999, Kolb, Rank et al. 2004).

Our hypothesis proposes that a robust ex vivo differentiation of leukemic cells into DCleu may reflect a productive in vivo transformation, enhancing antileukemic immune responses mediated by both innate and adaptive immune cells. Consequently, this process could be associated with an improved response to SCT.

The aims of this cumulative thesis were to study:

- The individual dendritic cell antigen expression profiles on naïve blasts, as well as assessing how these profiles changed after culture in order to refine culture methods that can generate substantial quantities of DC capable of eliciting antileukemic IEC reactions (**publication I**).
- The ability of ex vivo generated DC to prime T cells for antileukemic activity and to assess DC quality and subtype distribution as predictive indicators of T cell cytotoxic capability (**publication II**).
- The significance of ex vivo cultivated DC in predicting the clinical outcomes of patients with AML and undergoing SCT-based immunotherapy (**publication III**).

2.4. Publication I: DC Antigens are Expressed on Myeloid Blasts

Publication I (as co-author) ***"Various 'dendritic cell antigens' are already expressed on uncultured blasts in acute myeloid leukemia and myelodysplastic syndromes"*** (Dreyssig, Kremser et al. 2011)

Background: DC, as professional APC, have a high potential to stimulate IEC, making them promising candidates for antileukemic vaccination strategies. In that context, there is a need to provide sufficient amounts of DC that can initiate antileukemic IEC activity. Therefore, we investigated the expression of DCA on uncultured blast-containing MNC and DC, derived from these MNC. The generated DC were evaluated for their capability to activate (T) cells against leukemic blasts using a fluorolysis assay after MLC.

Methods: DC were generated from MNC in 186 patients (137 with AML and 49 with MDS) via six distinct approaches, and expression profiles were assessed pre- and post-culture via flow cytometry employing a special gating strategy. The specific DCA that were evaluated included CD1a, CD1b, CD1c (involved in presenting lipid antigens), CD25 (IL2 receptor), CD40 (interacts with CD4+ Th cells), CD83 (maturation marker) CD80, CD86, CD137L (costimulatory molecules), CD206 (mannose receptor). The T cell enriched MLC involved coculturing T cells from AML/MDS samples or the physiological reference group with leukemia-derived DC or blast-containing MNC, followed by twofold restimulation prior to cytotoxicity assays.

Results: The results showed that DCA are consistently detectable to varied extents on pre-culture blasts. A shift in expression profiles could be observed after culture, particularly an upregulation of DCA in most instances. Especially undifferentiated cases classified as M0 and M1 exhibited elevated mean expression levels of DCA after culture, particularly CD1b and CD206, while the overall expression of DCA was generally higher in cases with adverse cytogenetic profiles. Different DC-generating methods resulted in varying success rates in producing viable and mature DC. Nevertheless, at least one method was effective in producing DC in each patient case. It was possible to identify at least one individual DC marker for each patient that was absent in blasts before culture but reached peak levels in differentiated DC post culture, enabling identification and quantification of the generated DC.
In the fluorolysis assay DC activated T cells showed cytotoxic potential against leukemic cells whereas MNC stimulation mostly induced blast proliferation.

Summary: DCA are consistently expressed at varying levels pre-culture on leukemic cells and upregulated after culture. A combination of three methods ensures reliable generation of substantial amounts of DC. The DC generated can induce antileukemic immune reactions mediated by T cells.

Conclusion: Overall, the findings suggest that assessing patients' specific DCA expression patterns of uncultured blasts is crucial for optimizing DC generation and quantification.

2.5. Publication II: Quality and Quantity of DCleu Predict Lytic Potential of DC-Primed T cells

Publication II (as co-author), ***"The Quality and Quantity of Leukemia-derived Dendritic Cells from Patients with Acute Myeloid Leukemia and Myelodysplastic Syndrome are a Predictive Factor for the Lytic Potential of Dendritic Cell-primed Leukemia-Specific T Cells"*** (Grabrucker, Liepert et al. 2010)

Background: The high relapse rates of conventional AML treatment may result from compromised APC and effector cell functionality. DC-mediated antileukemic IEC reactions exhibit varying intensity and can also have contradictory effects. Therefore, we analyzed the T cells' cytotoxic capability after DC stimulation and contributed to establishing predictive factors in patients with AML and MDS.

Methods: MNC isolates from 17 AML and 2 MDS samples were analyzed. We generated and quantified DC and different subsets of DC using three different methods and an optimized gating strategy applied to the flow cytometric analysis. A functional fluorolysis assay was used to evaluate the cytotoxic capacity of primed (DC or MNC) or unprimed (control group) CD3+ T cells following T cell enriched MLC. The lysis efficiency was estimated by comparing the frequencies of viable blasts prior to and following IEC interaction.

Results: The results generally indicate that DC-stimulated T cells have higher cytotoxic potential potential in comparison to MNC-stimulated or unprimed T cells. Frequencies of certain DC subsets were found to be predictive for lytic efficiency, specifically DCleu in the DC fraction (DCleu+/DC), mature (CD83+) DC, and mature migratory (chemokine receptor 7 positive (CCR7+)) DC. Cutoff values could be established to separate samples with blast lysis/non-lysis, in particular frequencies higher than 65% of DCleu+/DC, more than 45% mature CD83+ DC, and more than 25% mature migratory CCR7+ DCs were linked to leukemia-specific lytic capacity of DC primed IEC. Moreover, improved response to SCT was seen in cases with elevated levels of DCleu+/DC, mature DC, and CCR7+ DC.

Summary: These findings indicate the pivotal role of DC's composition and quality after MLC priming for effective ex vivo immune reactions. T cells primed by high levels of DCleu and mature/mature migratory DC have an up to 100% probability of being primed against leukemic antigens. These ex vivo processes may simulate in vivo long-term immunological control of MRD by residual myeloid blasts differentiating towards DCleu, thereby initiating antileukemic IEC responses.

Conclusion: Functional fluorolysis assays of ex vivo DCleu primed T cells can be an indicator for patients' DC functionality and help to develop novel treatment approaches to overcome impaired DC-mediated anti-tumor immune reactions.

2.6. Publication III: Quality and Quantity of In Vitro Generated DC Predict Response to Allogeneic SCT-Based Immunotherapy

Publication III (as first author) ***"In Vitro Generated Dendritic Cells of Leukemic Origin Predict Response to Allogeneic Stem Cell Transplantation in Patients with AML and MDS"*** (Freudenreich, Tischer et al. 2022)

Background: AlloSCT is regarded as the sole potential curative treatment approach, harnessing the GvL effect. However, GvL based immunotherapies are ineffective in some patients, possibly due to insufficient antigen presentation.
Therefore, we analyzed the correlation between the ex vivo differentiation of malignant blasts into DC and the clinical outcome of SCT.

Methods: Up to six methods were applied to culture DC ex vivo from 69 samples obtained from 47 patients prior to and 22 patients after alloSCT in active stages of disease. DC and different subsets of DC (DC in MNC fraction (DC+/MNC), DCleu in MNC fraction (DCleu+/MNC), DCleu+/DC, mature migratory DC in DC fraction (CCR7+DC+/DC)) were evaluated and characterized via flow cytometry and correlated with patients' clinical outcomes, in particular long-term response, time to relapse, leukemia-free survival (LFS) and OS after SCT. We defined long-term response to SCT as initial response followed by one year of sustained CR.

Results: The findings demonstrate a highly significant positive correlation between higher frequencies of the DCleu+/DC subset and improved long-term response to immunotherapy, a longer time to relapse, and longer LFS and, partially, OS.
DCleu+/DC rates of long-term responders were significantly higher than those of non-responders to allogeneic SCT (76.8% vs. 58.8%, P=0.006, all patients before and after SCT pooled). To predict patients' outcomes, cutoff values for specific DC subtypes, particularly DCleu+/DC, could be established, enabling the categorization of cases into long-term responders or non-responders to SCT, as well as those with extended OS, LFS, or time to relapse. When pooling all patients it was found that frequencies of DCleu+/DC higher than 50%, were associated with better outcomes in terms of long-term response (54% responders in >50% DCleu+/DC section vs. 8% responders in ≤ 50% DCleu+/DC section, P=0.004), LFS (1015 versus 393 days, P = 0.005), and longer time to relapse (62% later than 105 days in >50% DCleu+/DC section vs. 25% in ≤50% DCleu+/DC section, P = 0.04) post-SCT. Mean OS was significantly higher in patients with more than 70% DCleu+/DC than in patients with lower frequencies (1409 vs. 903 days, P=0.048). The results in patients prior to SCT were comparable, and in some cases, even more significant.
Although the subset of mature migrating DC (CCR7+) was a marginally significant predictor of clinical outcomes, overall DC rates were not predictive.

Summary: Higher proportions of DCleu+/DC are significantly associated with improved clinical outcomes. The findings suggest that while the DC rates do not differ significantly across patients, the quality

of the DC population, particularly the presence of DCleu, is crucial for mediating effective antileukemic responses and predicting patient outcomes after SCT-based immunotherapy.

Conclusion: Response to immunotherapy seems to be dependent on frequencies and quality of leukemia-specific DC, in particular the presence of DCleu. Ex vivo generation and quantification of DC and their subtypes may serve in addition to known factors as an indicator to predict clinical outcome post-SCT.

2.7. Conclusion

Our studies support the concept of specific subtypes of DC (DCleu, mature/mature-migratory DC) playing a crucial role in anti-leukemic immunity.

In **publication I,** we were able to demonstrate that DCA are already consistently detectable to varied extents on pre-culture blasts. We conclude that it is crucial to assess patients' specific DCA signature prior to culture to optimize DC generation and quantification. We could show that a combination of three DC-generating methods facilitates the reliable generation of sufficient amounts of DC in any patient with AML and MDS, e.g., for vaccination purposes. These DC can elicit IEC responses against leukemic cells ex vivo after MLC (**publication I and II**).

Our findings substantiate the hypothesis that the presentation of leukemia-specific antigens by DCleu and mature/ mature migratory DC plays a significant role in stimulating strong antileukemic immune responses in vivo based on activation of T-cells as well as other effectors of the innate and adoptive immune system. We could show that frequencies and quality of DC are indicative for ex vivo immune reactions of DC-stimulated IEC (**publication II**) as well as for long-term SCT outcomes (**publication III**). These results indicate that DC, specifically DCleu and mature DC, induce the differentiation of immunocompetent antileukemic cells in vitro and, most likely, in vivo and lead to better outcomes post-SCT.

The generation and quantification of DC, especially DCleu, and functional fluorolysis assays of DC-stimulated T cells may not only serve as an indicator to assess antileukemic immune reactions and DC functionality ex vivo, but also as an additional parameter for predicting the long-term response to SCT and evaluating patients' therapy options.

These and other studies conducted by our workgroup led to the creation of clinically validated response modifier formulations (kits), designed to induce in vivo differentiation of DC from leukemic blasts. These have shown promising results in terms of induction of specific immune reactions in whole blood culture, animal studies and first individual clinical trials. This is of particular relevance for future in vivo DC-based immunotherapies, as they could significantly improve patients' long-term response rates and prolong remission and survival rates while minimizing adverse side effects.

3. Zusammenfassung

Die allogene Stammzelltransplantation (SCT) ist die einzige potenziell kurative Behandlungsoption für Patienten mit akuter myeloischer Leukämie (AML) und myelodysplastischem Syndrom (MDS), jedoch sind die Erfolgsraten trotz erheblicher Fortschritte in den letzten Jahrzehnten aufgrund hoher Rezidivraten und schwerer Nebenwirkungen nach wie vor unbefriedigend. Der wichtigste Mechanismus der SCT für die Eradikation maligner Zellen ist der Graft-versus-Leukemia-Effekt (GvL), der durch immunkompetente Effektorzellen (IEC) wie T- und NK-Zellen des Spenders vermittelt wird. Die Präsentation tumorassoziierter oder tumorspezifischer Antigene durch professionelle antigenpräsentierende Zellen (APC) kann effektive antileukämische Immunantworten auslösen. Unzureichende Antigenpräsentation durch leukämische Blasten, eingeschränkte Kostimulation und Überproduktion inhibitorischer Zytokine verhindern eine effiziente Immunantwort gegen aberrante Zellen. Dendritische Zellen (DC) sind potente APC und verbinden angeborene und adaptive Immunität, indem sie IEC und andere Komponenten des Immunsystems aktivieren. Leukämische Blasten können ex vivo in DC leukämischer Herkunft (DCleu) differenziert werden, die potenziell das gesamte Repertoire bekannter und unbekannter individueller leukämischer Antigene präsentieren. Diese DCleu können ex vivo und vermutlich auch in vivo potente IEC-vermittelte antileukämische Immunreaktionen auslösen.

Diese kumulative Dissertation umfasst drei Publikationen, die darauf abzielen, die Rolle von DC in antileukämischen Immunreaktionen besser zu verstehen.

In **Publikation I** (als Co-Autor) wurden die Expressionsprofile von DC Antigenen (DCA) auf naiven Blasten sowie die Veränderung dieser Profile nach der Zellkultur untersucht. Ziel war es, bestehende DC Generierungsmethoden so zu optimieren, dass zuverlässig ausreichende Mengen an DC produziert werden können, die antileukämische IEC-Reaktionen auslösen. Es wurden sechs verschiedene Methoden angewandt, um DC von 186 Patienten mit AML und MDS zu generieren. Die Ergebnisse zeigten, dass DCA regelmäßig in unterschiedlichem Ausmaß auf unkultivierten Blasten exprimiert werden und nach der Kultur hochreguliert werden. Mit mindestens einer Methode konnten bei jedem Patienten ausreichende Mengen an DC erzeugt werden. Immunzellen, die von diesen DC (in T-Zell angereicherter gemischter Lymphozytenkultur (MLC)) stimuliert wurden, zeigten in einem Fluorolyse-Assay lytische Aktivität gegen leukämische Blasten.

In **Publikation II** (als Co-Autor) untersuchten wir die Fähigkeit von ex vivo erzeugten DC, Effektorzellen für antileukämische Reaktionen zu stimulieren, und ermittelten prädiktive Faktoren für das lytische Potenzial von DC-stimulierten IEC. Wir generierten und quantifizierten DC und verschiedene DC-Subtypen aus Proben von 19 AML- und MDS-Patienten, und verwendeten einen funktionellen Fluorolyse-Assay, um die lytische Kapazität von DC bzw. MNC stimulierten IEC zu bewerten. Es konnte festgestellt werden, dass DC-stimulierte im Vergleich zu MNC- oder unstimulierten IEC ein höheres lytisches Potenzial aufweisen. Ein höherer Anteil von DCleu in der DC-Fraktion, von reifen/migratorischen (CD83+, CCR7+) korrelierte mit blastenlytischer Effektivität.

In **Publikation III** (als Erstautor) untersuchten wir einen möglichen Zusammenhang einer ex vivo DC Generierbarkeit mit dem klinischen Verlauf der Patienten nach SCT. DC wurden aus 69 Proben von AML- und MDS-Patienten erfolgreich ex vivo kultiviert und quantifiziert. Die Häufigkeit von DC (-Subtypen) wurde mit dem klinischen Verlauf der Patienten nach SCT korreliert. Es zeigte sich, dass ein höherer Anteil von DCleu in der DC-Fraktion signifikant mit langfristigem Ansprechen auf SCT, dem leukämiefreien und dem Gesamtüberleben, sowie längeren Intervallen bis zum Rezidiv nach der SCT korreliert. Zur Vorhersage des klinischen Verlaufs konnten Cutoff-Werte berechnet werden, die eine Abgrenzung von Langzeit-Respondern zu Non-Respondern auf SCT oder von Patienten mit verlängertem leukämiefreien- oder Gesamtüberleben oder längerer Zeit bis zum Rezidiv ermöglichen.

Daraus schließen wir, dass DC, insbesondere DCleu und reife DC, die Differenzierung von immunkompetenten antileukämischen Zellen nicht nur in vitro, sondern auch in vivo induzieren können. Ex vivo Generierung und Quantifizierung von DC, insbesondere von DCleu und reifen DC, sowie funktionelle Fluorolyse-Assays von DC-stimulierten IEC können nicht nur zur Vorhersage und Bewertung von antileukämischen Immunreaktionen und DC-Funktionalität ex vivo dienen, sondern auch als zusätzlicher Parameter für die Vorhersage des langfristigen Ansprechens auf SCT und hilfreich bei der Evaluierung der patientenindividuellen Therapieoptionen helfen. Darüber hinaus konnten wir zeigen, dass es möglich ist, bei jedem Patienten mit AML und MDS ausreichende Mengen an DC (z. B. für DC basierte Impfungen) zu erzeugen, indem man die beste von drei zuvor getesteten Methoden anwendet.

Unsere Forschung hat zur Entwicklung neuartiger Therapien geführt, bei denen zugelassene Medikamente (Response Modifier: Zytokine, Chemokine und Danger Signals) eingesetzt werden, um eine in vivo Differenzierung von leukämischen Blasten zu DC zu induzieren. Diese Ansätze haben in ex vivo-Experimenten und individuellen Heilversuchen vielversprechende Ergebnisse gezeigt.

4. Abstract

For many patients suffering from acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), allogeneic hematopoietic stem cell transplantation (SCT) is the sole potential curative treatment approach, but outcomes are limited by high relapse rates and severe side effects. The primary pathway of SCT for eliminating malignant cells is the graft-versus-leukemia (GvL) effect, driven by immune effector cells (IEC) of donor origin, such as cytotoxic T lymphocytes (T cells) and natural killer (NK) cells. Presentation of leukemia-specific antigens by professional antigen-presenting cells (APC) is essential for triggering effective anti-leukemic immune responses. Leukemic blasts exhibit insufficient stimulatory capacity due to impaired antigen-presentation, costimulatory signaling, and overexpression of inhibitory cytokines.
Dendritic cells (DC) are potent APC that bridge innate and adoptive immunity by activating IEC and other components of the immune system. Myeloid tumor cells can be induced to differentiate ex vivo towards leukemia-derived DC (DCleu) that potentially display the full spectrum of known and unidentified individual leukemic antigens. These DCleu can elicit potent IEC-mediated antileukemic immune reactions ex vivo and presumably in vivo.

This cumulative thesis includes three studies aimed at gaining deeper insights into the contribution of DC to antileukemic immune responses.

In **publication I** (as co-author), the individual dendritic cell antigen (DCA) expression profiles on naïve blasts, as well as how these profiles changed after culture, were assessed in order to refine culture methods that can generate substantial quantities of DC capable of inducing antileukemic T cell reactions. Six different methods were used to generate DC from 186 patients with AML and MDS. The results showed that DCA were consistently expressed at varying levels pre-culture on leukemic cells and were upregulated after culture. At least one method was effective in producing adequate amounts of DC in each patient case. IEC stimulated by these DC in a T cell enriched mixed lymphocyte culture (MLC) demonstrated cytotoxic potential against leukemic cells in a fluorolysis assay.

In **publication II** (as co-author), we evaluated the capacity of ex vivo-generated DC to prime IEC for antileukemic activity and established predictive factors for the lytic potential of DC-primed IEC. We generated and quantified DC and various subtypes of DC from 19 AML and MDS patients and used a functional fluorolysis assay following MLC to assess the lytic capacity of primed (DC/MNC) allogeneic and autologous IEC. The results showed that DC-primed IEC exhibit superior lytic potential compared to MNC-primed or unprimed T cells. Higher frequencies of certain DC subsets were found to be linked with blast-lytic efficiency, specifically DCleu in the DC fraction (DCleu+/DC), mature (CD83+) DC, and mature migratory (CCR7+) DC.

In **publication III** (as first author), we investigated the predictive value of ex vivo-generated DC in assessing treatment outcomes of AML and MDS patients undergoing SCT-based immunotherapy.

DC were successfully cultured and quantified ex vivo from 69 samples of AML and MDS patients. Frequencies of DC and DC subsets were correlated with patients' outcomes after SCT. It was found that elevated rates of DCleu+/DC have a highly significant correlation with improved outcomes in terms of long-term response, leukemia-free and overall survival, and longer time to relapse post-SCT. To predict patients' outcomes, cutoff values could be established to categorize patients as long-term responders or non-responders to SCT, as well as those with extended overall and leukemia-free survival or time to relapse.

We conclude that DC, particularly DCleu and mature DC, can induce the differentiation of immunocompetent antileukemic cells ex vivo and, notably, in vivo.
Ex vivo generation and quantification of DC, particularly DCleu and mature DC, and functional fluorolysis assays of DC-stimulated IEC may not only serve as an indicator to assess antileukemic immune reactions and DC functionality ex vivo, but also as an additional parameter for predicting the long-term response to SCT and evaluating patients' therapy options. Furthermore, we demonstrated that it is feasible to generate substantial quantities of DC (e.g., for vaccination) in any AML and MDS patient, using the most effective of three previously tested methods.
Our research has led to the development of novel therapies utilizing clinically approved cytokines, chemokines, and danger signals aiming at the in vivo generation of DC from leukemic blasts. These approaches have shown promising results in ex vivo experiments and initial clinical trials on individual patients.

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